The Genetic and Phenotypic Diversity of *Salmonella* Strains Associated with the U.S. Pistachio Environment

By

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Dissertation Abstract

The genetic diversity of *Salmonella* isolated associated with U.S. pistachios was determined by using the U.S. Food and Drug Administration's Center for Food Safety and Applied Nutrition Single Nucleotide Polymorphism (SNP) Pipeline. Additionally, representative *Salmonella* isolates obtained from pistachios stored in silos were selected to evaluate phenotypical characteristics that may play a role in persistence in pistachio orchards or during postharvest handling. A total of 11 *Salmonella* serovars and 15 strains have been isolated from U.S. pistachios from 2008 to 2018. Seven *Salmonella* strains have persisted in the California pistachio environment for ≥3 years, and some of these strains have been reported exclusively in association with pistachios. The copper homeostasis and silver resistant island sequence was present in three of the persistent strains and was associated with an increase in tolerance to $CuSO_4$ under anaerobic but not aerobic conditions. Growth of all strains except for *Salmonella* Enteritidis strain A (sporadic) was similar in pistachio hull slurry held at 30°C over 48 h. All *Salmonella* strains produced weak to strong biofilms after 4 days at 25°C, with seven strains, including two sporadic strains, producing moderate biofilms and one persistent strain (*Salmonella* Liverpool) producing a strong biofilm. The rdar+ and rdar- morphotypes were observed in both persistent and sporadic *Salmonella* strains. All *Salmonella* strains contained nine of the 10 genes previously associated with desiccation tolerance. The fate of *Salmonella* strains during simulated desiccation and subsequent dry storage was evaluated by inoculating sterile filters with individual *Salmonella* strains, which were dried overnight, and then stored at 24 ± 1 °C and 35% relative humidity for 50 days. After drying, population reductions of 0.50–1.25 logs were observed for eight of the nine *Salmonella* strains. The population reductions (3.98–5.12 log) of eight strains were not significantly different at day 50. All but one of the

Salmonella strains isolated from California pistachios were able to survive desiccation and storage, irrespective of their multicellular morphology or the presence of genes thought to aid in their survival during these processes. No single characteristic studied here fully explained the association of specific *Salmonella* strains with pistachios. Instead, it appears that multiple factors, including phenotypic and genotypic features and environmental factors, may be involved in the persistence of this foodborne pathogen in this environment.

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Chapter I. Background Information

Food safety is a crucial aspect of public health and the economy in the United States. The Centers for Disease Control and Prevention estimates that every year, one in six Americans gets sick from consuming contaminated food (Centers for Disease Control and Prevention, 2018). This amounts to approximately 48 million people becoming ill, 128,000 hospitalizations, and 3,000 deaths (Centers for Disease Control and Prevention, 2018). The economic burden of foodborne illnesses in the United States is estimated to be over $15 billion annually in medical costs and lost productivity (U.S. Department of Agriculture, 2023). Moreover, outbreaks of foodborne illnesses can have severe consequences for the food industry, including reduced consumer confidence, loss of sales, and expensive recalls. Therefore, ensuring the safety of the food supply chain is critical to protect public health and the economy.

Food illnesses are often caused by the contamination of bacterial pathogens such as *Campylobacter, Clostridium perfringens, Escherichia coli* O157:H7 and *Salmonella* (Centers for Disease Control and Prevention, 2018). These enteric bacteria are often found in raw or undercooked meats, poultry, and eggs, as well as unpasteurized dairy products. However, any food can become contaminated with bacterial pathogens via cross-contamination at any point in the supply chain (food production, harvest, processing, handling, storage, and preparation) (Centers for Disease Control and Prevention, 2022a). Therefore, identifying contamination sources, understanding modes of contamination, and the potential hazards associated with a contamination event is essential to develop mitigation strategies that will effectively reduce the overall burden of foodborne illnesses.

Salmonella enterica is one of the leading causes of foodborne illnesses in the United States accounting for approximately 1.5 million infections, 26,500 hospitalization and 420 deaths annually (Centers for Disease Control and Prevention, 2018). Between, 2017 and 2020, *Salmonella* was responsible for 83 (64%) out of the 130 multistate outbreaks associated with contaminated food, resulting in over 6,000 cases of salmonellosis and 1,489 hospitalizations (Centers for Disease Control and Prevention, 2022b). Recalls and outbreaks related to *Salmonella* have affected various food categories including fresh and frozen fruits and vegetables, fish, eggs, chicken, dairy, and low-moisture foods (LMF) such as nuts and seeds. LMFs (foods with a water activity (a_w) <0.85) were once generally regarded as safe because the growth of bacterial foodborne pathogens was prevented. Prior to 1990, a total of 13 outbreaks associated with LMFs were reported in USA, Canada, the UK, Australia, New Zealand, and across Europe (Acuff et al., 2023). However, from 2012 to 2020, there were 54 outbreaks, and 1,659 recalls linked to LMFs, with *Salmonella* contamination accounting for 81.5% and 84.0% of the total outbreaks and recalls, respectively. Contaminated nuts, nut products, and seeds accounted for most outbreaks (51.9%) and recalls (56.9%) during this period (Acuff et al., 2023).

The United States is the largest producer of pistachios in the world with a commercial production of 401,038 metric tons of pistachios in 2022 (Administrative Committee For Pistachios, 2022), more than double the production in 2014 (186,906 metric tons). Most of the U.S. pistachio volume (99%) is grown in California's Central Valley. The harvest of California pistachios typically takes place in the autumn, spanning from late August to early October. During harvest, pistachios fruits are mechanically shaken from the trees and collected onto catch frames. Subsequently, pistachios are transported in bins or bottom dump trailers to a facility where the hulls are removed using mechanical force. Hulled pistachios are then rinsed to remove

the loosely adhering hull and then hulled pistachios are separated based on buoyancy in a tank of water. The underdeveloped, damaged, and partially in-hull pistachios (floaters) are separated from fully developed and hulled pistachios (sinkers). After separation, the floaters and sinkers are dried separately to achieve moisture levels between 8–15% and are then stored in silos (500,000- to 750,000-kg capacity) (Harris and Ferguson 2013). In the silos, pistachios are further dried using warm, dry ambient air for several days to moisture levels of <7% (a_w of <0.70). Fully dry pistachios can be stored for up to 18 months in the silo (Harris and Ferguson 2013).

U.S. pistachios have been involved in three multistate outbreaks, all of which were linked to *Salmonella* contamination (Harris et al., 2022). The first outbreak occurred between 2008 and 2009, resulting in 83 salmonellosis cases across 21 states (Whitham et al., 2021). This outbreak was associated with pistachio and pistachio products contaminated with *Salmonella* Montevideo, *Salmonella* Senftenberg, and *Salmonella* Newport (Centers for Disease Control and Prevention, 2009). In 2013, eight cases of salmonellosis in six states were linked to roasted pistachios contaminated with *Salmonella* Senftenberg (U.S. Food and Drug Administration, 2014). Between 2015 and 2016, 11 cases of salmonellosis in nine states were linked to roasted inshell and shelled California pistachios contaminated with *Salmonella* Montevideo and *Salmonella* Senftenberg (Centers for Disease Control and Prevention, 2016). Furthermore, since 2009, there have been 16 recalls associated with raw and roasted U.S. pistachios due to the *Salmonella* contamination (Yada & Harris, 2021).

A previous 3-year study analyzing California inshell pistachio samples collected from storage silos during 2010 to 2012, indicated the presence of six *Salmonella* serovars (Agona, Liverpool, Montevideo, Tennessee, Senftenberg, and Worthington) with nine pulsed-field gel electrophoresis fingerprints (Harris et al., 2016). The *Salmonella* prevalence (0.6%) and levels

(<1 MPN/100 g) in the silo samples were consistent with those observed from other tree nuts (Bansal et al., 2010; Brar et al., 2016; Danyluk et al., 2007). However, compared to the diversity of other U.S. tree nuts, the diversity of *Salmonella* serovars in pistachio was limited. In 2016, six *Salmonella* serovars (Duisburg, Liverpool, Mbandaka, Montevideo, Senftenberg, Worthington), were isolated from raw, shelled pistachios at retail markets (Zhang et al., 2021). Both surveys, along with recalls and outbreaks investigations provided further evidence of limited *Salmonella* diversity in U.S. pistachios and highlighted the repeated isolation of *Salmonella* Montevideo and *Salmonella* Senftenberg in pistachios between 2008–2018.

Whole genome sequence analysis combined with metadata analysis provided evidence of the persistence of *Salmonella* strains in the U.S. pistachio supply chain (Haendiges et al., 2021). That study was limited to *Salmonella* Montevideo and *Salmonella* Senftenberg isolates recovered from pistachio and pistachio environments that were available in the NCBI Pathogen Detection database. *Salmonella* isolates from the silo survey were not analyzed in that study. Therefore, it remains unknown whether there are additional persistent *Salmonella* strains present in the California pistachio environment. Haendigens et al., (2021) identified the presence of the copper homeostasis and silver resistance island (CHASRI) in some of the persistent *Salmonella* Montevideo and *Salmonella* Senftenberg strains. This finding lead to the hypothesis that CHASRI enables strain adaptation to the pre-harvest environment, potentially explaining the limited diversity and persistence of *Salmonella* strains in the California pistachio environment (Haendiges et al., 2021). However, the phenotypic advantage conferred by CHASRI has not been evaluated, and therefore, it remains unknown whether the presence of this sequence fully explains the limited *Salmonella* diversity or if there are other genotypic and phenotypic

advantages of *Salmonella* that account for the persistence of certain strains in the pistachio environment.

Our study aimed to further explore the diversity and persistence of *Salmonella* in the California pistachio environment. In Chapter II, the sequences of *Salmonella* isolates obtained from the pistachio silo survey conducted in 2010, 2011 and 2012 (Harris et al. 2016) are analyzed to determine phylogenic relationships, and to determine the presence of antibiotic genes, and the CHASRI sequence. Additionally, we conduct SNP analyses among *Salmonella* isolates from the silo survey and isolates from U.S. pistachios deposited in the NCBI database to determine the number of unique *Salmonella* strains isolated from California pistachios since 2008. Furthermore, a SNP analysis between *Salmonella* strains isolated from California pistachios and California almonds is conducted to determine the uniqueness and distribution of *Salmonella* strains associated with pistachios.

Chapter III focuses on investigating the phenotypical responses of *Salmonella* strains during simulated conditions during the production, harvest, and postharvest handling of California pistachios. One common practice in pistachio cultivation is the application of copper to promote tree health. Genetic analysis identified the presence of CHASRI in some strains suggesting that persistent *Salmonella* strains may have adapted to the presence of copper in the environment (Haendiges et al., 2021). In this chapter we evaluate the copper sensitivity of individual *Salmonella* strains associated with California pistachios. Additionally, we assess the growth behavior of *Salmonella* strains using pistachio hull nutrients, as transportation delays from the orchard to the hulling facility can cause an increase in *Salmonella* populations (Moussavi et al., 2019). Lastly, we evaluate the biofilm-forming potential of *Salmonella* strains as the postharvest environment poses a risk for biofilm formation. The aim of this chapter is to

determine whether copper sensitivity, ability to grow in hull extracts, and biofilm-forming ability play a role in *Salmonella* persistence in the pistachio environment.

Salmonella is a pathogen known for its adaptability, allowing it to thrive under various stress conditions (Norberto et al., 2022). During the drying process of pistachios, *Salmonella* cells on contaminated pistachios undergo desiccation, followed by exposure to a low-moisture and low-nutrient environment during storage. In Chapter IV, we evaluate the impact of phenotypic or genotypic characteristics associated with desiccation tolerance and storage survival. The first part of Chapter IV focuses on investigating the expression of the rough, dry, and red morphotype, a phenotype known to enhance desiccation resistance (White et al., 2006) Furthermore, we identify the presence of genes linked to *Salmonella* survival on pistachios during both desiccation and storage. Finally, we evaluate the survival of individual *Salmonella* strains during a simulated drying and storage study. This chapter aims to determine the *Salmonella* characteristics displayed during the drying and storage steps, and their role in the persistence and diversity of *Salmonella* strains found in the later stages of the pistachio production chain.

Lastly, chapter V summarizes the overall findings and limitations of our studies and provides suggestions for future research that may shed light on the diversity and persistence of *Salmonella* strains in the California pistachio production environment. Additionally, the results of our study may improve and expand on the understanding of the phenotypic and genotypic characteristic of the *Salmonella* strains associated with California pistachios which may allow for the development of effective *Salmonella* control strategies.

References

Acuff, J. C., Dickson, J. S., Farber, J. M., Grasso-Kelley, E. M., Hedberg, C., Lee, A., & Zhu, M.-J. (2023). Practice and progress: updates on outbreaks, advances in research, and processing technologies for low-moisture food safety. *Journal of Food Protection*, *86*(1), 100018. https://doi.org/10.1016/j.jfp.2022.11.010

Administrative Committee for Pistachios. (2022). 2022 Pistachio bearing acreage, production and yield per acreage. Available at: https://acpistachios.wpenginepowered.com/wp-content/uploads/2023/01/2022-Pistachio-Statistics.pdf. Accessed 5 February 2023.

Bansal, A., Jones, T. M., Abd, S. J., Danyluk, M. D., & Harris, L. J. (2010). Most-probable-number determination of *Salmonella* levels in naturally contaminated raw almonds using two sample preparation methods. *Journal of Food Protection*, *73*(11), 1986–1992. https://doi.org/10.4315/0362-028x-73.11.1986

Brar, P. K., Strawn, L. K., & Danyluk, M. D. (2016). Prevalence, level, and types of *Salmonella* isolated from North American in-shell pecans over four harvest years. *Journal of Food Protection*, *79*(3), 352–360. https://doi.org/10.4315/0362-028X.JFP-15-365

Centers for Disease Control and Prevention. (2018). Burden of foodborne illness: findings. Available at: https://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html. Accessed on 5 May 2023

Centers for Disease Control and Prevention. (2022a). Foods that can cause food poisoning. Available at: https://www.cdc.gov/foodsafety/foods-linked-illness.html. Accessed on 10 May 2023

Centers for Disease Control and Prevention. (2009). Multistate outbreak of *Salmonella* infections linked to pistachio nuts (final update). Available at: https://www.cdc.gov/salmonella/2009/pistachio-nuts-4-14-2009.html. Accessed 27 February 2023

Centers for Disease Control and Prevention. (2016). Multistate outbreak of *Salmonella* Montevideo and *Salmonella* Senftenberg infections linked to Wonderful pistachios. Available at: https://www.cdc.gov/salmonella/montevideo-03-16/index.html. Accessed 10 November

Centers for Disease Control and Prevention. (2019). Reports of selected *Salmonella* outbreak investigations. Available at https://www.cdc.gov/salmonella/outbreaks.html. Accessed 29 April 2023

Centers for Disease Control and Prevention. (2022b). Summary of possible multistate enteric (Intestinal) disease outbreaks in 2017–2020. Available at: https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/annual-summaries/annual-summaries-2017-2020.html. Accessed 9 June 2023

Danyluk, M. D., Jones, T. M., Abd, S. J., Schlitt-Dittrich, F., Jacobs, M., & Harris, L. J. (2007). Prevalence and amounts of *Salmonella* found on raw California almonds. *Journal of Food Protection, 70*(4), 820–827. https://doi.org/10.4315/0362-028x-70.4.820

Haendiges, J., Davidson, G. R., Pettengill, J. B., Reed, E., Ramachandran, P., Blessington, T., Miller, J. D., Anderson, N., Myoda, S., Brown, E. W., Zheng, J., Tikekar, R., & Hoffmann, M. (2021). Genomic evidence of environmental and resident *Salmonella* Senftenberg and Montevideo contamination in the pistachio supply-chain. *Plos One*, *16*(11), e0259471. https://doi.org/10.1371/journal.pone.0259471

Harris, & Ferguson. (2013a). Improving the safety of almonds and pistachios, p. 350–378. In L. J. Harris (ed.), Improving the safety and quality of nuts. Woodhead Publishing Ltd., Cambridge.

Harris, Linda J, Lieberman, V., Mashiana, R. P., Atwill, E., Yang, M., Chandler, J. C., Bisha, B., & Jones, T. (2016). Prevalence and amounts of *Salmonella* found on raw California inshell pistachios. *Journal of Food Protection*, *79*(8), 1304–1315. https://doi.org/10.4315/0362-028X.JFP-16-054

Harris, L J, Yada, S., Beuchat, L. R., & Danyluk, M. D. (2022). Outbreaks of foodborne illness associated with the consumption of tree nuts, peanuts, and sesame seeds (version 2) [Table and references]. In Outbreaks from tree nuts, peanuts, and sesame seeds. Available at: https://ucfoodsafety.ucdavis.edu/low-moisture-foods/nuts-and-nut-pastes. Accessed 10 May 2023

Moussavi, M., Lieberman, V., Theofel, C., Barouei, J., & Harris, L. J. (2019). Growth of *Salmonella* on inoculated inhull pistachios during postharvest handling. *Journal of Food Protection*, *82*(2), 217–225. https://doi.org/10.4315/0362-028X.JFP-18-351

Norberto, A. P., Alvarenga, V. O., Hungaro, H. M., & Sant'Ana, A. S. (2022). Desiccation resistance of a large set of *Salmonella* enterica strains and survival on dry- and wet-inoculated soybean meal through storage. *LWT*, *158*, 113153. https://doi.org/10.1016/j.lwt.2022.113153

U.S. Department of Agriculture. (2023). Cost estimates of foodborne illnesses. Available at: https://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses.aspx. Accessed on May 5 2023

U.S. Food and Drug Administration. (2014). FDA investigation summary—multistate outbreak of *Salmonella* Senftenberg infections associated with pistachios from a California roaster. Available at: http://wayback.archive-it.org/7993/20171114154922/https://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm386377.htm. Accessed 1 October 2022

White, A. P., & Surette, M. G. (2006). Comparative genetics of the rdar morphotype in *Salmonella*. *Journal of Bacteriology*, 188(24), 8395–8406. https://doi.org/10.1128/JB.00798-06

Whitham, H. K., Sundararaman, P., Dewey-Mattia, D., Manikonda, K., Marshall, K. E., Griffin, P. M., Gleason, B. L., Subramhanya, S., & Crowe, S. J. (2021). Novel outbreak-associated food vehicles, United States. *Emerging Infectious Diseases*, *27*(10), 2554–2559. https://doi.org/10.3201/eid2710.204080

Yada, S., & Harris, L. J. (2021). Recalls of tree nuts and peanuts in the U.S., 2001 to present (version 2) [Table and references]. In U.S. recalls of nuts. Available at:

https://ucfoodsafety.ucdavis.edu/low-moisture-foods/nuts-and-nut-pastes. Accessed April 27 2023

Zhang, G., Hu, L., Luo, Y., Santillana Farakos, S. M., Johnson, R., Scott, V. N., Curry, P., Melka, D., Brown, E. W., Strain, E., Bunning, V. K., Musser, S. M., & Hammack, T. S. (2021). Survey of *Salmonella* in raw tree nuts at retail in the United States. *Journal of Food Science*, *86*(2), 495–504. https://doi.org/10.1111/1750-3841.15569

Chapter II. Characterizing the Genetic Diversity of *Salmonella* Isolated from U.S. Raw Inshell Pistachios Using Whole Genome Sequencing

Abstract

A total of seven *Salmonella* serovars were isolated from raw inshell pistachio samples collected from California storage silos during the 2010, 2011, and 2012 harvests (silo survey isolates). The genetic diversity of 169 isolated samples was determined by analyzing the whole genome sequence data using the U.S. Food and Drug Administration's Center for Food Safety and Applied Nutrition Single Nucleotide Polymorphism (SNP) Pipeline. *Salmonella* isolates clustered by serovars Agona, Enteritidis, Montevideo, Sandiego, Senftenberg, Liverpool, Tennessee, and Worthington in the phylogenetic tree. Within each serovar, isolates grouped into one or two clusters (≤14 SNPs). Two distinct clusters (>14 SNPs) were identified for *Salmonella* Enteritidis, Montevideo and Liverpool for a total of 11 unique strains. Sequences of representative silo survey isolates clustered with sequences of *Salmonella* strains isolated from U.S. pistachio–associated samples collected between 2008–2018 available on the National Center for Biotechnology Information database, and, in all but two cases, not with sequences of *Salmonella* strains recovered from raw California almonds from 2001 through 2013. The genomic evidence suggests that strains of *Salmonella* Agona, Liverpool A, Montevideo A and B, Senftenberg, and Worthington have persisted in the California pistachio environment for ≥3 years and some of these strains have been reported exclusively in association with pistachios.

Introduction

The United States is the leading supplier of pistachios, accounting for 47% of worldwide production (International Nut and Dried Fruit Council, 2021). Over the past four decades, U.S. pistachio production has increased rapidly and, in 2022 (401,039 metric tons) was 30 times the volume produced in 1979 (Administrative Committee for Pistachios, 2023). This accelerated growth led to a rapid development of infrastructure, and to a geographical localization for pistachio production. Most U.S. pistachios are grown in California, with Arizona and New Mexico making up just 1% of the total production volume. Roughly 96% of California's pistachios comes from five contiguous counties—Kern, Fresno, Tulare, Madera, and Kings—all located in the Central Valley (Administrative Committee for Pistachios, 2023).

In California, pistachios are harvested from late August to early October (American Pistachio Growers, 2017). The trees are mechanically shaken and the pistachio fruits fall into catch frames (Harris & Ferguson, 2013). The pistachios are subsequently transported in bins or bottom dump trailers to processors, and, upon arrival, debris is removed. Pistachio hulls are detached from the inshell pistachios in a wet, physically abrasive process. Hulled pistachios are deposited into a water tank where mature, fully developed nuts or "sinkers" are separated from damaged or underdeveloped or adhering hull "floaters." Hulled pistachios are dried to 10□15% moisture content in forced air dryers, and then moved to large (450 to 680 metric ton volume) storage silos where ambient air is introduced until the pistachios have achieved a final moisture level of <7%, typically within a few days.

Dry pistachios, like other low-moisture foods, do not support bacterial growth (Podolak et al., 2010). However, foodborne pathogens such as *Salmonella* can survive in low-moisture environments, including on pistachios, for extended periods, with slow reductions at ambient

temperatures (Blessington et al., 2012; Danyluk et al., 2008; Kimber et al., 2012; Limcharoenchat et al., 2019). Isolation of *Salmonella* from raw and roasted pistachios has led to several major recalls of the nut products in the United States (Yada & Harris, 2022). Pistachios have been linked to three reported outbreaks of salmonellosis in the United States. In 2009, pistachios were recalled after *Salmonella* serovars Montevideo, Newport, and Senftenberg were isolated from commercial products (Centers for Disease Control and Prevention, 2009). The pulsed-field gel electrophoresis (PFGE) fingerprints for *Salmonella* Montevideo isolated from the pistachio samples were matched to a *Salmonella* isolate from a single salmonellosis case reported at that time (Centers for Disease Control and Prevention, 2009). This case was subsequently linked to an outbreak of 83 salmonellosis cases in 21 U.S. states (Whitham et al., 2021). In 2013, *Salmonella* Senftenberg contamination of California pistachios resulted in a multistate outbreak, with eight cases from six states (U.S. Food and Drug Administration, 2014a). In 2015–2016, roasted unshelled and shelled pistachios were associated with a multistate outbreak; two and nine cases of salmonellosis caused by *Salmonella* Senftenberg and *Salmonella* Montevideo, respectively, were reported (Centers for Disease Control and Prevention, 2016).

The prevalence, levels, and distribution of *Salmonella* from raw inshell California pistachios was determined for pistachios collected from storage silos during or shortly after the 2010, 2011, and 2012 harvests (Harris et al., 2016). *Salmonella* was isolated from 32 of 3,966 100-g pistachio samples (0.61%) (Harris et al., 2016). A similar prevalence of *Salmonella* was reported for raw California almond kernels (~1% of 14,949 100-g samples) (Bansal et al., 2010; Danyluk et al., 2007; Lambertini et al., 2012), inshell U.S. pecans (0.95% of 4,641 100-g samples) (Brar et al., 2016), and inshell California walnuts (0.14% of 2,903 375-g samples) (Davidson et al., 2015) sampled after harvest and before further processing. Over 30 *Salmonella*

serotypes were identified in each of the almond and pecan surveys. In contrast, seven *Salmonella* serovars (Agona, Enteritidis, Liverpool, Montevideo, Senftenberg, Tennessee, and Worthington) with nine PFGE "fingerprints" were identified among the 323 *Salmonella* isolates from the 3-year pistachio survey, suggesting a more narrow and persistent contamination source (Harris et al., 2016).

Whole genome sequencing (WGS) has replaced PFGE fingerprinting as the gold standard for strain characterization in outbreak investigations (Ribot et al., 2019). WGS analysis provides information that PFGE fingerprinting does not, such as the presence of bacteriophage, pathogenicity genes, antimicrobial resistance genes, and more robust information of the genetic relatedness of isolates (Didelot et al., 2012; Kozyreva et al., 2016). In addition, WGS allows for single nucleotide polymorphism (SNP) analysis, which has been used by food regulatory and public agencies to compare the genetic relationship among bacterial isolates, for source tracking or tracing transmission routes, and for grouping isolates from different time periods and environments (Brown et al., 2019; Rantsiou et al., 2018; Taylor et al., 2015). The genetic relatedness of *Salmonella* Senftenberg and *Salmonella* Montevideo isolates associated with pistachios was evaluated using the Center for Food Safety and Applied Nutrition (CFSAN) SNP Pipeline and whole genome multi-locus sequence typing (wgMLST) (Haendiges, Davidson, Pettengill, Reed, Blessington, et al., 2021). The authors suggested that *Salmonella* Senftenberg and *Salmonella* Montevideo strains were persistent in pistachio production environments due, in part, to the presence of the copper homeostasis and silver resistance island (CHASRI) identified in several strains. They hypothesized that adaptation to extrinsic pressures in the pistachio supply chain might have selected for, and may explain, the increased recovery of strains containing this

island (Haendiges et al., 2021). Sequences of two of the seven *Salmonella* serovars identified in the pistachio survey (Harris et al., 2016) were included but none of the specific isolates.

In the present study, the WGS data from *Salmonella* isolates obtained from the 2010, 2011, and 2012 pistachio silo surveys (Harris et al., 2016) were analyzed using the CFSAN SNP Pipeline (Davis et al., 2015) to determine the genetic relatedness among isolates. WGS sequences were used to confirm serovar and to identify antimicrobial resistance (AMR) genes and the presence of the CHASRI. Additionally, a SNP analysis among *Salmonella* isolates obtained from the pistachio silo survey, as well as those linked to pistachio outbreaks (Harris et al., 2022), a retail survey (Zhang et al., 2021), and other isolates found on pistachios or in pistachio environments during U.S. Food and Drug Administration (FDA) investigations (Haendiges et al., 2021; Harris et al., 2022), was performed to evaluate the persistence of these strains in the pistachio supply chain between 2007 and 2018. A SNP comparison analysis was performed among isolates from the pistachio silo survey and *Salmonella* isolates obtained from California almond surveys between 2001 and 2013 (Bansal et al., 2010; Danyluk et al., 2007; Lambertini et al., 2012; Santillana Farakos et al., 2017) to determine whether strains found on pistachios are also found on almonds.

Materials and Methods

Inclusion criteria for selecting Salmonella isolates for WGS

Salmonella isolates from raw pistachios harvested in 2010, 2011, and 2012 were previously characterized by classical agglutination serology (serovar) and PFGE fingerprints (Harris et al., 2016). Briefly, 3,966 raw inshell pistachios samples (~1 kg) were collected within 4 months of harvest from storage silos at seven California pistachio processors representing ~98% of California production. An initial 100-g subsample of each was analyzed using Association of Official Agricultural Chemists (AOAC) official method 2001.9 to detect *Salmonella*. Additional enrichments (50 g, 25 g, 2.5 g, and 0.25 g) were conducted for some samples. At least three colonies were selected from each positive enrichment. All isolates were stored at −80 °C in tryptic soy broth (TSB) supplemented with 15% glycerol.

Salmonella isolates were categorized based on the original ~1-kg samples (n = 69) that were positive by any method used. For each 1-kg sample, up to three isolates per *Salmonella* serovar were selected for sequencing (n = 169). When available, the isolates for each sample included one isolate from each positive enrichment method (AOAC, secondary MPN, three-tube MPN) employed. These 169 *Salmonella* isolates are denoted as silo survey isolates.

Whole genome sequencing

Silo survey isolates were streaked onto tryptic soy agar (TSA: tryptic soy broth [TSB; BD] plus 1.5% granulated agar [Millipore]), and then incubated for 24 h at 37 °C. A single colony was selected and inoculated into 2 ml of TSB at 37°C for 24 h. *Salmonella* cells were collected by centrifugation at 18,210 × g for 2 min. The supernatant was discarded, and the DNA was extracted with the QIAamp DNA minikit (Qiagen) following the manufacturer's directions. Paired-end libraries were constructed using a TruSeq DNA library kit (Illumina Inc.) following

the manufacturer's directions or by the DNA Technologies and Expression Analysis Core at University of California Davis using Seqwell plexWell LP384 Library Preparation Kit for Illumina Sequencing Platforms (seqWell, Beverly, MA). Pooled libraries were sequenced with MiSeq, HiSeq, or the NovaSeq Illumina system by the DNA Technologies and Expression Analysis Core (Davis, CA). Sequences were submitted to NCBI under BioProject ID PRJNA976331 (Supplemental Table S1).

Quality control, assembly core SNP typing

The quality of the raw sequences was assessed using the FastQC analysis tool (Andrews 2010). The ends of the sequences, including Illumina adapters, were trimmed using Trimmomatic software (version 0.36) (Blodgett, 2010). Forward and reverse reads were assembled de novo with SPAdes (version 3.14.1) genome assembler (Bankevich et al., 2012). *Salmonella* genome assemblies were serotyped using SeqSero2 (Zhang et al., 2019). Additionally, the NCBI BLASTn tool was used to identify the correct serotype for isolates with inconsistent serotyping results by searching for highly similar isolates matching the first 10,000 base pairs (bp) of the longest contig assembled. *De novo* genomes were aligned and a core genome single nucleotide tree was built using the default parameters of Parsnp (version 1.2) (Treangen et al., 2014). *Salmonella enterica* subsp. *enterica* (GenBank sequence CP026379.1) was used as the reference genome for the core genome alignment.

Phylogenetic SNP analysis using the CFSAN SNP Pipeline

The CFSAN SNP Pipeline is a robust tool that produces a SNP matrix from WGS data of a given set of samples, particularly samples involved in a foodborne outbreak (Davis et al., 2015). All software required to execute the CFSAN SNP Pipeline (version 2.2.0) was installed on a local ubuntu platform. After functionality verification of the pipeline, a phylogenetic SNP

analysis for each serotype was performed. Using the CFSAN SNP Pipeline, SNP matrices were generated for isolates belonging to each serovar. Additionally, the CFSAN SNP Pipeline was used to determine the SNP differences between *Salmonella* isolates from the pistachio survey, *Salmonella* sequences available on the National Center for Biotechnology Information (NCBI) database, and *Salmonella* sequences from almond isolates (Moyne et al., 2023). Five closed genomes available on the NCBI database were used as references: CP011259.1 (*Salmonella* Agona), CP018637.1 (*Salmonella* Enteritidis), CP039509.1 (*Salmonella* Worthington), CP007530.1 (*Salmonella* Montevideo), and CP029036.1 (*Salmonella* Senftenberg). At the time of the analysis, no closed genome for *Salmonella* Liverpool was available on the NCBI database. Colleagues from the Institute of Integrative Biology and Systems at the University of Laval, Quebec, shared a closed genome for *Salmonella enterica* serovar Liverpool (Isolate ID S1713) isolated from ground chicken, and available on the *Salmonella* Foodborne Syst-OMICS database (SalFoS) (salfos.ibis.ulaval.ca). After clusters (groups of *Salmonella* isolates separated by >14 SNPs) were identified, one isolate within each cluster (≤14 SNP differences) and each year was selected for further analysis, when available. Isolates within a cluster were considered a strain.

NCBI isolates for SNP analysis and comparison

Previously sequenced and published outbreak-associated isolates (denoted as outbreak isolates) were selected: *Salmonella* Montevideo isolates associated with the outbreak in 2009 (*n* = 1) (Bakker et al., 2011; Whitham et al., 2021) and 2016 (*n* = 2) (Haendiges et al., 2018) and *Salmonella* Senftenberg isolates associated with the outbreak in 2013 (*n* = 1) (Haendiges et al., 2021) and 2016 (*n* = 2) (Haendiges et al., 2018). Sequences for *Salmonella* serovars Liverpool, Montevideo, Senftenberg, and Worthington from isolates obtained from pistachio samples during a retail survey conducted by the FDA (Zhang et al., 2021) were selected (denoted as retail survey

isolates). *Salmonella* Montevideo isolate CFSAN010209 was chosen to ensure complete representation of the two multi-locus sequence types previously associated with *Salmonella* Montevideo isolated from pistachios (Haendiges, Davidson, Pettengill, Reed, Blessington, et al., 2021). Using the NCBI Pathogen Detection tool, additional *Salmonella enterica* serovar Agona, Liverpool, and Worthington isolates were selected. SNP clusters with isolates of interest were identified based on the following search criteria: "Salmonella" AND "serovar (Agona, Enteritidis, Liverpool or Worthington)" AND "pistachio." A total of five NCBI SNP clusters was identified: one Agona, PDS000106143.1; two Enteritidis, PDS000026955.58 and PDS000029794.4; one Liverpool, PDS000001378.248; and one Worthington, PDS000031070.33. Only isolates from pistachios collected in the United States were considered for analysis. All *Salmonella* Agona (three), and *Salmonella* Worthington (three) isolates matching the criteria were selected. Fifteen *Salmonella* Liverpool isolates matched the query; one isolate per collection year was selected (total of three isolates, Supplemental Table 2). The genome sequences of the selected isolates were downloaded for analysis. Two *Salmonella* Enteritidis isolates from pistachios identified in the NCBI database search were not included, as these isolates were not from U.S. locations.

Salmonella isolated from raw almond selected for SNP analysis and comparison

Based on phylogenetic trees obtained by Moyne et al., (2023), single isolates of *Salmonella* serovars Agona, Enteritidis, Montevideo, Liverpool, Senftenberg, Tennessee, and Worthington from each cluster separated by ≥20 SNPs were selected for comparison to the pistachio silo survey isolates (Supplemental Table 3). The CFSAN SNP Pipeline was used to obtain SNP matrices, and phylogenetic trees were constructed as described below. Additionally, if *Salmonella* isolates from the almond and pistachio surveys formed a single cluster with <14

SNP differences, the CFSAN SNP Pipeline was executed only for isolates in the cluster. A total of 21 *Salmonella* isolates obtained from the California almond surveys was selected for SNP analysis and comparison.

Phylogeny and tree visualizations

Phylogenetic neighbor-joining trees were built with MEGA X (Kumar et al., 2018) using SNP matrices generated for each CFSAN SNP Pipeline analysis. All phylogenetic trees were created using Interactive Tree Of Life (iTOL), an online tool for phylogenetic tree display and annotation (Letunic & Bork, 2021).

In silico identification of antimicrobial resistance genes

The ResFinder 4.1 database was used to identify acquired antimicrobial resistance genes in the pistachio silo isolates (Bortolaia et al., 2020). The sequences of the assembled draft genomes were compared against the antimicrobial resistance genes on the ResFinder 4.1 database. The selected resistance genes met the following criteria: a minimum 90% identity, and a minimum length of 80% coverage overlap between the best matching resistance gene sequence in the database and the input sequences.

Analysis of the CHASRI

The sequences of the assembled draft genomes were compared against an annotated reference sequence of *Salmonella enterica* serovar Typhimurium (GenBank accession number CP019649.1) to identify the copper homeostasis and silver resistance island (CHASRI) (Staehlin et al., 2016). Using the "graphic" option on the NCBI database, the CHASRI was located on the annotated sequence (4,682,799 bp to 4,703,795 bp). NCBI BLASTn tool was used to find regions of similarity between pistachio draft genomes and the CHASRI sequence on the annotated

reference sequence. The CHASRI sequence was determined to be present when a contig of the draft sequence showed 100% coverage and over 95% identity against the annotated sequence.

Results

Phylogenic analysis of representative Salmonella isolates

The 169 representative *Salmonella* silo survey isolates selected for WGS analysis are listed in Table 1. The predicted antigenic profile of 20 isolates initially serotyped by agglutination as Liverpool, Montevideo, or Senftenberg (Harris et al., 2016) were not found in the SeqSero2 database (Table 1). In two cases, the agglutination and SeqSero2 serovar did not match; initially, both isolates LJH1292-1 and LJH1610 were serotyped as Senftenberg, but SeqSero2 predicted Sandiego and Westhampton, respectively. Highly similar isolates were identified using NCBI BLASTn. The LJH1292-1 sequence was a 100% match to *Salmonella enterica* subspecies *enterica* serovar Sandiego strain CFSAN012509 (isolated in California from ground annatto seed), while the LJH1610 sequence was a 100% match to several *Salmonella* Senftenberg isolates, including strains CFSAN004025, and SA20061017 and SA20130280 (two avian isolates from Canada). Thus, LJH1292-1 was excluded from the list of *Salmonella* Senftenberg isolates and treated as a single *Salmonella* Sandiego isolate whereas isolate LJH1610 was treated as a *Salmonella* Senftenberg isolate (Table 1).

The genomes of the representative isolates were aligned, and a maximum-likelihood phylogenetic tree was constructed using Parsnp (Fig. 1). All isolates except LJH1292-1 (Sandiego), grouped by serovars identified using a standard agglutination-based serotyping (Table 1) and the SeqSero2 method. The phylogenetic tree was made up of 10 clusters. Two clusters were observed for both *Salmonella* Enteritidis (two and four isolates) and *Salmonella* Montevideo (20 and 58 isolates). Single clusters were identified for *Salmonella* serovars Agona

(two isolates), Liverpool (26 isolates), Sandiego (one isolate), Senftenberg (16 isolates), Tennessee (one isolate), and Worthington (39 isolates).

SNP CFSAN Pipeline analysis

Initially, the CFSAN SNP Pipeline was used for each of the serotypes with two or more isolates (Agona, Enteritidis, Montevideo, Liverpool, Senftenberg, and Worthington). The two S*almonella* Montevideo clusters were separated by 428 SNPs and were denoted Cluster A (58 isolates) and Cluster B (20 isolates) (Fig. 2). A second CFSAN SNP Pipeline analysis that included isolates within each cluster indicated that *Salmonella* Montevideo Cluster A isolates differed by ≤7 SNPs and *Salmonella* Montevideo Cluster B isolates were identical (0 SNP differences) for 18 of the 20 isolates. *Salmonella* Montevideo isolate LJH1287-2 and isolate LJH1525 differed from other Cluster B isolates by ≤2 SNPs (Supplemental Tables 4 and 5).

The *Salmonella* Enteritidis isolates in Cluster A (four isolates) and Cluster B (two isolates) were separated by 764 SNP differences (Fig. 3). *Salmonella* Enteritidis Cluster A isolates differed by ≤5 SNPs while Cluster B isolates differed by 14 SNPs. Isolates in Cluster A included those previously phage typed as rough dry nonconforming (RDNC) and phage type (PT) 37 while those in Cluster B were identified as PT 9c (Harris et al., 2016).

All but one *Salmonella* Liverpool isolate, LJH1509, differed by ≤7 SNPs (Fig. 3). Isolate LJH1509 was separated from the other cluster of *Salmonella* Liverpool isolates by at least 29 SNP differences and was considered a unique strain. *Salmonella* serovars Agona, Senftenberg, and Worthington each grouped into single clusters. The two *Salmonella* Agona isolates differed by three SNPs (Supplemental Table 6). Within each serovar cluster, *Salmonella* Senftenberg and

Salmonella Worthington isolates differed by ≤8 and ≤5 SNPs, respectively (Fig. 3). When available, one isolate from each cluster and each year was selected for further analysis.

Antimicrobial resistance profile and CHASRI sequence identification

All pistachio survey isolates contained the ACC(6')-Iaa genotype presumptively conferring resistance to amikacin and tobramycin. In addition, the fosA7 gene (presumptive fosfomycin resistance) was identified in *Salmonella* Agona and *Salmonella* Tennessee (Table 2). The CHASRI was present in *Salmonella* Montevideo isolates belonging to Cluster A, *Salmonella* Senftenberg, *Salmonella* Tennessee and *Salmonella* Worthington (Table 2). These isolates contained 100% of the CHARSI sequence, with over 95.6% similarity to the CHARSI sequence of *Salmonella enterica* serovar Typhimurium (GenBank accession number CP019649.1).

SNP analysis among Salmonella isolates from the pistachio silo survey, pistachio-linked outbreaks, and retail survey

Salmonella Montevideo Cluster A isolates grouped with isolate 531954 recovered by the FDA from pistachios associated with the 2009 outbreak and associated recall (Bakker et al., 2011; Whitham et al., 2021) and with isolates from the 2016 outbreak (Haendiges, Davidson, Pettengill, Reed, Blessington, et al., 2021) and a 2015–2017 retail survey (Zhang et al., 2021) (Fig. 4). Isolates in this cluster covered a 7-year period and differed by ≤4 SNPs. *Salmonella* Montevideo isolate LJH1289-1 collected in 2010 from the silo survey had 0 SNP differences from a 2016 pistachio retail survey isolate (CFSAN051296) that was also associated with the 2016 outbreak (Haendiges et al., 2018). Isolate FCC0123, obtained from a pistachio facility in 2009 (Haendiges, Davidson, Pettengill, Reed, Blessington, et al., 2021), was the only NCBI-selected isolate that clustered with *Salmonella* Montevideo pistachio survey isolates in Cluster B.

All but one of the *Salmonella* Senftenberg isolates (FSW0103) grouped in a single cluster (Fig. 4). Isolates in this cluster, associated with the 2016 outbreak, the 2016 retail survey, and the 2010 and 2012 silo survey, differed by ≤10 SNPs. Isolate LJH1501, from a 2012 silo survey sample, differed by 1 SNP from an isolate associated with the 2016 outbreak (CFSANN045763). Isolate LJH1310 differed by 4 and 6 or 7 SNPs from the 2016 retail isolate CFSAN058295, and isolates associated with the 2016 outbreak, CFSAN045763 and CFSAN047866 (Supplemental Table 7). Isolate FSW0103 associated with the 2013 outbreak had over 114 SNP differences from *Salmonella* Senftenberg isolates from the silo survey or associated with other outbreaks and surveys (Fig. 4). All selected NCBI *Salmonella* Agona and *Salmonella* Worthington isolates clustered with isolates obtained from the silo survey. Isolates in each of the *Salmonella* Agona and *Salmonella* Worthington clusters differed by ≤5 SNPs (Fig. 4). Similarly, *Salmonella* Liverpool A and B isolates from the silo survey differed by ≤5 SNPs and 7 SNPs from selected NCBI *Salmonella* isolates. Both *Salmonella* Liverpool A and *Salmonella* Worthington isolates obtained from the retail survey clustered closely (≤5 SNPs) with the corresponding *Salmonella* isolates obtained from the silo survey (Fig. 4).

SNP analysis among Salmonella isolates from the pistachio and almond surveys

Salmonella Agona isolates from the pistachio silo survey differed by ≥45 SNPs from the isolates collected in the almond survey (Fig. 5). Selected *Salmonella* Enteritidis isolates formed three distinct clusters separated by over 450 SNPs (Fig. 5). *Salmonella* Enteritidis PT 9c and PT 30 isolates clustered based on phage type. *Salmonella* Enteritidis isolates PT 37 and RDNC from the pistachio silo survey (Cluster A) clustered with *Salmonella* Enteritidis PT 8 from the almond survey. No *Salmonella* isolates from the pistachio silo survey clustered with *Salmonella* Enteritidis PT 30 collected from almond samples. SNP analysis among all the selected almond

and pistachio silo survey isolates of *Salmonella* Enteritidis indicated that PT 9c isolate LJH1028 (almonds, 2005) differed by 5 and 13 SNPs from pistachio survey isolates LJH1275 (2010) and LJH1349 (2011) (Cluster B; Supplemental Table 8). The same results were obtained when the CFSAN SNP Pipeline was run using only *Salmonella* Enteritidis PT 9c isolates. Isolate LJH1028 (almonds, 2005) differed from LJH1272 (almonds, 2010) by 10 SNPs (Moyne et al., 2023) and these isolates clustered with LJH1024, a 2004 almond outbreak strain by 10 and 13 SNPs, respectively. The initial analysis including all selected *Salmonella* Enteritidis isolates from the almond and pistachio surveys indicated that *Salmonella* Enteritidis PT 8 isolate LJH1046 (almonds, 2005) differed by ≤3 SNP differences from the Cluster A pistachio silo survey isolates LJH1351 (2011), LJH1438 (2012) and LJH1297 (2010) (Fig. 5). The same results were obtained when the CFSAN SNP Pipeline was run using only isolates in this cluster.

Initial CFSAN SNP Pipeline analysis among all selected *Salmonella* Montevideo isolates indicated that three different clusters were formed, separated by more than 250 SNPs (Fig. 5). Two of these clusters contained isolates collected from both the pistachio and almond surveys. *Salmonella* isolates belonging to the pistachio silo survey Cluster B (Fig. 5) differed by at least 25 SNPs from almond survey isolates. The initial CFSAN analysis indicated that isolate LJH0653 (almonds, 2001) differed by ≤4 SNPs from pistachio isolates belonging to pistachio silo survey Cluster A (Supplemental Table 9). A secondary CFSAN SNP Pipeline analysis was done to compare isolate LJH0653 and the pistachio silo survey isolates that clustered with it (LJH1289-1, 2010; LJH1347-1, 2011; LJH1453, 2012). *Salmonella* Montevideo LJH0653 from the almond survey differed by at least 15 SNPs from the Cluster A pistachio silo survey isolates (Fig. 5).

Selected *Salmonella* Senftenberg isolates from the almond and pistachio surveys formed five different clusters that differed by ≥50 SNPs (Fig. 5). *Salmonella* isolates from the pistachio silo survey clustered with isolate LJH0713 (almonds, 2002). This was the only almond survey isolate in this cluster (Moyne et al., 2023). Initial CFSAN SNP Pipeline analysis indicated that LJH0713 isolate differed by ≤5 SNPs from pistachio isolates LJH1310 (2010) and LJH1501 (2012). The same results were obtained when the CFSAN SNP Pipeline was run using only isolates in this cluster. A single *Salmonella* Liverpool (LJH0783), isolated from almonds differed by ≥46 and 34 SNPs from isolates belonging to Liverpool Clusters A and B, respectively (Supplemental Table 10). Likewise, single *Salmonella* Tennessee and Worthington isolates obtained from almonds differed by ≥115 and ≥63 SNPs, respectively, from pistachio isolates (Supplemental Tables 11 and 12).

Discussion

Several multi-year surveys for *Salmonella* in tree nuts and peanuts have been conducted at different points of the supply chain, from preharvest to retail (Brar et al., 2016; Danyluk et al., 2007; Davidson et al., 2015; Harris et al., 2016; Miksch et al., 2013; Zhang et al., 2017, 2021). In surveys of almond kernels, inshell hazelnuts, pecans, peanuts, and walnuts, samples were collected after postharvest handling but before entry into processing facilities. A wide range of *Salmonella* serovars were isolated both within and between collection years, which is consistent with general environmental contamination where significant serovar diversity would be expected (Gorski et al., 2011, 2022; Rodriguez et al., 2006).

In contrast, limited *Salmonella enterica* strains have been isolated from California pistachios at different points of the production and supply chain, including postharvest processing environments, storage silos, and at retail. In the present study, WGS analysis of 169

Salmonella enterica isolates from pistachios collected from storage silos from 2010 to 2012 yielded eight serovars (Agona, Enteritidis, Sandiego, Senftenberg, Montevideo, Liverpool, Tennessee, and Worthington) and 11 strains, expanding upon nine PFGE fingerprints described in a previous analysis (Harris et al., 2016).

These same strains have been associated with retail surveys, outbreaks, and recalls for over a decade. In 2009, *Salmonella* Montevideo, Newport, and Senftenberg were isolated from finished product associated with a multistate (21 states) salmonellosis outbreak linked to pistachios (Centers for Disease Control and Prevention, 2009; Whitham et al., 2021). *Salmonella* Senftenberg was isolated from a California pistachio processing facility in a 2013 outbreak investigation (U.S. Food and Drug Administration, 2014a). In 2016, *Salmonella* Montevideo and *Salmonella* Senftenberg were linked to a nine-state salmonellosis outbreak associated with roasted inshell and shelled pistachios (Centers for Disease Control and Prevention, 2016). In response to the 2016 outbreak, FDA inspected the California processor implicated in the outbreak and isolated *Salmonella* Senftenberg and *Salmonella* Liverpool from raw in-shell pistachios collected from silos (U.S. Food and Drug Administration, 2016). *Salmonella* serovars Liverpool, Montevideo, Senftenberg, and Worthington were also isolated from raw shelled pistachios collected at retail outlets along with *Salmonella* serovars Duisburg and Mbandaka, (Zhang et al., 2021).

It was previously proposed that California pistachios may be contaminated with persistent or resident *Salmonella* strains (Bakker et al., 2011; Haendiges, Davidson, Pettengill, Reed, Blessington, et al., 2021; Harris et al., 2016). The term “persistence” has been used to describe long-term survival of bacterial foodborne pathogens in food (Patel et al., 2013; Shi et al., 2007), on a simple and defined matrix (e.g., stainless steel, water, or soil) (De Cesare et al.,

2003; Islam et al., 2004), or in complex environments (e.g., processing plant) (Estrada et al., 2020; Holch et al., 2013). For this discussion, strains that were isolated from two or more sampling events (silo survey, retail survey, FDA investigations, or routine samplings) and isolated in three or more years were considered persistent strains. By this definition, *Salmonella* Agona, Montevideo Clusters A and B, Senftenberg, Liverpool Cluster A, and Worthington strains are persistent strains. Although *Salmonella* Liverpool Cluster B was isolated from the silo survey in 2011 and from a pistachio sample in 2014, it did not meet the definition of persistent strain. This strain, along with *Salmonella* Enteritidis Clusters A and B, Sandiego, Duisburg, Mbandaka, Tennessee, and Newport (Harris et al., 2016; Zhang et al., 2021) were considered sporadic pistachio strains.

Bakker et al. (2011) identified multiple clinical isolates of *Salmonella* Montevideo collected in 2007, 2008, and 2009 that clustered exclusively with a *Salmonella* isolate obtained from a 2009 pistachio sample; this cluster of isolates was referred to as the "pistachio clade" (Bakker et al., 2011). The "pistachio clade" included *Salmonella* isolate 531954, which was associated with the 2009 recall and outbreak (Bakker et al., 2011; Whitham et al., 2021). In the present SNP analysis, pistachio silo isolates from *Salmonella* Montevideo Cluster A clustered with the isolate associated with the 2009 (531954) and the 2016 (CFSAN051296 and CFSAN045764) outbreaks and isolates from each of the years of the silo survey (2010–2012), indicating that the *Salmonella* Montevideo Cluster A persisted in the pistachio environment for at least 10 years (2007 to 2016). *Salmonella* Montevideo Cluster B silo survey isolates from 2010, 2011, and 2012 clustered with isolate FCC0123 collected in 2009 from a pistachio facility. Isolate FCC0123 clustered (<15 SNPs) with isolates recovered in 2014 and 2017 from two different pistachio facilities (Haendiges, Davidson, Pettengill, Reed, Blessington, et al., 2021),

providing evidence that *Salmonella* Montevideo B has persisted in the pistachio environment for at least 9 years.

Isolation of persistent strains from pistachios recovered from storage silos suggest that these *Salmonella* were introduced prior to or at the silo. After pistachios are harvested, they are transported to hulling facilities in plastic totes or large trailers. In some cases the humidity, temperature, and transport duration may be sufficient to support significant multiplication of *Salmonella* on the inhull pistachios (Moussavi et al., 2019). Pistachio hulls are removed by physical means and then the inshell nuts are separated by density in a tank of water. Floater pistachios tend to be smaller, damaged nuts with adhering hull material whereas sinkers are fully formed, heavier, and lack adhering hulls. Sinkers and floaters are separately partially dried using forced hot air (70 to 105 °C) and then transferred to silos for further ambient air drying and storage. Further multiplication of *Salmonella* may be possible in the early stages of drying, especially on floater pistachios (Moussavi et al., 2019). However, isolates from persistent *Salmonella* strains showed limited SNP changes (≤10 SNPs) over their period of isolation suggesting that these strains multiply sporadically or are in an environment of relatively low environmental stress (Haendiges, Davidson, Pettengill, Reed, Blessington, et al., 2021).

During harvest and postharvest handling, pistachios come in to contact with multiple surfaces (e.g., harvesting equipment, plastic bins, trailers, conveyor belts). Contaminated harvesting equipment, float tanks, and other food contact surfaces have been identified as potential sources of pathogen cross contamination and harborage (Beuchat and Ryu, 1997; Centers for Disease Control and Prevention, 1998; Estrada et al., 2020; Kusumaningrum et al., 2003; Meyer and Vaughn, 1969; Veluz et al., 2012). *Salmonella* can survive on contaminated inshell pistachios for long periods of time (Haendigens et al., 2021; Kimber et al., 2012). At 24

°C storage, *Salmonella* levels declined on inshell pistachios by an average of 0.15 log per month; at storage temperatures of −19 and 4 °C no significant reduction was observed over a year of storage. Isolation of *Salmonella* from both raw and roasted pistachios at retail (Zhang et al., 2021; U.S. Food and Drug Administration, 2020) suggests that both pre- and post-roasting contamination can occur.

There are other examples of long-term persistence of *Salmonella* in food processing environments. In 1998, consumption of oat cereal contaminated with *Salmonella* Agona caused a multistate salmonellosis outbreak (Centers for Disease Control and Prevention, 1998). In 2008, the same cereal-processing facility was linked to a second *Salmonella* Agona outbreak with the same PFGE pattern (Russo et al., 2013), suggesting this strain persisted in the processing environment for 10 years. The persistence of *Salmonella* in the tree nut production environment has been well documented (Arthurson et al., 2011; Gorski et al., 2011; Uesugi et al., 2007). *Salmonella* Enteritidis PT 30, associated with a 2001 salmonellosis outbreak linked to raw California almonds, was isolated from an outbreak-associated orchard over 5 years (Moyne et al., 2023; Uesugi et al., 2007) and multiple times from survey almonds from 2001 through 2013. Although there are no reports of *Salmonella* isolation directly from pistachio orchards, these data demonstrate the potential for long-term environmental persistence of *Salmonella* in tree nut orchards.

Haendiges et al. (2021) suggested that persistence of *Salmonella* in pistachio production environments (orchards) may be related to copper resistance conferred by the CHASRI sequence, a gene island that emerged in response to copper in the environment (Staehlin et al., 2016). Most California pistachio orchards are located in the San Joaquin Valley (SJV) where deficiencies of micronutrients such as boron, copper, and zinc are common (Beede, 2017). The

foliar application of this micronutrient as Cu-EDTA and $CuSO_4$ is recommended and sometimes used for pistachio trees (Beede et al., 2005). In the present study, three of six persistent strains (*Salmonella* Montevideo Cluster A, *Salmonella* Senftenberg, and *Salmonella* Worthington) contained the CHASRI sequence. These strains were isolated in the silo survey more frequently than strains without the CHASRI (*Salmonella* Agona, Enteritidis Clusters A and B, Tennessee, and Sandiego) (Harris et al., 2016). However, persistent *Salmonella* Liverpool Cluster A, a strain found more frequently than *Salmonella* Senftenberg in the silo survey, was CHASRI negative, whereas sporadic strain *Salmonella* Tennessee, isolated on a single occasion, was CHASRI positive.

Individual pistachio samples yielded from one to five strains that were often a mixture of CHASRI positive and negative strains of *Salmonella* (Harris et al., 2016; Supplemental Table 12). For example, CHASRI-positive *Salmonella* Worthington was isolated from the same sample as CHASRI-negative *Salmonella* Liverpool Clusters A and B and *Salmonella* Montevideo Cluster B. Strains of *Salmonella* Montevideo Cluster A (CHASRI-positive) and Cluster B (CHASRI-negative) were often isolated from the same sample. These results suggest that the presence of this gene island does not fully drive the isolation frequency and persistence of *Salmonella* in the pistachio production environment. Further phenotypical tests of *Salmonella* strains exposed to the copper source and concentrations used in orchards simulating the growing and harvesting environment are needed to determine the impact of CHARSI on selection for specific *Salmonella* strains.

In California, the top pistachio-producing counties—Kern, Fresno, Tulare, Madera, and Kings—also produced over 100 million pounds of almonds in 2021–2022 (Administrative Committee for Pistachios, 2023; Almond Board of California, 2021). In these counties, almond

and pistachio orchards may be adjacent or separated by short distances. *Salmonella* serovars Agona, Liverpool, Montevideo, and Worthington have been isolated from both almonds and pistachios. Isolates from *Salmonella* Enteritidis Cluster A and B and *Salmonella* Senftenberg were isolated from both almond and pistachio samples. However, *Salmonella* Agona and *Salmonella* Montevideo isolates from almonds differed by >14 SNPs from pistachio isolates suggesting that persistent *Salmonella* Agona and *Salmonella* Montevideo strains found in pistachios have not been found in almonds. These results highlight the discriminatory power of whole genome sequencing to differentiate among closely related strains.

The present work provides additional evidence for a unique pathogen (*Salmonella*)-product (pistachio) association. Isolates that cluster in the NCBI Pathogen Database with silo survey isolates *Salmonella* Montevideo strain A (≤16 SNPs) and B (≤8 SNPs) and *Salmonella* Senftenberg (≤15 SNPs) have exclusively been found in pistachios and pistachio environments (Bakker et al., 2011; Haendiges, Davidson, Pettengill, Reed, Blessington, et al., 2021). Similarly, all neighboring (≤15 SNPs) food isolates (as of March 2023) for *Salmonella* Agona, Worthington, and Liverpool isolates that cluster with isolates from the silo survey, were exclusively associated with pistachios (Supplemental Table 13).

The current study demonstrates that, unlike California almonds, *Salmonella* isolated from California pistachios has been consistently limited to a small number of strains since at least 2008. Strains of *Salmonella* Agona, *Salmonella* Montevideo, *Salmonella* Senftenberg, *Salmonella* Liverpool and *Salmonella* Worthington have persisted in the California pistachio production chain for at least 10 years and some of these have been exclusively associated with pistachios. The contamination sources, and reasons persistence, and association of these strains to California pistachios remains unknown. Further research investigating the genotypic and

phenotypic differences of sporadic and persistent *Salmonella* strains may provide insight to identify the mechanisms of persistence and environmental condition rendering strains the ability to persist in the pistachio environment.

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Tables and Figures

Table 1. *Salmonella enterica* isolates (n = 169) from a pistachio silo survey (Harris et al., 2016) selected for WGS analysis

Serotyping method			
Agglutination	**SeqSero2**	**Collection year**	**Isolate ID**
Agona	Agona	2010	LJH1308
	Agona	2012	LJH1429
Enteritidis	Enteritidis	2010	LJH1274-1[c], LJH1275-1[d], LJH1297[e]
	Enteritidis	2011	LJH1349-1[d], LJH1351-1[c]
	Enteritidis	2012	LJH1438-1[c]
Liverpool	Liverpool	2011	LJH1350-1
	Liverpool	2012	LJH1466-1, LJH1467-1, LJH1473, LJH1475, LJH1483, LJH1489, LJH1496, LJH1500, LJH1509, LJH1510, LJH1515, LJH1523, LJH1553, LJH1559, LJH1560, LJH1566, LJH1587, LJH1595, LJH1598, LJH1601, LJH1603, LJH1609.
	NA[a]	2012	LJH1547, LJH1567, LJH1576
Montevideo	Montevideo	2010	LJH1279-2, LJH1281-1, LJH1287-2, LJH1289-1, LJH1289-4, LJH1295-1, LJH1299, LJH1301, LJH1309.
	Montevideo	2011	LJH1347-1, LJH1348-1, LJH1352-1, LJH1358, LJH1359, LJH1362, LJH1363, LJH1364, LJH1373, LJH1384, LJH1392, LJH1393, LJH1397 LJH1405, LJH1407, LJH1410, LJH1411, LJH1417, LJH1418, LJH1419
	Montevideo	2012	LJH1428-1, LJH1430, LJH1432-2, LJH1433, LJH1434, LJH1435, LJH1439-1, LJH1440, LJH1442, LJH1446, LJH1450, LJH1452, LJH1455, LJH1456, LJH1457, LJH1464-1, LJH1468-1, LJH1478, LJH1480, LJH1486, LJH1494, LJH1498, LJH1504, LJH1511, LJH1516, LJH1522, LJH1525, LJH1530, LJH1537-1, LJH1548, LJH1572, LJH1577, LJH1583, LJH1588, LJH1590, LJH1602, LJH1606
	NA[a]	2010	LJH1291, LJH1296-1, LJH1303
	NA[a]	2011	LJH1383, LJH1398
	NA[a]	2012	LJH1443, LJH1448, LJH1453, LJH1471, LJH1491, LJH1534, LJH1585
Tennessee	Tennessee	2012	LJH1280-1
Senftenberg	Sandiego	2010	LJH1292-1[b]
Senftenberg	Senftenberg	2010	LJH1293-1, LJH1306, LJH1310
Senftenberg	Senftenberg	2012	LJH1437-1, LJH1447, LJH1470, LJH1485, LJH1501, LJH1538-1, LJH1539-1, LJH1554, LJH1561, LJH1564, LJH1579, LJH1610[b],
Senftenberg	NA[a]	2012	LJH1505
Worthington	Worthington	2010	LJH1288, LJH1290, LJH1298, LJH1302, LJH1304, LJH1305-1
Worthington	Worthington	2011	LJH1406
Worthington	Worthington	2012	LJH1462-1, LJH1463-1, LJH1465-1, LJH1469, LJH1477, LJH1481, LJH1490, LJH1497, LJH1499, LJH1503, LJH1507, LJH1508, LJH1512, LJH1514, LJH1517, LJH1520, LJH1529, LJH1535, LJH1562, LJH1565, LJH1578, LJH1580, LJH1584, LJH1589, LJH1594, LJH1596, LJH1597, LJH1608.
Worthington	NA[a]	2012	LJH1519, LJH1552, LJH1569, LJH1586

[a] NA, not available; predicted antigenic profile was not found in SeqSero2 v1.1.0 database.
[b] Serotype prediction was confirmed by identifying highly similar isolates in the NCBI database using the NCBI BLASTn tool.
[c] *Salmonella* Enteritidis phage type (PT) RDNC.
[d] *Salmonella* Enteritidis PT 9c.
[e] *Salmonella* Enteritidis PT 37.

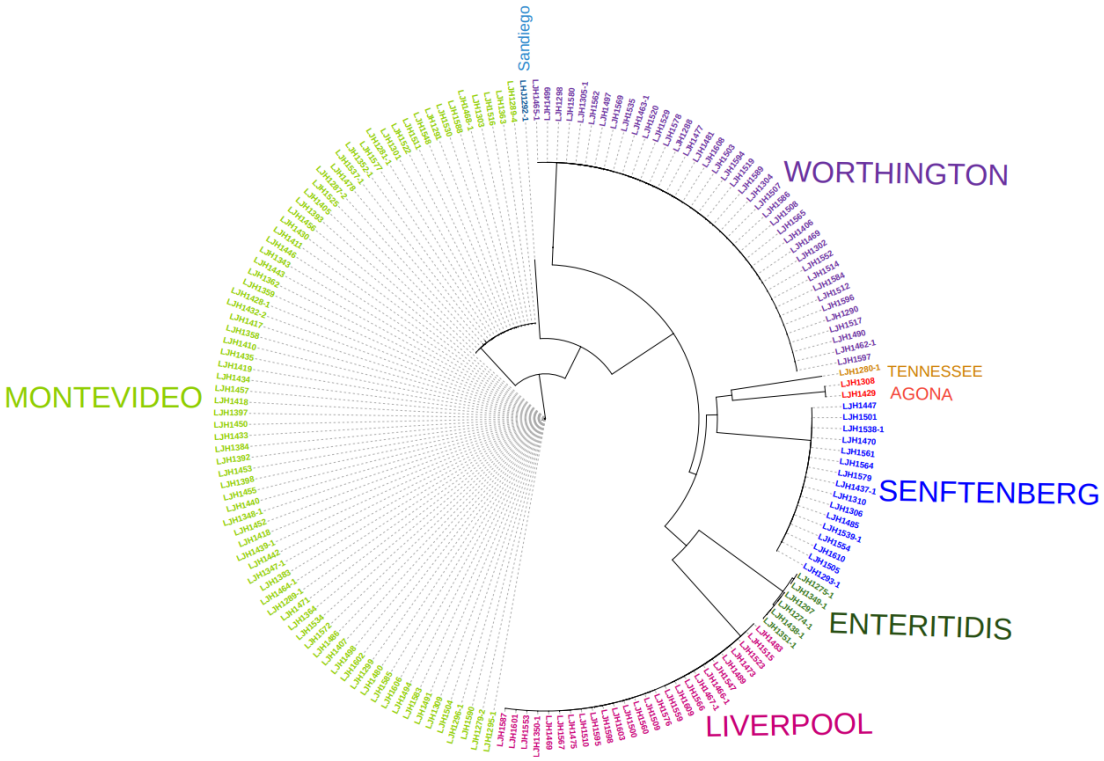

Figure 1. Phylogenetic tree of *Salmonella* isolates (*n* = 169) inferred by Parnsp using all SNP, indel, and structural variation within core genomes. *Salmonella* enterica serovar Senftenberg, assembly ID ASM295317v1, was used as the reference genome.

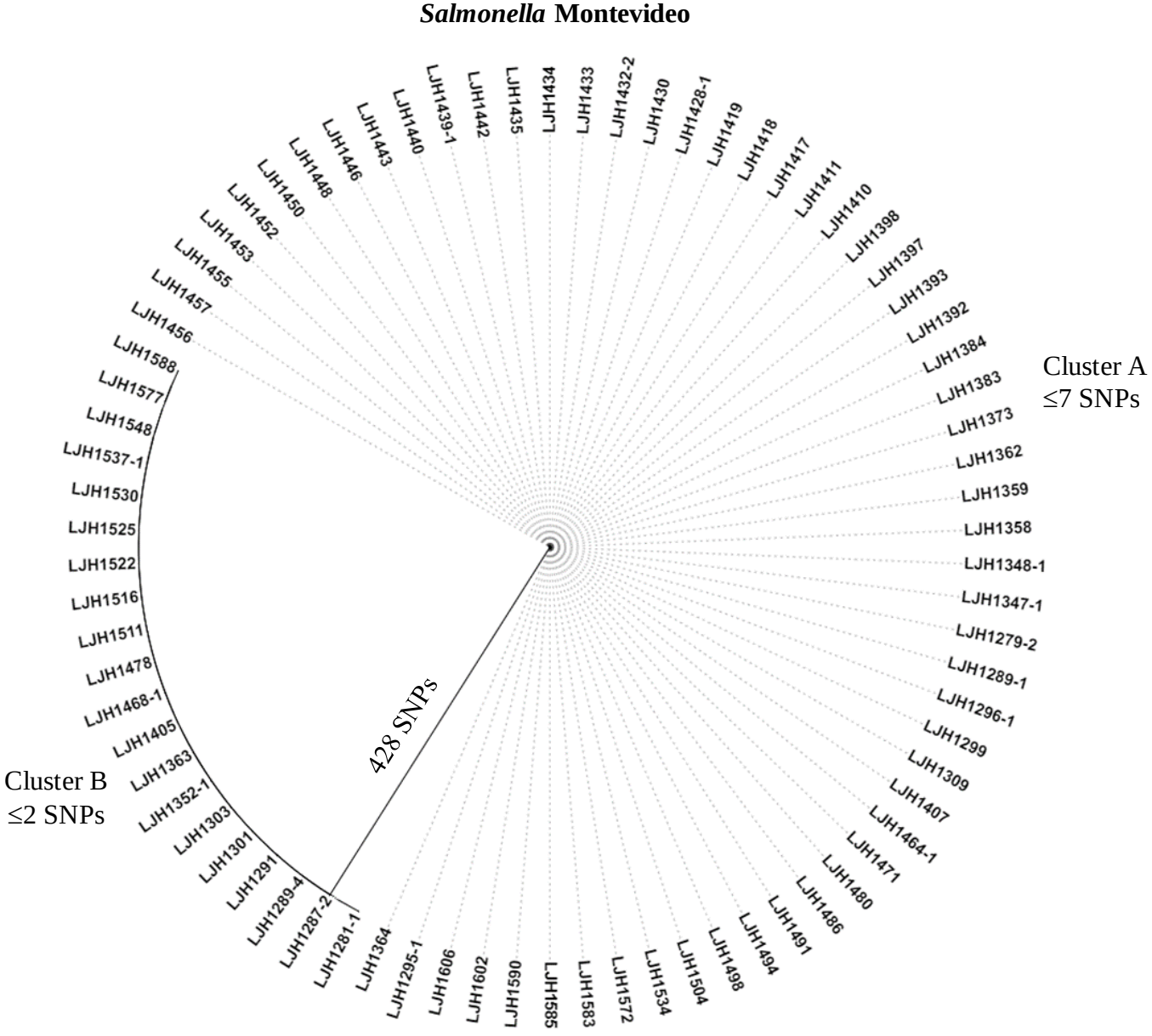

Figure 2. Phylogenetic tree based on the SNPs found using the CFSAN Pipeline among selected *Salmonella* Montevideo isolates from the pistachio silo survey (Harris et al., 2016

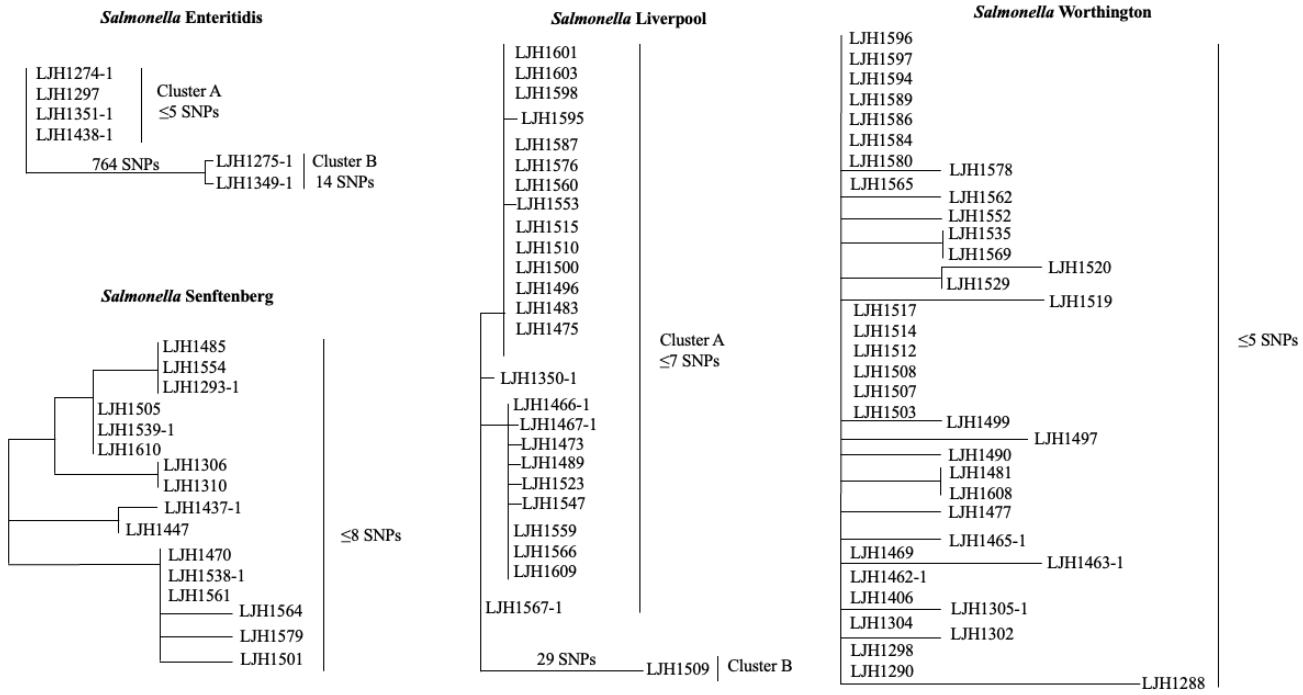

Figure 3. Phylogenetic trees based on the SNPs found using the CFSAN pipeline among selected *Salmonella* isolates from the pistachio silo survey (Harris et al., 2016).

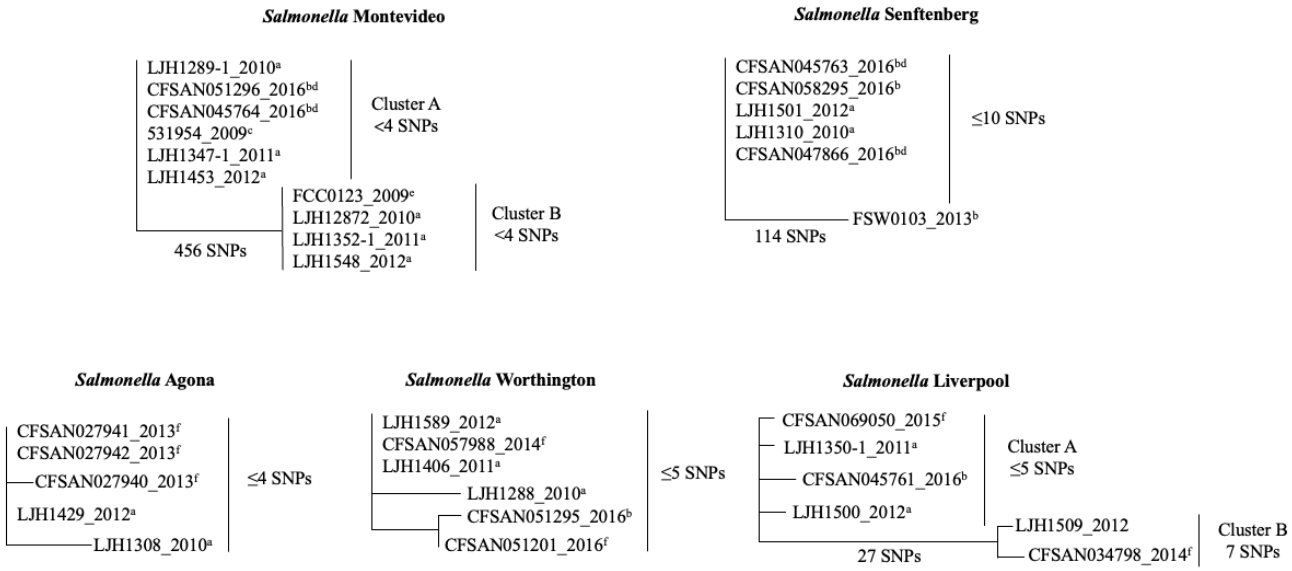

Figure 4. Phylogenetic trees based on SNPs found using the SNP CFSAN Pipeline among selected *Salmonella* isolates from the pistachio silo survey and NCBI database. Isolate label format represents "isolate ID_collection year". [a] Isolates obtained from pistachios during the silo survey (Harris et al., 2016). [b] Isolates obtained from pistachios collected during a retail survey of raw tree nuts (Zhang et al., 2021). [c] Isolate associated with a 2009 outbreak linked to pistachios (Whitham et al. 2021; Bakker et al., 2011).[d] Isolates associated with 2016 outbreaks linked to pistachios (Haendiges et al., 2018). [e] Isolate from the sequence type (ST) 138 (Haendiges et al., 2021). [f] Isolates identified from NCBI using the query: "Salmonella" AND "serotype (Agona, Enteritidis, Liverpool or Worthington)" AND "pistachio".

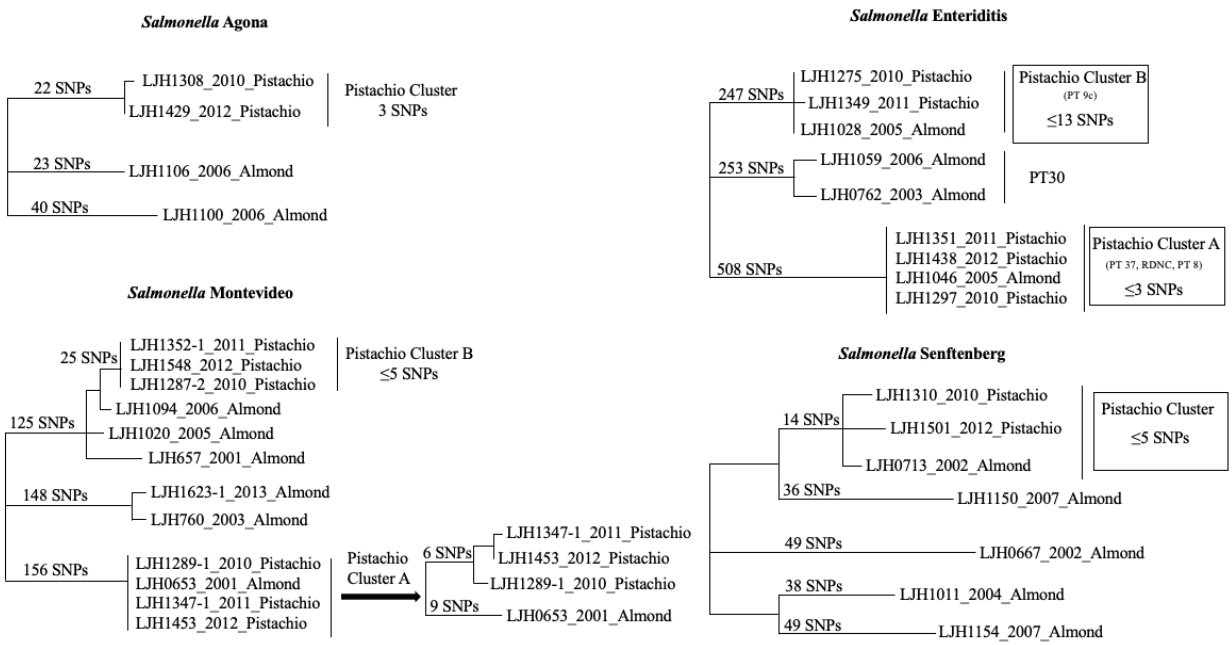

Figure 5. Phylogenetic trees based on SNPs found using the SNP CFSAN Pipeline among selected *Salmonella* isolates from the pistachio silo survey and selected *Salmonella* isolates obtained from almond survey (Moyne, et al 2023). Isolate label format represents "isolate ID_collection year_survey of origin". Squares are around clusters containing *Salmonella* isolates from almonds and pistachios.

References

Administrative Committee for Pistachios (2023). 2022 pistachio bearing acreage, production and yield per acreage. Available at: https://acpistachios.wpenginepowered.com/wp-content/uploads/2023/01/2022-Pistachio-Statistics.pdf. Accessed 5 February 2023.

Almond Board of California (2021). 2021 Almond Almanac. Available at: https://www.almonds.com/sites/default/files/2022-12/2022_Almanac.pdf. Accessed 8 February 2023.

American Pistachio Growers (2017). Growing and harvesting American pistachios. Available at: https://www.americanpistachios.org/growing-and-harvesting. Accessed 1 October 2021.

Andrews, S. (2010). FastQC: A quality control tool for high throughput sequence data. Available at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed 5 November 2022

Arthurson, V., Sessitsch, A., & Jäderlund, L. (2011). Persistence and spread of *Salmonella enterica* serovar Weltevreden in soil and on spinach plants. *FEMS Microbiology Letters*, *314*(1), 67–74. https://doi.org/10.1111/j.1574-6968.2010.02140.x

Bakker, H. C. den, Switt, A. I. M., Cummings, C. A., Hoelzer, K., Degoricija, L., Rodriguez-Rivera, L. D., Wright, E. M., Fang, R., Davis, M., Root, T., Schoonmaker-Bopp, D., Musser, K. A., Villamil, E., Waechter, H., Kornstein, L., Furtado, M. R., & Wiedmann, M. (2011). A whole-genome single nucleotide polymorphism-based approach to trace and identify outbreaks linked to a common *Salmonella enterica* subsp. *enterica* serovar Montevideo

pulsed-field gel electrophoresis type. *Applied and Environmental Microbiology*, *77*(24), 8648–8655. https://doi.org/10.1128/AEM.06538-11

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*, *19*(5), 455–477. https://doi.org/10.1089/cmb.2012.0021

Bansal, A., Jones, T. M., Abd, S. J., Danyluk, M. D., & Harris, L. J. (2010). Most-probable-number determination of *Salmonella* levels in naturally contaminated raw almonds using two sample preparation methods. *Journal of Food Protection*, *73*(11), 1986–1992. https://doi.org/10.4315/0362-028x-73.11.1986

Beede, R. H., Brown, P. H., Kallsen, C., & Weinbaum, S. A. (2005). Diagnosing and correcting nutrient deficiencies. Available at: https://ucanr.edu/sites/fruitandnut/files/73696.pdf. Accessed 1 February 2023.

Beede, R. H. (2017). Pistachio micronutrient management. Available at: https://ucanr.edu/sites/PistachioShortCourse/files/274450.pdf. Accessed 8 February 2023.

Beuchat, L. R., & Ryu, J. H. (1997). Produce handling and processing practices. *Emerging Infectious Diseases*, *3*(4), 459–465. https://doi.org/10.3201/eid0304.970407

Blessington, T., Mitcham, E. J., & Harris, L. J. (2012). Survival of *Salmonella* enterica, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on inoculated walnut kernels during

storage. *Journal of Food Protection*, *75*(2), 245–254. https://doi.org/10.4315/0362-028X.JFP-11-278

Blodgett, R. (2010). Most probable number from serial dilutions, appendix 2. *In* U.S. Food and Drug Administration bacteriological analytical manual. Available at: https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions. Accessed 5 September 2022.

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R. L., Rebelo, A. R., Florensa, A. F., Fagelhauer, L., Chakraborty, T., Neumann, B., Werner, G., Bender, J. K., Stingl, K., Nguyen, M., Coppens, J., Xavier, B. B., Aarestrup, F. M. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *The Journal of Antimicrobial Chemotherapy*, *75*(12), 3491–3500. https://doi.org/10.1093/jac/dkaa345

Brar, P. K., Strawn, L. K., & Danyluk, M. D. (2016). Prevalence, level, and types of *Salmonella* isolated from North American in-shell pecans over four harvest years. *Journal of Food Protection*, *79*(3), 352–360. https://doi.org/10.4315/0362-028X.JFP-15-365

Brown, E., Dessai, U., McGarry, S., & Gerner-Smidt, P. (2019). Use of whole-genome sequencing for food safety and public health in the United States. *Foodborne Pathogens and Disease*, *16*(7), 441–450. https://doi.org/10.1089/fpd.2019.2662

Centers for Disease Control and Prevention (1998). Multistate outbreak of *Salmonella* serotype Agona infections linked to toasted oats cereal–United States, April–May, 1998. *Morbidity and Mortality Weekly Report*, *47*(22), 462–464.

Centers for Disease Control and Prevention (2009). Multistate outbreak of *Salmonella* infections linked to pistachio nuts (final update). Available at: https://www.cdc.gov/salmonella/2009/pistachio-nuts-4-14-2009.html. Accessed 10 April 2022.

Centers for Disease Control and Prevention (2016). Multistate outbreak of *Salmonella* Montevideo and *Salmonella* Senftenberg infections linked to Wonderful Pistachios. Available at: https://www.cdc.gov/salmonella/montevideo-03-16/index.html. Accessed 10 November 2022

Danyluk, M. D., Jones, T. M., Abd, S. J., Schlitt-Dittrich, F., Jacobs, M., & Harris, L. J. (2007). Prevalence and amounts of *Salmonella* found on raw California almonds. *Journal of Food Protection*, *70*(4), 820–827. https://doi.org/10.4315/0362-028x-70.4.820

Danyluk, M. D., Nozawa-Inoue, M., Hristova, K. R., Scow, K. M., Lampinen, B., & Harris, L. J. (2008). Survival and growth of *Salmonella* Enteritidis PT 30 in almond orchard soils. *Journal of Applied Microbiology*, *104*(5), 1391–1399. https://doi.org/10.1111/j.1365-2672.2007.03662.x

Davidson, G. R., Frelka, J. C., Yang, M., Jones, T. M., & Harris, L. J. (2015). Prevalence of *Escherichia coli* O157:H7 and *Salmonella* on inshell California walnuts. *Journal of Food Protection*, *78*(8), 1547–1553. https://doi.org/10.4315/0362-028X.JFP-15-001

Davis, S., Pettengill, J. B., Luo, Y., Payne, J., Shpuntoff, A., Rand, H., & Strain, E. (2015). CFSAN SNP Pipeline: an automated method for constructing SNP matrices from next-generation sequence data. *PeerJ Computer Science*, *1*, e20. https://doi.org/10.7717/peerj-cs.20

De Cesare, A., Sheldon, B. W., Smith, K. S., & Jaykus, L.-A. (2003). Survival and persistence of *Campylobacter* and *Salmonella* species under various organic loads on food contact surfaces. *Journal of Food Protection*, *66*(9), 1587–1594. https://doi.org/10.4315/0362-028X-66.9.1587

Didelot, X., Bowden, R., Wilson, D. J., Peto, T. E. A., & Crook, D. W. (2012). Transforming clinical microbiology with bacterial genome sequencing. *Nature Reviews. Genetics*, *13*(9), 601–612. https://doi.org/10.1038/nrg3226

Estrada, E. M., Hamilton, A. M., Sullivan, G. B., Wiedmann, M., Critzer, F. J., & Strawn, L. K. (2020). Prevalence, persistence, and diversity of *Listeria monocytogenes* and *Listeria* species in produce packinghouses in three U.S. states. *Journal of Food Protection*, 277–286. https://doi.org/10.4315/0362-028X.JFP-19-411

Gorski, L., Liang, A. S., Walker, S., Carychao, D., Aviles Noriega, A., Mandrell, R. E., & Cooley, M. B. (2022). *Salmonella* enterica serovar diversity, distribution, and prevalence in public-access waters from a Central California coastal leafy green-growing region from 2011 to 2016. *Applied and Environmental Microbiology*, *88*(3), e0183421. https://doi.org/10.1128/AEM.01834-21

Gorski, L., Parker, C. T., Liang, A., Cooley, M. B., Jay-Russell, M. T., Gordus, A. G., Atwill, E. R., & Mandrell, R. E. (2011). Prevalence, distribution, and diversity of *Salmonella enterica* in a major produce region of California. *Applied and Environmental Microbiology*, *77*(8), 2734–2748. https://doi.org/10.1128/AEM.02321-10

Haendiges, J., Blessington, T., Zheng, J., Davidson, G., Miller, J. D., & Hoffmann, M. (2018). Complete genome sequences of four *Salmonella enterica* subsp. *enterica* serovar Senftenberg

and Montevideo isolates associated with a 2016 multistate outbreak in the United States. *Genome Announcements*, *6*(26). https://doi.org/10.1128/genomeA.00630-18

Haendiges, J., Davidson, G. R., Pettengill, J. B., Reed, E., Ramachandran, P., Blessington, T., Miller, J. D., Anderson, N., Myoda, S., Brown, E. W., Zheng, J., Tikekar, R., & Hoffmann, M. (2021). Genomic evidence of environmental and resident *Salmonella* Senftenberg and Montevideo contamination in the pistachio supply-chain. *Plos One*, *16*(11), e0259471. https://doi.org/10.1371/journal.pone.0259471

Harris, L. J, Lieberman, V., Mashiana, R. P., Atwill, E., Yang, M., Chandler, J. C., Bisha, B., & Jones, T. (2016). Prevalence and amounts of *Salmonella* found on raw California inshell pistachios. *Journal of Food Protection*, *79*(8), 1304–1315. https://doi.org/10.4315/0362-028X.JFP-16-054

Harris, L.J., & Ferguson, L. (2013). Improving the safety of almonds and pistachios (Chapter 15). In L. J. Harris (ed.), *Improving the safety and quality of nuts* (pp. 350–378). Woodhead Publishing Ltd., Cambridge.

Harris, L J, Yada, S., Beuchat, L. R., & Danyluk, M. D. (2022). Outbreaks of foodborne illness associated with the consumption of tree nuts, peanuts, and sesame seeds (version 2) [Table and references]. *In* Outbreaks from tree nuts, peanuts, and sesame seeds. Available at: https://ucfoodsafety.ucdavis.edu/low-moisture-foods/nuts-and-nut-pastes. Accessed 10 March 2022.

Holch, A., Webb, K., Lukjancenko, O., Ussery, D., Rosenthal, B. M., & Gram, L. (2013). Genome sequencing identifies two nearly unchanged strains of persistent *Listeria*

monocytogenes isolated at two different fish processing plants sampled 6 years apart. *Applied and Environmental Microbiology, 79*(9), 2944–2951. https://doi.org/10.1128/AEM.03715-12

International Nut and Dried Fruit Council (2021). Statistical yearbooks. Materials for the nut and dried fruit industry. *Statistical yearbook 2020/2021*. Available at: https://www.nutfruit.org/industry/technical-resources?category=statistical-yearbooks. Accessed 16 February 2023.

Islam, M., Doyle, M. P., Phatak, S. C., Millner, P., & Jiang, X. (2004). Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *Journal of Food Protection, 67*(7), 1365–1370. https://doi.org/10.4315/0362-028x-67.7.1365

Kimber, M. A., Kaur, H., Wang, L., Danyluk, M. D., & Harris, L. J. (2012). Survival of *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on inoculated almonds and pistachios stored at -19, 4, and 24° C. *Journal of Food Protection*, *75*(8), 1394–1403. https://doi.org/10.4315/0362-028X.JFP-12-023

Kozyreva, V. K., Crandall, J., Sabol, A., Poe, A., Zhang, P., Concepción-Acevedo, J., Schroeder, M. N., Wagner, D., Higa, J., Trees, E., & Chaturvedi, V. (2016). Laboratory investigation of *Salmonella enterica* serovar Poona outbreak in California: comparison of pulsed-field gel electrophoresis (PFGE) and whole genome sequencing (WGS) results. *PLoS Currents. Influenza, 8*. https://doi.org/10.1371/currents.outbreaks.1bb3e36e74bd5779bc43ac3a8dae52e6

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C., & Beumer, R. R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology*, *85*(3), 227–236. https://doi.org/10.1016/S0168-1605(02)00540-8

Lambertini, E., Danyluk, M. D., Schaffner, D. W., Winter, C. K., & Harris, L. J. (2012). Risk of salmonellosis from consumption of almonds in the North American market. *Food Research International*, *45*(2), 1166–1174. https://doi.org/10.1016/j.foodres.2011.05.039

Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Research*, *49*(W1), W293–W296. https://doi.org/10.1093/nar/gkab301

Limcharoenchat, P., James, M. K., & Marks, B. P. (2019). Survival and thermal resistance of *Salmonella* Enteritidis PT 30 on almonds after long-term storage. *Journal of Food Protection*, *82*(2), 194–199. https://doi.org/10.4315/0362-028X.JFP-18-152

Meyer, M. T., & Vaughn, R. H. (1969). Incidence of *Escherichia coli* in black walnut meats. *Applied Microbiology*, *18*(5), 925–931. https://doi.org/10.1128/am.18.5.925-931.1969

Miksch, R. R., Leek, J., Myoda, S., Nguyen, T., Tenney, K., Svidenko, V., Greeson, K., & Samadpour, M. (2013). Prevalence and counts of *Salmonella* and enterohemorrhagic

Escherichia coli in raw, shelled runner peanuts. *Journal of Food Protection, 76*(10), 1668–1675. https://doi.org/10.4315/0362-028X.JFP-13-047

Moussavi, M., Lieberman, V., Theofel, C., Barouei, J., & Harris, L. J. (2019). Growth of *Salmonella* on inoculated inhull pistachios during postharvest handling. *Journal of Food Protection, 82*(2), 217–225. https://doi.org/10.4315/0362-028X.JFP-18-351

Moyne, A. M., Lawal, O., Goodridge, L., & Harris, L. J. (2023). Genetic diversity of *Salmonella enterica* isolated from raw almonds and an almond orchard. *Submitted to PLOSOne 2023-05-12.*

Patel, J., Singh, M., Macarisin, D., Sharma, M., & Shelton, D. (2013). Differences in biofilm formation of produce and poultry *Salmonella* enterica isolates and their persistence on spinach plants. *Food Microbiology, 36*(2), 388–394. https://doi.org/10.1016/j.fm.2013.06.019

Podolak, R., Enache, E., Stone, W., Black, D. G., & Elliott, P. H. (2010). Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. *Journal of Food Protection, 73*(10), 1919–1936. https://doi.org/10.4315/0362-028X-73.10.1919

Rantsiou, K., Kathariou, S., Winkler, A., Skandamis, P., Saint-Cyr, M. J., Rouzeau-Szynalski, K., & Amézquita, A. (2018). Next generation microbiological risk assessment: opportunities of whole genome sequencing (WGS) for foodborne pathogen surveillance, source tracking and risk assessment. *International Journal of Food Microbiology, 287*, 3–9. https://doi.org/10.1016/j.ijfoodmicro.2017.11.007

Ribot, E. M., Freeman, M., Hise, K. B., & Gerner-Smidt, P. (2019). PulseNet: entering the age of next-generation sequencing. *Foodborne Pathogens and Disease*, *16*(7), 451–456. https://doi.org/10.1089/fpd.2019.2634

Rodriguez, A., Pangloli, P., Richards, H. A., Mount, J. R., & Draughon, F. A. (2006). Prevalence of *Salmonella* in diverse environmental farm samples. *Journal of Food Protection*, *69*(11), 2576–2580. https://doi.org/10.4315/0362-028x-69.11.2576

Russo, E. T., Biggerstaff, G., Hoekstra, R. M., Meyer, S., Patel, N., Miller, B., Quick, R., & *Salmonella* Agona Outbreak Investigation Team. (2013). A recurrent, multistate outbreak of *Salmonella* serotype Agona infections associated with dry, unsweetened cereal consumption, United States, 2008. *Journal of Food Protection*, *76*(2), 227–230. https://doi.org/10.4315/0362-028X.JFP-12-209

Santillana Farakos, S., Pouillot, R., Johnson, R., Spungen, J., Son, I., Anderson, N., & Doren, J. M. V. (2017). A quantitative assessment of the risk of human salmonellosis arising from the consumption of almonds in the United States: the impact of preventive treatment levels. *Journal of Food Protection*, 863–878. https://doi.org/10.4315/0362-028X.JFP-16-403

Shi, X., Namvar, A., Kostrzynska, M., Hora, R., & Warriner, K. (2007). Persistence and growth of different *Salmonella* serovars on pre- and postharvest tomatoes. *Journal of Food Protection*, *70*(12), 2725–2731. https://doi.org/10.4315/0362-028x-70.12.2725

Staehlin, B. M., Gibbons, J. G., Rokas, A., O'Halloran, T. V., & Slot, J. C. (2016). Evolution of a heavy metal homeostasis resistance island reflects increasing copper stress in enterobacteria. *Genome Biology and Evolution*, *8*(3), 811–826. https://doi.org/10.1093/gbe/evw031

Taylor, A. J., Lappi, V., Wolfgang, W. J., Lapierre, P., Palumbo, M. J., Medus, C., & Boxrud, D. (2015). Characterization of foodborne outbreaks of *Salmonella enterica* serovar Enteritidis with whole-genome sequencing single nucleotide polymorphism-based analysis for surveillance and outbreak detection. *Journal of Clinical Microbiology*, *53*(10), 3334–3340. https://doi.org/10.1128/JCM.01280-15

Treangen, T. J., Ondov, B. D., Koren, S., & Phillippy, A. M. (2014). The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biology*, *15*(11), 524. https://doi.org/10.1186/s13059-014-0524-x

Uesugi, A. R., Danyluk, M. D., Mandrell, R. E., & Harris, L. J. (2007). Isolation of *Salmonella* Enteritidis phage type 30 from a single almond orchard over a 5-year period. *Journal of Food Protection*, *70*(8), 1784–1789. https://doi.org/10.4315/0362-028x-70.8.1784U.S. Food and Drug Administration (2014a). FDA investigation summary—Multistate outbreak of *Salmonella* Senftenberg infections associated with pistachios from a California roaster. Available at: https://wayback.archive-it.org/7993/20171114154922/https://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm386377.htm. Accessed 3 March 2023.

U.S. Food and Drug Administration (2014b). Warning Letter: ARO Pistachio, Inc. Available at: http://wayback.archive-it.org/7993/20171115095458/https://www.fda.gov/ICECI/EnforcementActions/WarningLetters/2014/ucm381450.htm. Accessed 3 March 2023.

U.S. Food and Drug Administration (2016). Paramount Farms 10/7/16 warning letter, October 7, 2016. Available at: https://wayback.archive-it.org/7993/20190424200426/https://www.fda.gov/ICECI/EnforcementActions/WarningLetters/2016/ucm524491.htm. Accessed 22 March 2022.

U.S. Food and Drug Administration (2020). Barcelona Nut Company Recalls Roasted and Salted in Shell Pistachios Because of Possible Health Risk. Available at: https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/barcelona-nut-company-recalls-roasted-and-salted-shell-pistachios-because-possible-health-risk. Accessed 15 November 2022.

Veluz, G. A., Pitchiah, S., & Alvarado, C. Z. (2012). Attachment of *Salmonella* serovars and *Listeria monocytogenes* to stainless steel and plastic conveyor belts. *Poultry Science*, *91*(8), 2004–2010. https://doi.org/10.3382/ps.2011-01689

Whitham, H. K., Sundararaman, P., Dewey-Mattia, D., Manikonda, K., Marshall, K. E., Griffin, P. M., Gleason, B. L., Subramhanya, S., & Crowe, S. J. (2021). Novel outbreak-associated food vehicles, United States. *Emerging Infectious Diseases*, *27*(10), 2554–2559. https://doi.org/10.3201/eid2710.204080

Yada, S., & Harris, L. J. (2022). Recalls of tree nuts and peanuts in the U.S., 2001 to present (version 2) [Table and references]. In U.S. recalls of nuts. Available at: https://ucfoodsafety.ucdavis.edu/low-moisture-foods/nuts-and-nut-pastes. Accessed 3 January 2023.

Zhang, G., Hu, L., Luo, Y., Santillana Farakos, S. M., Johnson, R., Scott, V. N., Curry, P., Melka, D., Brown, E. W., Strain, E., Bunning, V. K., Musser, S. M., & Hammack, T. S. (2021). Survey of *Salmonella* in raw tree nuts at retail in the United States. *Journal of Food Science*, *86*(2), 495–504. https://doi.org/10.1111/1750-3841.15569

Zhang, G., Hu, L., Melka, D., Wang, H., Laasri, A., Brown, E. W., Strain, E., Allard, M., Bunning, V. K., Musser, S. M., Johnson, R., Santillana Farakos, S., Scott, V. N., Pouillot, R., Doren, J. M. V., & Hammack, T. S. (2017). Prevalence of *Salmonella* in cashews, hazelnuts, macadamia nuts, pecans, pine nuts, and walnuts in the United States. *Journal of Food Protection*, *80*(3), 459–466. https://doi.org/10.4315/0362-028X.JFP-16-396

Zhang, S., den Bakker, H. C., Li, S., Chen, J., Dinsmore, B. A., Lane, C., Lauer, A. C., Fields, P. I., & Deng, X. (2019). SeqSero2: rapid and improved *Salmonella* serotype determination using whole-genome sequencing data. *Applied and Environmental Microbiology*, *85*(23). https://doi.org/10.1128/AEM.01746-19

Chapter II. Phenotypic characteristics that may contribute to persistence of *Salmonella* strains in the pistachio supply chain.

Abstract

From 2007 to 2018, six persistent *Salmonella* strains have been isolated from California pistachios or the pistachio supply chain multiple times (≥3 years apart, and from one or more surveys, outbreak investigations, or routine samplings). Representative isolates of these six persistent strains and three sporadic strains also isolated from pistachios collected from storage silos were selected to evaluate phenotypical characteristics that may play a role in persistence in pistachio orchards or during postharvest handling. Presence of a copper homeostasis and silver resistant island sequence in three of the persistent strains was associated with an increase in tolerance to $CuSO_4$ under anaerobic but not aerobic conditions; all isolates were resistant to ≥120 mM Cu-EDTA under both anerobic and aerobic conditions. Growth of all strains except for *Salmonella* Enteritidis strain A (sporadic) was similar in pistachio hull slurry at 30°C over 48 h; maximum populations of 8.70─8.85 CFU/mL were observed after ≥40 h of incubation. All *Salmonella* strains produced weak to strong biofilms after 4 days at 25°C, with seven strains, including two sporadic strains, producing moderate biofilms and one persistent strain (*Salmonella* Liverpool strain A) producing a strong biofilm. These findings suggest that resistance to copper, growth using pistachio hull nutrients, or biofilm forming ability do not independently account for *Salmonella* persistence in the California pistachio production chain.

Introduction

The United States is the top producer of pistachios in the world, and California-grown pistachios account for more than 99% of the country's production (American Pistachio Growers, 2021). *Salmonella* is the primary pathogen of concern for the pistachio industry as it has led to most bacteria-associated recalls and all reported outbreaks associated with U.S. pistachios. (Harris et al., 2022; Yada & Harris, 2021). Pistachios and other tree nuts are low-moisture foods that are not conducive to bacterial growth. However, *Salmonella* survives on pistachios with 1 to 3 log reductions observed over 12 months of ambient storage (Haendiges et al., 2021; Kimber et al., 2012). Between 2007–2018, a limited number of *Salmonella enterica* serovars were isolated from samples collected throughout the pistachio supply chain (Bakker et al., 2011; Haendiges et al., 2021; Harris et al., 2016; Zhang et al., 2021) and two of these serovars, *Salmonella* Montevideo and *Salmonella* Senftenberg, have been associated with three foodborne outbreaks in the United States linked to pistachios (Centers for Disease Control and Prevention, 2016; U.S. Food and Drug Administration, 2014; Whitham et al., 2021).

Haendiges et al. (2021) analyzed the genetic diversity of *Salmonella* Montevideo and *Salmonella* Senftenberg associated with pistachio outbreaks, recalls and regulatory investigations. The 95 *Salmonella* Montevideo isolates they evaluated formed two distinct multilocus sequence types (MLST). Both MLST (ST138 and ST316) had clinical, environmental, and pistachio isolates with collection dates that spanned a decade (2009–2017). The 106 *Salmonella* Senftenberg isolates also formed two MLST with clinical, environmental, and pistachio isolates and collection dates from 2009 to 2016 (ST14) or 2013 to 2018 (ST185). The authors described these isolates as persistent strains within the pistachio environment (Haendiges et al., 2021). Additional *Salmonella* Montevideo ST138 and ST316 and *Salmonella*

Senftenberg ST14 were identified by whole genome sequence analysis of isolates from a 3-year survey of raw pistachios collected from California storage silos between 2010 and 2012 (Chapter II). In addition to the *Salmonella* Montevideo (A and B) and Senftenberg strains, single strains belonging to *Salmonella* Agona, Liverpool (A), and Worthington were considered persistent, as they were isolated in ≥3 years and from more than one avenue (e.g., surveys, outbreak investigations, or routine sampling) (Chapter II). Other serovars isolated in the silo survey (*Salmonella* Enteritidis (two strains), Tennessee, Liverpool strain B (a single isolate), and Sandiego) did not meet the persistent definition and were thus considered sporadic strains. The underlying reasons that have led to the establishment of specific persistent strains of *Salmonella* associated with California pistachios remain unknown.

The copper homeostasis and silver resistance island (CHASRI) sequence was identified in pistachio strains *Salmonella* Montevideo ST316, *Salmonella* Senftenberg ST14 (Haendiges et al., 2021) and *Salmonella* Worthington (Chapter II). Haendiges et al. (2021) suggested that the CHASRI sequence might provide these *Salmonella* strains with a phenotypical advantage against copper sources used in the orchard environment. Most of California's pistachio orchards are located in five counties—Fresno, Kern, Kings, Madera, and Tulare, (Administrative Committee For Pistachios, 2022)—all located in the San Joaquin Valley where deficiencies of micronutrients, such as boron, copper and zinc, are common (Beede, 2017; Beede et al., 2005). As a result, the foliar application of copper is recommended for the production of healthy pistachio trees (Beede et al., 2005). The use of copper in agricultural production challenges the copper homeostasis mechanisms of bacteria in the environment and, as a consequence, different mechanisms have evolved to control the transport and detoxification of copper ions from bacterial cells (Staehlin et al., 2016).

In California, mature pistachio trees are mechanically shaken at harvest time, causing pistachio fruit to drop onto a catch frame that deposits pistachios into plastic bins or trailers. The pistachios are then transported to the perimeter of the orchard, where they are emptied into large (~25,000 Kg) bottom dump trailers (Moussavi et al., 2019). Trailers are driven to hulling facilities where the pistachios undergo hulling and then drying. Pistachios are dried to moisture levels between 8–15%, using forced hot air at temperatures between 70–105°C. The time between harvesting and hulling is dependent on a variety of factors, including the time of truck arrival at the orchard, loading time, the distance between the orchard and the huller, and the hold time between receiving and unloading the trailer (Harris & Ferguson, 2013). The temperature and humidity within transport trailers can vary depending on ambient temperature, sun exposure, location of the pistachios in the trailer, and time in the trailer (Moussavi et al., 2019). Significant growth of an inoculated *Salmonella* cocktail was observed on inhull pistachios during simulated postharvest transport (Moussavi et al., 2019)

Once California pistachios arrive at the huller facilities, the outer hulls are removed and the inshell nuts are sorted in a float tank based on density and buoyancy. The inshell pistachios are then dried to a moisture content of 8–15% before being transferred to storage silos (500,000 to 750,000-kg) where they are further dried, fumigated, and held for up to 18 months (Farakos et al., 2018). Adequate cleaning and sanitation practices in the postharvest environments are critical for reducing microbial levels and to prevention cross contamination (Finn et al., 2013). Such practices are limited in the pistachio hulling, drying and storage environments (dryers and silos) due to the nature of the semi-enclosed facilities and short harvesting season. In addition, cleaning and sanitation practices are challenged by the accumulation of organic matter in postharvest equipment and processing surfaces, potentially promoting the growth of bacteria and

encouraging the formation of biofilms (Srey et al., 2013). Biofilms are communities of microorganisms that are encased in a self-produced extracellular matrix, providing protection against environmental stresses (changes in pH, temperature, and nutrient availability) and antimicrobial agents (Aviles et al., 2013; Joseph et al., 2001). *Salmonella* cells present in biofilms may persist for extended periods and can serve as a reservoir for the bacteria, making them a potential source of contamination for food products (Kusumaningrum et al., 2003; Srey et al., 2013; Vestby et al., 2009). Biofilm formation differs among *Salmonella* serovars and strains (Akinola et al., 2020; Dantas et al., 2020; Jitendra Patel & Sharma, 2010; Silva et al., 2019).

The present study aims to evaluate the phenotypical responses of *Salmonella* strains associated with pistachios during simulated conditions throughout the first steps of the production of California pistachios, including growing, harvest, and postharvest handling. The study specifically investigates some of the phenotypical factors that may explain the persistence of some strains of *Salmonella* in the California pistachio production chain: impact of copper sensitivity, ability to grow in hull extracts, and biofilm-forming ability.

Materials and Methods

Salmonella strains

Eight *Salmonella* serovars were identified among a total of 169 isolates recovered from lots of raw California pistachios harvested in 2010, 2011, and 2012 (Chapter II; Harris et al., 2016); eleven *Salmonella enterica* strains were identified using SNPs matrixes generated by using the Center for Food Safety and Nutrition (CFSAN) SNP Pipeline (Chapter II). A representative isolate was selected from each *Salmonella* strain for which there were two or more isolates (n = 9): *Salmonella* Agona (LJH1308), *Salmonella* Enteritidis strain A (LJH1297),

Salmonella Enteritidis strain B (LJH1275-1), *Salmonella* Liverpool strain A (LJH1350-1), *Salmonella* Montevideo strain A (LJH1347-1), *Salmonella* Montevideo strain B (LJH1352-1), *Salmonella* Senftenberg (LJH1310), and *Salmonella* Worthington (LJH1288) (Table 1). The single isolate representing *Salmonella* Liverpool strain B (LJH1509) was also included. Six of the strains, excluding *Salmonella* Enteritidis strain A and B, and *Salmonella* Liverpool strain B, were considered persistent strains (isolated in ≥3 years and from more than one avenue [e.g., surveys, outbreak investigations, or routine sampling]) (Chapter II). *Salmonella* Typhimurium ATCC 14028 was included as a positive control for the biofilm experiment.

Growth conditions

Frozen (−80°C) *Salmonella* stock cultures were streaked onto tryptic soy agar (TSA; tryptic soy broth plus 1.5% granulated agar) and incubated at 37°C for 24 h. A single isolated colony was transfer to 3 mL of tryptic soy broth (TSB). After 24 h, a 10-µL aliquot was transferred into 10 mL of fresh TSB and incubated at 37°C for 24 h. For the biofilm experiments, TSB broth was replaced by Luria-Bertani (LB) broth (Fisher Scientific). The culture in the fresh TSB or LB broth tubes after incubation is referred as the *Salmonella* suspension. All culture media were Difco brand (BD) unless otherwise specified.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of copper was determined using the two-fold microdilution method in a 96-well plate format as previously described, with slight modifications (Sheng & Zhu, 2014). Each *Salmonella* suspension was diluted 1:10 (vol/vol) in 0.1% peptone and then diluted (1:100) in 9.9 mL of Muller Hinton broth (MHB, Sigma) to obtain a final concentration of 6 log CFU/mL (*Salmonella* inoculum).

Copper stock solutions were prepared by mixing copper (II) sulfate pentahydrate ($CuSO_4$; Sigma) or 15% copper EDTA (Cu-EDTA; Custom Hydronutrients), a chelated copper fertilizer, with sterile ultrapure water (Milli-Q Advantage A10, MilliporeSigma). Using flat bottom 96-well Nunc™ MicroWell™ polystyrene plate (Thermo Fisher Scientific), serial dilutions of the stock solutions were made by adding 100 µL of a copper solution of specific concentration (30 to 240 mM) to 100 µL MHB. Wells with copper solutions ranged from 15 to 120 mM of copper. Each *Salmonella* strain was tested at each concentration by adding 100 µL of a *Salmonell*a inoculum to each well containing 100 µL of a specific concentration of copper source ($CuSO_4$ or Cu-EDTA). Negative control wells were made by adding 100 µL of ultrapure water instead of a *Salmonella* inoculum to wells with different copper concentrations. Positive controls contained 100 µL of *Salmonella* inoculum and 100 µL of ultrapure water. The final *Salmonella* population in each well was 6.03 ± 0.13 log CFU/mL, which was confirmed by plating on TSA. Wells with 100 µL of copper dilutions and 100 µL of MHB were used as blanks. Well plates were incubated at 37°C for 24 h under aerobic or anaerobic conditions; anaerobic conditions were achieved by placing well plates in AnaeroPack™ regular jars each containing two AnaeroPack™ Anaero anaerobic gas generator sachets (Thermo Scientific) inside an incubator. After incubation, the optical density value of each well was measured at 600 nm (OD_{600}) using a Cytation 1 Imaging Reader (BioTek).

Pistachio hull slurry preparation and inoculation

Fresh in-hull pistachios were collected from a California pistachio processor in September 2022 and stored in an airtight bag (Ziploc) at 5°C for 72 h. The hulls were separated from the inshell nuts, and then mixed 1:2 (wt/vol) with 0.1% peptone and blended at high speed for 2 min in a commercial food processor (Waring). The mixture was filtered through

cheesecloth, and the filtered liquid was centrifuged at 3,220 × *g* for 5 min (Eppendorf 5018). To remove solids and native bacteria, the supernatant was sequentially filtered using 8, 3, and 1.2-µm MF-Millipore® membrane filters (MilliporeSigma) followed by a 0.8/0.2 µm Acrodisc® syringe filter (PALL). The filtered slurry was plated onto TSA to confirm the absence of native bacteria. The sterile slurry was stored in 50-mL plastic tubes at −20°C until used.

Salmonella suspensions were serially diluted (1:100) three times in sterile ultrapure water to obtain a final *Salmonella* population of 3 log CFU/mL (*Salmonella* inoculum). For each strain, 2 mL of *Salmonella* inoculum was mixed with 3 mL of sterile pistachio hull slurry in a 5-ml tube and incubated at 30°C, with shaking at 150 rpm, in a MaxQ 6000 incubator (Thermo Fisher Scientific). Additionally, 3 mL of uninoculated hull slurry was mixed into 2 mL of sterile ultrapure water and incubated at the same conditions (to serve as the negative control). *Salmonella* populations in each strain-slurry mixture were determined by plating onto TSA and CHROMagar™ Salmonella (CHROMSal; CHROMagar) at the time of the inoculation (0 h) and after 16, 20, 24, 28, 40, and 48 h of incubation.

Biofilm formation and quantification

To form biofilms, each prepared *Salmonella* suspension was mixed by vortexing, and 150 µL of the suspension or uninoculated sterile LB broth (negative control) was transferred into six wells of a flat bottom Nunc™ MicroWell™ 96-well polystyrene plate and then incubated at 25 or 37°C for 4 days. Biofilm formation was quantified as previously described (J Patel et al., 2011), with some modifications. After incubation, microplate cultures were aspirated using a HandE-Vac aspirator system (Argos Technologies) and washed three times with sterile ultrapure water to eliminate planktonic cells. The plates were air dried for 45 min and the remaining attached bacteria were fixed with 200 µL of 99% methanol for 15 min; then the plates were

emptied and left to air dry for 10 min. The fixed biofilms were stained for 45 min with 200 µL of crystal violet (0.5%) (Sigma), and then the crystal violet was removed via aspiration. The individual wells were then washed three times with sterile ultrapure water and left to air dry for 10 min. To resuspend the dye bound to the adherent cells on the well walls, 200 µL of 95% ethanol was added to each well and the contents of each well were mixed by pipette. After 5 min, the optical density value of each well was measured at 600 nm (OD_{600}) using a Cytation 1 Imaging Reader (BioTek).

Data analysis

For each MIC experiment, two wells with a specific concentration of $CuSO_4$ or Cu-EDTA with and without a single *Salmonella* strain were evaluated. The experiment was repeated three times (three biological replicates) to obtain a total of six OD_{600} measurements of uninoculated control samples and inoculated samples per copper concentration. From these data, the means and standard deviation were determined. MIC was defined as the lowest concentration of copper at which no bacterial growth was observed after 24h incubation; mathematically, MIC has been defined as the lowest concentration of copper with an average well OD_{600} within three standard deviations of the blank (Daly et al., 2017).

For the growth in pistachio hull slurry experiment, at each time point, one uninoculated (negative control) and one inoculated slurry per strain were evaluated. The experiment was repeated three times (three biological replicates) to obtain a total of three analytical units for the uninoculated control samples and inoculated samples per time point. From these data, the means and standard deviation were determined. Average population levels were log transformed and analysis of variance (ANOVA) followed by Tukey-Kramer tests were performed; differences between mean values were considered significant at $P \leq 0.05$. Differences between media types

were analyzed using a matched pairs test. All data were analyzed with JMP 16.0 software (SAS Institute Inc., Cary, NC).

For each biofilm experiment, two plates (technical replicates) were analyzed. Each plate contained six uninoculated (negative control) wells, and six inoculated wells per strain. The experiment was repeated three times (biological replicates) to obtain a total of 24 OD_{600} measurements for uninoculated control samples and inoculated samples. From these data, the means and standard deviation were determined. The biofilm formation potential of *Salmonella* isolates was determined using the following scale (where ODc = OD_{600} average of negative control, ODs = OD_{600} average of the sample): ODs < ODc = no biofilm formation; ODc < ODs < 2ODc = weak biofilm formation; 2ODc < ODs < 4ODc = moderate biofilm formation; and 4ODc < ODs = strong biofilm formation (Papa et al., 2018).

Results

Minimum inhibitory concentration (MIC)

The MIC for Cu-EDTA for all *Salmonella* strains was ≥120 mM under both aerobic and anaerobic conditions (Table 2). The MIC for $CuSO_4$ was 15 mM for all strains under aerobic conditions, and 15 mM and 7.5 mM for strains with and without CHASRI, respectively, under anaerobic conditions.

Growth in pistachio hull slurry

Salmonella populations on TSA were consistently but not always significantly ($P > 0.05$) higher than counts on CHROMSal (Table 3; statistics not shown). On average, TSA counts were 0.11 log CFU/mL higher than counts on CHROMSal for each strain at a specific incubation time (Table 3). For this reason, only TSA are described in the text. Samples of sterile pistachio hull

slurry were inoculated with individual *Salmonella* strains to achieve levels of 2.7–2.8 log CFU/mL (Table 3). After 16 h of incubation, *Salmonella* populations of all nine strains increased significantly ($P < 0.05$) by 2.2–4.3 log. However, at this sampling time, *Salmonella* Enteritidis strain A and *Salmonella* Montevideo strain A populations were significantly lower than the populations of other *Salmonella* strains with populations of 4.9 and 6.2 log CFU/mL, respectively (Table 3). Populations of all *Salmonella* strains continued to significantly ($P < 0.05$) increase after 20 and 24 h of incubation, with the exception of *Salmonella* Enteritidis A. Except for *Salmonella* Enteritidis strain A, populations increased to 7.60–8.07, 8.33–8.71 and 8.54–8.80 log CFU/mL after 20, 24 and 28 h of incubation, respectively (Table 3). Overall, the populations of *Salmonella* Enteritidis strain A were significantly ($P < 0.05$) lower at 16, 20, 24, and 28 h compared to other strains (Table 3). At 40 and 48 h, populations for all *Salmonella* strains were not significantly ($P < 0.05$) different from each other with levels between 8.48–8.75 log CFU/mL.

Biofilm formation

Biofilms were not formed by any *Salmonella* strain after 4 days of incubation at 37°C. After 4 days of incubation at 25°C, an average OD_{600} measurement of 0.085 was obtained for the negative control. The OD_{600} measurements for the biofilms of seven of the nine *Salmonella* strains were between two and four times higher than those for the negative control, classifying these strains as moderate biofilm formers (Table 4). *Salmonella* Enteritidis A was classified as a weak biofilm former as the average OD_{600} measurement was <2 times the OD_{600C}. *Salmonella* Liverpool A produced strong biofilms, with an average OD_{600} of 0.341. *Salmonella* Typhimurium, the positive control, formed moderate biofilms (Table 4).

Discussion

Salmonella has emerged as a significant foodborne pathogen of concern for California tree nuts, with recalls and reported outbreaks predominantly attributed to contamination by this pathogen (Harris et al., 2022; Yada & Harris, 2021). Despite similarities in the cultivation for almonds and pistachios, including geographic region and season, the contamination profile of *Salmonella* in these two commodities is very different. Thirty-two different *Salmonella* serovars were isolated from raw California almonds from more than 14,000 lots collected from nine harvests over 13 years (2001─2013) (Bansal et al., 2010; Danyluk et al., 2007; Moyne et al., 2023). In contrast, 11 serovars were isolated from pistachios between 2007─2018 (Bakker et al., 2011; Centers for Disease Control and Prevention, 2009; Chapter II; Haendiges et al., 2021; Harris et al., 2016; Zhang et al., 2021). *Salmonella* Montevideo and *Salmonella* Senftenberg have garnered particular attention as they have been linked to reported outbreaks associated with U.S. pistachios in 2009, 2013, and 2016 (Centers for Disease Control and Prevention, 2016; U.S. Food and Drug Administration, 2014; Whitham et al., 2021). These two serovars, along with one of two strains of *Salmonella* Liverpool and one strain each of *Salmonella* Agona and Worthington (total of five serovars) were considered persistent strains that may have adapted to and become established within the California pistachio supply chain (Chapter II; Haendiges et al., 2021). Isolation of these five serovars from pistachios collected from different storage silos over 3 years suggest that at least one reservoir for these isolates is in the orchard, the silo, or somewhere between the orchard and the silo. Downstream isolation of the same *Salmonella* strains from pistachios collected at retail or isolation from roasted product suggests that the roasting step applied at that time was inadequate to sufficiently reduce populations of *Salmonella*

or that recontamination occurred between roasting and packaging (Chapter II; Farakos et al., 2018; Haendiges et al., 2021).

A pistachio orchard reservoir for *Salmonella* might be explained by selective pressure within that production system. The CHASRI sequence has been identified in different Enterobacteria, including some strains of *Escherichia coli, Klebsiella pneumoniae*, and *Salmonella enterica* (Haendiges et al., 2021) and has been shown to confer increased copper resistance aerobically, anaerobically, and during shifts between aerobic and anaerobic environments (Staehlin et al., 2016). The presence of the CHASRI sequence in persistent *Salmonella* Montevideo ST316 and Senftenberg ST14 led Haendiges et al., (2021) to hypothesize that this sequence might confer a competitive advantage in the production environment, thereby limiting the diversity of *Salmonella* serovars in the production and, consequently, in the postharvest environment (Haendiges et al., 2021). However, the CHASRI sequence was not found in all persistent strains (Chapter II; Haendiges et al., 2021). In addition, several serovars including both CHASRI positive and negative strains were often isolated from the same lot of pistachios (Chapter II; Harris et al., 2016) suggesting that even if CHASRI plays a role, other factors are involved in driving *Salmonella* diversity and persistence.

The copper availably in the San Joaquin Valley soils is approximately 1.5 parts per million (ppm) (Beede et al., 2005); however, the suggested level of copper in pistachio leaves is between 6–10 ppm. Approximately 75% of California growers apply copper to pistachio orchards at least one time per year (Brown et al., 2008).The post-bloom foliar application of both chelated copper (Cu-EDTA) and copper sulfate ($CuSO_4$) has been recommended to correct the copper deficiency in pistachio orchards (Beede et al., 2005). EDTA has been used as a chelating agent for supplying micronutrients to plants (Norvell & Lindsay, 1969). The chelating

effectivity of EDTA depends on the ability to keep metals in solution by complexing the metal ion (Norvell & Lindsay, 1969). Recommended mixtures of copper solutions for foliar applications combine approximately 340 g of 15% Cu-EDTA or 450 g of $CUSO_4$ into 378 L of water (Beede et al., 2005), achieving concentrations of 2.5 mM of Cu-EDTA and 7.5 mM of $CuSO_4$. Evaporation of water after application would increase these concentrations.

In the present study, the growth of CHASRI-positive and -negative *Salmonella* strains was not inhibited under either aerobic or anaerobic conditions at ≤120 mM Cu-EDTA. This finding may be explained by the exceptional ability of EDTA to bind to copper (Maketon et al., 2008); the formation of such copper-EDTA complexes is known to inhibit or, in some instances, prevent cell death (Mathews et al., 2013). In natural environments, the formation of the copper-EDTA complex is less stable because EDTA is absorbed by soil and the copper ions may be displaced from EDTA by other ligands (Norvell & Lindsay, 1969). Additionally, soil pH has been shown to influence copper displacement from EDTA, and it is known that soils in the San Joaquin Valley, where California pistachios are produced, are alkaline (Norvell & Lindsay, 1969). In the orchard environment, chelated copper complexes may be depleted, increasing the concentration of free copper ions, therefore Cu-EDTA solutions may have similar effects in *Salmonella* cells, as seen in the present study when $CuSO_4$ was used as the copper source.

Under anaerobic but not aerobic conditions, the MIC of $CuSO_4$ was greater for *Salmonella* strains with CHASRI (15 mM) than without (7.5 mM), providing some evidence to support the hypothesis that CHASRI confers a survival advantage when copper is present (Haendiges et al., 2021). Anaerobic conditions in orchard soils occur in the presence of excess water and poor drainage (DuPont, 2020). Localized excess water may occur when using drip

irrigation, a widely used irrigation strategy among California pistachio growers (Mohammadi Mohammadabadi et al., 2020; Zaccaria, 2017).

Copper foliar application and drip irrigation are also common practices in California almond production (Almond Board of California, 2018; Brown et al., 2008). Additionally, anaerobic soil disinfestation (ASD), an alternative to chemical fumigation for the control of soil pathogen and pests, is used in the process of soil remediation during almond orchard replacement in California (Browne et al., 2018; Strauss & Kluepfel, 2015; Yaghmour et al., 2019). During ASD, anaerobic conditions are generated after soil is irrigated, amended with a carbon source and then covered with a plastic tarp (anaerobic soil disinfestation). This approach has been shown to effectively control soil plant pathogens (Poret-Peterson et al., 2020); however, it does not eliminate *Salmonella* in the treated soil and, in some cases, facilitates *Salmonella* survival (Murphy et al., 2022). The establishment of anaerobic environments in copper-amended pistachio and almond orchards may select for the survival of *Salmonella* strains with CHASRI sequences but does not account for the presence of these isolates in pistachios but not almonds (Chapter II; Moyne et al., 2023).

Postharvest environmental conditions also may play a role in the diversity and persistence of *Salmonella* strains in the pistachio supply chain. The temperature and humidity in trailers during transport from the orchard to the hulling facility can differ depending on ambient temperature, time of harvest, sun exposure, location of the pistachios in the trailer, and time in the trailer (Moussavi et al., 2019). Temperatures from 22–41°C have been reported in the trailers (Harris & Ferguson, 2013; Moussavi et al., 2019). Significant growth of a five- to six-strain cocktail of *Salmonella* that included representative isolates of Enteritidis strain B (sporadic), and Montevideo strain A (persistent) was observed on inhull pistachios after 3 to 6 h of incubation at

23, 35, or 37°C and 50 or 90% RH. *Salmonella* lag times, growth rates and final populations differed significantly among independent replicate experiments conducted with different hull material sources (Moussavi et al., 2019), highlighting the heterogeneity among harvested inhull pistachios.

In the present study, individual *Salmonella* strains were inoculated into a sterile pistachio hull slurry and growth was measured at 30□C. The single hull slurry represented nutrients found in pistachio hulls that might be available to microorganisms during harvest or postharvest handling environments. Similar approaches have been used to determine the growth of *Salmonella* Enteritidis PT 30 (a persistent strain in almonds (Moyne et al., 2023)) on almond and walnut hulls (Blessington et al., 2014; Uesugi & Harris, 2006). The maximum population increases of *Salmonella* on inoculated inhull pistachios ranged from 0.15 to 6.49 log CFU/g with estimated lag phases of 0 to 7 h and maximum concentrations between 1.67 and 8.41 log CFU/g depending on the year or stage of harvest when the pistachios were collected, and pistachio characteristics (presence of adhering hull material), incubation temperature, and relative humidity (Moussavi et al., 2019). In the present study, the maximum population increase for all strains was ≥5.85 log CFU/mL after 28 or 40 h of incubation. Smaller population increases were observed for *Salmonella* Enteritidis strain A and Montevideo strain A after 16 h suggesting a longer lag phase, a slower growth rate or both, however, further studies with additional sampling points between inoculation and 16 h would be necessary to confirm hypothesis. Results shown here provide additional evidence that *Salmonella* can multiply using the nutrients in pistachio hulls under conditions relevant during postharvest handling and transportation. Additionally, the data presented indicates that *Salmonella* strains associated with California pistachios have comparative growth capability and achieve comparable maximum populations.

Throughout postharvest handling, in-hull, hulled wet, and hulled dry pistachios contact different surfaces which, if contaminated, may become cross-contamination sites. Pistachios are harvested over a 2.5-month period; thus, processing lines may run day and night, restricting time for thorough cleaning and sanitation. Poor sanitation practices, improper equipment design, and maintenance are some of the main causes of contamination of *Salmonella* in low-moisture foods (Finn et al., 2013; Podolak et al., 2010). The accumulation of organic matter, pistachio hulls, pistachio dust, and moisture during hulling, drying and storage (e.g., on plastic, stainless steel, conveyer belts) may promote the formation of biofilms, serving as *Salmonella* reservoirs and as a potential venue for food contamination (Carrasco et al., 2012; Dantas et al., 2020; Lee et al., 2020; Paz-Méndez et al., 2017). In the present study, seven (78%) of the nine *Salmonella* strains evaluated displayed a moderate biofilm potential with one persistent strain (Liverpool strain A) forming a strong biofilm, indicating that these strains may establish in the processing environment. *Salmonella* isolates from poultry environments displayed weak to strong biofilm potential, and biofilm potential differed among isolates belonging to the same serotype (Obe et al., 2021). The degree of biofilm formation may be impacted by incubation temperature (Akinola et al., 2020). *Salmonella* isolates evaluated in the present study produced weak to strong biofilms at 25°C but no biofilms at 37°C after 4 days of incubation. Ambient temperatures during pistachio harvest can range from 13 to 40 °C and the hulling operation occurs under these conditions (Moussavi et al., 2019). Dryers are continuously filled with freshly wet hulled pistachios; biofilm formation may be likely to occur at this step. In addition to temperature, biofilm production is dependent on surface type (De Oliveira et al., 2014). Further biofilm assays mimicking environmental condition and surface types in the pistachio post harvesting

environment are needed to understand the role biofilm potential may play in *Salmonella* persistence.

The present study evaluated different phenotypical traits that might confer a selective advantage for persistent *Salmonella* strains during production, harvest, and post-harvest handling of California pistachios. Three of the six persistent strains; *Salmonella* Montevideo strain A, *Salmonella* Senftenberg and *Salmonella* Worthington contained the CHASRI sequence displaying an enhanced resistance to copper in anaerobic conditions, grew well on pistachio hull material and formed moderate biofilm formers. The reminding persistent strains and sporadic strains *Salmonella* Enteritis strain B and *Salmonella* Liverpool strain B did not contain the CHASRI sequence but were moderate to strong biofilm formers and grew well utilizing pistachio hull nutrients. These results indicate that the CHASRI sequence (decreased copper sensitivity phenotype), the ability to form biofilms, or growth in pistachio hull extracts during simulated harvest-to-hulling delays cannot independently account for the narrow range of *Salmonella* strains that have been repeatedly isolated from within the California pistachio production chain. The findings of this study along with the isolation of different strains repeatedly found from the same sample suggest that one strain might not dominate over others (Chapter II), and that other phenotypical characteristics may be driving the persistence of *Salmonella* strains isolated from California pistachios. Further studies investigating other phenotypic traits such as desiccation tolerance, long-term survival during storage, and thermal tolerance may help elucidate drivers of persistence in the California pistachio supply chain.

Tables

Table 1. *Salmonella* enterica isolates used to determine phenotypic characteristics of persistent and sporadic strains from the 2010–2012 pistachio silo survey.

Occurrence in the pistachio environment [a]	***Salmonella* serovar**	**Strain** [b]	**Isolate ID**	**Collection year**
Persistent	Agona	N/A [c]	LJH1308	2010
	Liverpool	A	LJH1350-1	2011
	Montevideo	A	LJH1347-1	2011
	Montevideo	B	LJH1352-1	2011
	Senftenberg	N/A	LJH1310	2010
	Worthington	N/A	LJH1288	2010
Sporadic	Enteritidis [d]	A	LJH1297	2010
	Enteritidis [e]	B	LJH1275-1	2010
	Liverpool	B	LJH1509	2012

[a] Occurrence as described in Chapter II. Persistent strains were isolated in ≥ 3 years from more than one avenue (e.g., surveys, outbreak investigations, or routine sampling)].
[b] Strain designation based on Chapter II.
[c] N/A; not applicable
[d] *Salmonella* Enteritidis phage type (PT) 37.
[e] *Salmonella* Enteritidis PT 9c.

Table 2. Minimum inhibitory concentration (MIC) of 15% copper EDTA (Cu-EDTA) and copper sulfate ($CuSO_4$) against selected *Salmonella enterica* isolates under aerobic and anaerobic conditions.

CHASRI profile[a]	*Salmonella* serovar (strain)	Isolate ID	Minimal inhibitory concentration			
			Cu-EDTA		$CuSO_4$	
			Aerobic	Anaerobic	Aerobic	Anaerobic
Present	Montevideo (A)	LJH1347-1	>120 mM	>120 mM	15 mM	15 mM
	Senftenberg	LJH1310	>120 mM	120 mM	15 mM	15 mM
	Worthington	LJH1288	>120 mM	>120 mM	15 mM	15 mM
Absent	Agona	LJH1308	>120 mM	120 mM	15 mM	7.5 mM
	Enteritidis (A)	LJH1297	>120 mM	120 mM	15 mM	7.5 mM
	Enteritidis (B)	LJH1275-1	>120 mM	120 mM	15 mM	7.5 mM
	Liverpool (A)	LJH1350-1	>120 mM	>120 mM	15 mM	7.5 mM
	Liverpool (B)	LJH1509	>120 mM	120 mM	15 mM	7.5 mM
	Montevideo (B)	LJH1352-1	>120 mM	120 mM	15 mM	7.5 mM

[a] Copper homeostasis and silver resistance island (CHASRI) profile was previously identified (Chapter II).

Table 3. *Salmonella* populations in pistachio hull slurry plated onto tryptic soy agar (TSA) or CHROMagar™ Salmonella (CHROMSal) and incubated at 30°C for up to 48 h.

Media type	*Salmonella* serovar (strain)	*Salmonella* (log CFU/mL) [a]						
		Incubation time (h)						
		0 [b]	16	20	24	28	40	48
TSA	Agona	2.68 ± 0.07 Aa	6.84 ± 0.16 ABb	8.04 ± 0.10 ABc	8.44 ± 0.22 ABd	8.67 ± 0.05 ABe	8.58 ± 0.04 ABde	8.58 ± 0.13 ABde
	Enteritidis (A)	2.72 ± 0.03 Aa	4.93 ± 0.16 Db	5.93 ± 0.13 Cc	7.25 ± 0.20 Cd	8.20 ±0.10 Ce	8.67 ± 0.12 ABf	8.74 ± 0.24 Af
	Enteritidis (B)	2.74 ± 0.10 Aa	6.46 ± 0.23 ABCb	7.69 ± 0.11 ABc	8.33 ± 0.14 Bd	8.54 ± 0.10 Bd	8.62 ± 0.23 ABd	8.58 ± 0.14 ABd
	Liverpool (A)	2.72 ± 0.09 Aa	7.02 ± 0.65 Ab	7.98 ± 0.23 ABc	8.48 ± 0.12 ABd	8.66 ± 0.08 ABd	8.52 ± 0.04 Bd	8.48 ± 0.07 Bd
	Liverpool (B)	2.76 ± 0.07 Aa	6.81 ± 0.30 ABb	7.94 ± 0.24 ABc	8.52 ± 0.05 ABd	8.66 ± 0.08 ABd	8.54 ± 0.03 ABd	8.58 ± 0.08 ABd
	Montevideo (A)	2.78 ± 0.05 Aa	6.17 ± 0.30 Cb	7.60 ± 0.26 Bc	8.41 ± 0.11 ABd	8.68 ± 0.04 ABd	8.63 ± 0.10 ABd	8.66 ± 0.10 ABd
	Montevideo (B)	2.81 ± 0.09 Aa	6.85 ± 0.33 ABb	8.05 ± 0.49 ABc	8.71 ± 0.11 ABd	8.80 ± 0.04 Ad	8.73 ± 0.12 Ad	8.75 ± 0.15 Ad
	Senftenberg	2.72 ± 0.06 Aa	6.76 ± 0.15 ABCb	8.07 ± 0.11 Ac	8.44 ± 0.22 ABd	8.67 ± 0.12 ABd	8.54 ± 0.09 ABd	8.57 ± 0.14 ABd
	Worthington	2.76 ± 0.07 Aa	6.72 ± 0.20 ABCb	7.98 ± 0.13 ABc	8.53 ± 0.10 ABd	8.68 ± 0.05 ABd	8.56 ± 0.03 ABd	8.57 ± 0.05 ABd
CHROMSal	Agona	2.51 ± 0.05 Aa	6.79 ± 0.27 ABCb	7.88 ± 0.07 ABCc	8.33 ± 0.17 ABd	8.31 ± 0.11 Bd	8.28 ±0.03 Bd	8.27 ± 0.11 Ad
	Enteritidis (A)	2.61 ± 0.21 Aa	4.91 ± 0.18 Db	5.97 ± 0.13 Dc	7.26 ± 0.15 Cd	8.12 ± 0.07 Ce	8.32 ± 0.42 ABe	8.41 ± .69 Ae
	Enteritidis (B)	2.64 ± 0.04 Aa	6.34 ± 0.31 BCb	7.66 ± 0.18 BCc	8.20 ± 0.15 ABd	8.42 ± 0.12 ABd	8.35 ± 0.17 ABd	8.36 ± 0.19 Ad
	Liverpool (A)	2.63 ± 0.07 Aa	6.82 ± 0.49 ABb	8.08 ± 0.20 Ac	8.41 ± 0.13 ABc	8.46 ± 0.10 ABc	8.36 ± 0.12 ABc	8.25 ± 0.09 Ac
	Liverpool (B)	2.55 ± 0.20 Aa	6.80 ± 0.40 ABb	8.04 ± 0.15 ABc	8.36 ± 0.18 ABcd	8.50 ± 0.08 Acd	8.36 ± 0.10 ABcd	8.36 ± 0.06 Ad
	Montevideo (A)	2.52 ± 0.19 Aa	6.15 ± 0.27 Cb	7.56 ± 0.35 Cc	8.41 ± 0.12 ABd	8.56 ± 0.14 Ad	8.57 ± 0.06 ABd	8.70 ± 0.13 Ad
	Montevideo (B)	2.72 ± 0.08 Aa	7.06 ± 0.56 Ab	8.10 ± 0.11 Ac	8.46 ± 0.03 Acd	8.58 ± 0.06 Ad	8.66 ± 0.15 Ad	8.66 ± 0.13 Ad
	Senftenberg	2.56 ± 0.07 Aa	6.75 ± 0.15 ABCb	8.03 ± 0.11 ABc	8.37 ± 0.09 ABd	8.44 ± 0.09 ABd	8.41 ± 0.11 ABd	8.42 ± 0.17 Ad
	Worthington	2.56 ± 0.03 Aa	6.73 ± 0.23 ABCb	7.99 ± 0.19 ABc	8.43 ± 0.10 ABd	8.54 ± 0.06 Ad	8.46 ± 0.04 ABd	8.43 ± 0.09 Ad

[a] Values are means ± standard deviation, $n = 3$. Within media type, within columns mean values with different uppercase letters are significantly different ($P < 0.05$); within rows, mean values with different lowercase letters are significantly different ($P < 0.05$).
[b] Values at 0 h represent populations immediately after inoculation.

Table 4. Biofilm formation potential of selected *Salmonella enterica* strains isolated from raw pistachios in a 2010–2012 pistachio silo survey.

Occurrence in pistachio [a]	***Salmonella* serovar (strain)**	**Isolate ID**	**OD_{600}**	**Biofilm potential**
Persistent	Agona	LJH1308	0.197 ± 0.055	Moderate
	Liverpool (A)	LJH1350-1	0.341 ± 0.145	Strong
	Montevideo (A)	LJH1347-1	0.215 ± 0.087	Moderate
	Montevideo (B)	LJH1352-1	0.185 ± 0.077	Moderate
	Senftenberg	LJH1310	0.309 ± 0.122	Moderate
	Worthington	LJH1288	0.237 ± 0.127	Moderate
Sporadic	Enteritidis (A)	LJH1297	0.103 ± 0.032	Weak
	Enteritidis (B)	LJH1275-1	0.264 ± 0.102	Moderate
	Liverpool (B)	LJH1509	0.226 ± 0.066	Moderate
N/A [b]	Typhimurium [c]	ATCC 14028	0.310 ± 0.157	Moderate
N/A	N/A	Negative control	0.085 ± 0.015	None

[a] Occurrence as described in Chapter II. Persistent strains were isolated in ≥ 3 years from more than one avenue (e.g., surveys, outbreak investigations, or routine sampling)].
[b] N/A; not applicable
[c] Positive control

References

Administrative Committee for Pistachios (2023). 2022 pistachio bearing acreage, production and yield per acreage. Available at: https://acpistachios.wpenginepowered.com/wp-content/uploads/2023/01/2022-Pistachio-Statistics.pdf. Accessed 5 February 2023.

Akinola, S. A., Tshimpamba, M. E., Mwanza, M., & Ateba, C. N. (2020). Biofilm production potential of *Salmonella* serovars isolated from chickens in North West Province, South Africa. *Polish Journal of Microbiology, 69*(4), 427–439. https://doi.org/10.33073/pjm-2020-046

Almond Board of California (2018). Irrigation innovations help farmers take care of the land and its resources. Available at: https://www.almonds.com/why-almonds/almond-living-magazine/irrigation-innovations-help-farmers-take-care-land-and-its. Accessed 28 February 2023

American Pistachio Growers (2021). California pistachio growers and processors create a $5.2 billion impact on the state's economy. Available at: https://americanpistachios.org/about-us/pistachio-power-unshelled/press-releases/california-economic-impact. Accessed 28 April 2023.

Aviles, B., Klotz, C., Eifert, J., Williams, R., & Ponder, M. (2013). Biofilms promote survival and virulence of *Salmonella enterica* sv. Tennessee during prolonged dry storage and after passage through an in vitro digestion system. *International Journal of Food Microbiology*, *162*(3), 252–259. https://doi.org/10.1016/j.ijfoodmicro.2013.01.026

Bakker, H. C. den, Switt, A. I. M., Cummings, C. A., Hoelzer, K., Degoricija, L., Rodriguez-Rivera, L. D., Wright, E. M., Fang, R., Davis, M., Root, T., Schoonmaker-Bopp, D., Musser, K. A., Villamil, E., Waechter, H., Kornstein, L., Furtado, M. R., & Wiedmann, M. (2011). A whole-genome single nucleotide polymorphism-based approach to trace and identify outbreaks linked to a common *Salmonella enterica* subsp. enterica serovar Montevideo pulsed-field gel electrophoresis type. *Applied and Environmental Microbiology*, *77*(24), 8648–8655. https://doi.org/10.1128/AEM.06538-11

Bansal, A., Jones, T. M., Abd, S. J., Danyluk, M. D., & Harris, L. J. (2010). Most-probable-number determination of *Salmonella* levels in naturally contaminated raw almonds using two sample preparation methods. *Journal of Food Protection*, *73*(11), 1986–1992. https://doi.org/10.4315/0362-028x-73.11.1986

Beede, R. H., Brown, P. H., Kallsen, C., & Weinbaum, S. A. (2005). Diagnosing and correcting nutrient deficiencies. Available at: https://ucanr.edu/sites/fruitandnut/files/73696.pdf. Accessed 1 February 2023.

Beede, R. H. (2017). Pistachio micronutrient management. Available at: https://ucanr.edu/sites/PistachioShortCourse/files/274450.pdf. Accessed 8 February 2023. 8th Advances in pistachio production. Accessed 19 February 2023.

Blessington, T., Mitcham, E. J., & Harris, L. J. (2014). Growth and survival of Enterobacteriaceae and inoculated *Salmonella* on walnut hulls and maturing walnut fruit. *Journal of Food Protection*, *77*(9), 1462–1470. https://doi.org/10.4315/0362-028X.JFP-14-075

Brown, P., Trexler, C., Lopus, S., & Santibáñez, M. P. (2008). Updating our knowledge and planning for future research, education and outreach activities to optimize the management of nutrition in almond and pistachio production. Available at: https://www.cdfa.ca.gov/is/ffldrs/frep/pdfs/completedprojects/06-0625Brown.PDF. Accessed 20 April 2023

Browne, G., Ott, N., Poret-Peterson, A., Gouran, H., & Lampinen, B. (2018). Efficacy of anaerobic soil disinfestation for control of prunus replant disease. *Plant Disease*, *102*(1), 209–219. https://doi.org/10.1094/PDIS-09-16-1392-RE

Carrasco, E., Morales-Rueda, A., & García-Gimeno, R. M. (2012). Cross-contamination and recontamination by *Salmonella* in foods: a review. *Food Research International*, *45*(2), 545–556. https://doi.org/10.1016/j.foodres.2011.11.004

Centers for Disease Control and Prevention (2009). Multistate outbreak of *Salmonella* infections linked to pistachio nuts (final update). Available at: https://www.cdc.gov/salmonella/2009/pistachio-nuts-4-14-2009.html. Accessed April 1 2023

Centers for Disease Control and Prevention (2016). Multistate outbreak of *Salmonella* Montevideo and *Salmonella* Senftenberg infections linked to wonderful pistachios. Available at: https://www.cdc.gov/salmonella/montevideo-03-16/index.html. Accessed 3 April 2022

Daly, S. M., Sturge, C. R., & Greenberg, D. E. (2017). Inhibition of bacterial growth by peptide-conjugated morpholino oligomers. *Methods in Molecular Biology*, *1565*, 115–122. https://doi.org/10.1007/978-1-4939-6817-6_10

Dantas, S. T. A., Camargo, C. H., Tiba-Casas, M. R., Vivian, R. C., Pinto, J. P. A. N., Pantoja, J. C. F., Hernandes, R. T., Fernandes Júnior, A., & Rall, V. L. M. (2020). Environmental persistence and virulence of *Salmonella* spp. isolated from a poultry slaughterhouse. *Food Research International (Ottawa, Ont.), 129*, 108835. https://doi.org/10.1016/j.foodres.2019.108835

Danyluk, M. D., Jones, T. M., Abd, S. J., Schlitt-Dittrich, F., Jacobs, M., & Harris, L. J. (2007). Prevalence and amounts of *Salmonella* found on raw California almonds. *Journal of Food Protection, 70*(4), 820–827. https://doi.org/10.4315/0362-028x-70.4.820

De Oliveira, D. C. V., Fernandes Júnior, A., Kaneno, R., Silva, M. G., Araújo Júnior, J. P., Silva, N. C. C., & Rall, V. L. M. (2014). Ability of *Salmonella* spp. to produce biofilm is dependent on temperature and surface material. *Foodborne Pathogens and Disease, 11*(6), 478–483. https://doi.org/10.1089/fpd.2013.1710

DuPont, T. (2020). Soil health in orchards. Available at: https://treefruit.wsu.edu/orchard-management/soils-nutrition/soil-health-in-orchards. Accessed March 27 2023

Farakos, S. M. S., Pouillot, R., Davidson, G. R., Johnson, R., Spungen, J., Son, I., Anderson, N., & Doren, J. M. V. (2018). A quantitative risk assessment of human salmonellosis from consumption of pistachios in the United States. *Journal of Food Protection*, *81*(6), 1001–1014. https://doi.org/10.4315/0362-028X.JFP-17-379

Finn, S., Condell, O., McClure, P., Amézquita, A., & Fanning, S. (2013). Mechanisms of survival, responses, and sources of *Salmonella* in low-moisture environments. *Frontiers in Microbiology, 4*, 331. https://doi.org/10.3389/fmicb.2013.00331

Haendiges, J., Davidson, G. R., Pettengill, J. B., Reed, E., Ramachandran, P., Blessington, T., Miller, J. D., Anderson, N., Myoda, S., Brown, E. W., Zheng, J., Tikekar, R., & Hoffmann, M. (2021). Genomic evidence of environmental and resident *Salmonella* Senftenberg and Montevideo contamination in the pistachio supply-chain. *Plos One*, *16*(11), e0259471. https://doi.org/10.1371/journal.pone.0259471

Harris, L. J., Lieberman, V., Mashiana, R. P., Atwill, E., Yang, M., Chandler, J. C., Bisha, B., & Jones, T. (2016). Prevalence and amounts of *Salmonella* found on raw California inshell pistachios. *Journal of Food Protection*, *79*(8), 1304–1315. https://doi.org/10.4315/0362-028X.JFP-16-054

Harris, L. J., & Ferguson, L. (2013). Improving the safety of almonds and pistachios (Chapter 15). In L. J. Harris (ed.), *Improving the safety and quality of nuts* (pp. 350–378). Woodhead Publishing Ltd., Cambridge

Harris, L. J., Yada, S., Beuchat, L. R., & Danyluk, M. D. (2022). Outbreaks of foodborne illness associated with the consumption of tree nuts, peanuts, and sesame seeds (version 2) [Table and references]. In outbreaks from tree nuts, peanuts, and sesame seeds. Available at: https://ucfoodsafety.ucdavis.edu/low-moisture-foods/nuts-and-nut-pastes. Accessed April 25 2023

Joseph, B., Otta, S. K., Karunasagar, I., & Karunasagar, I. (2001). Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *International Journal of Food Microbiology*, *64*(3), 367–372. https://doi.org/10.1016/S0168-1605(00)00466-9

Kimber, M. A., Kaur, H., Wang, L., Danyluk, M. D., & Harris, L. J. (2012). Survival of *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on inoculated almonds and pistachios stored at -19, 4, and 24° C. *Journal of Food Protection*, *75*(8), 1394–1403. https://doi.org/10.4315/0362-028X.JFP-12-023

Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C., & Beumer, R. R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology*, *85*(3), 227–236. https://doi.org/10.1016/S0168-1605(02)00540-8

Lee, K.-H., Lee, J.-Y., Roy, P. K., Mizan, M. F. R., Hossain, M. I., Park, S. H., & Ha, S.-D. (2020). Viability of *Salmonella* Typhimurium biofilms on major food-contact surfaces and eggshell treated during 35 days with and without water storage at room temperature. *Poultry Science*, *99*(9), 4558–4565. https://doi.org/10.1016/j.psj.2020.05.055

Maketon, W., Zenner, C. Z., & Ogden, K. L. (2008). Removal efficiency and binding mechanisms of copper and copper-EDTA complexes using polyethyleneimine. *Environmental Science & Technology*, *42*(6), 2124–2129. https://doi.org/10.1021/es702420h

Mathews, S., Hans, M., Mücklich, F., & Solioz, M. (2013). Contact killing of bacteria on copper is suppressed if bacterial-metal contact is prevented and is induced on iron by copper ions. *Applied and Environmental Microbiology*, *79*(8), 2605–2611. https://doi.org/10.1128/AEM.03608-12

Mohammadi Mohammadabadi, A., Hosseinifard, S. J., Sedaghati, N., & Nikooei Dastjerdi, M. (2020). Pistachio (Pistachia vera L.) seedling growth response to irrigation method and

volume in Iran. *Agricultural Water Management*, *240*, 106287. https://doi.org/10.1016/j.agwat.2020.106287

Moussavi, M., Lieberman, V., Theofel, C., Barouei, J., & Harris, L. J. (2019). Growth of *Salmonella* on inoculated inhull pistachios during postharvest handling. *Journal of Food Protection*, *82*(2), 217–225. https://doi.org/10.4315/0362-028X.JFP-18-351

Moyne, A. M., Lawal, O., Goodridge, L., & Harris, L. J. (2023). Genetic diversity of *Salmonella* enterica isolated from raw almonds and in almond orchards. *Journal of Food Protection*

Murphy, C. M., Weller, D. L., Reiter, M. S., Bardsley, C. A., Eifert, J., Ponder, M., Rideout, S. L., & Strawn, L. K. (2022). Anaerobic soil disinfestation, amendment-type, and irrigation regimen influence *Salmonella* survival and die-off in agricultural soils. *Journal of Applied Microbiology*, *132*(3), 2342–2354. https://doi.org/10.1111/jam.15324

Norvell, W. A., & Lindsay, W. L. (1969). Reactions of EDTA complexes of Fe, Zn, Mn, and Cu with soils. *Soil Science Society of America Journal*, *33*(1), 86–91. https://doi.org/10.2136/sssaj1969.03615995003300010024x

Obe, T., Nannapaneni, R., Schilling, W., Zhang, L., & Kiess, A. (2021). Antimicrobial tolerance, biofilm formation, and molecular characterization of *Salmonella* isolates from poultry processing equipment. *Journal of Applied Poultry Research*, *30*(4), 100195. https://doi.org/10.1016/j.japr.2021.100195

Papa, R., Bado, I., Iribarnegaray, V., Gonzalez, M. J., Zunino, P., Scavone, P., & Vignoli, R. (2018). Biofilm formation in carbapenemase-producing *Pseudomonas* spp. and *Acinetobacter*

baumannii clinical isolates. *International Journal of Infectious Diseases*, *73*, 119–120. https://doi.org/10.1016/j.ijid.2018.04.3688

Patel, J, Sharma, M., & Ravishakar, S. (2011). Effect of curli expression and hydrophobicity of *Escherichia coli* O157:H7 on attachment to fresh produce surfaces. *Journal of Applied Microbiology*, *110*(3), 737–745. https://doi.org/10.1111/j.1365-2672.2010.04933.x

Patel, J., & Sharma, M. (2010). Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. *International Journal of Food Microbiology*, *139*(1–2), 41–47. https://doi.org/10.1016/j.ijfoodmicro.2010.02.005

Paz-Méndez, A. M., Lamas, A., Vázquez, B., Miranda, J. M., Cepeda, A., & Franco, C. M. (2017). Effect of food residues in biofilm formation on stainless steel and polystyrene surfaces by *Salmonella enterica* strains isolated from poultry houses. *Foods*, *6*(12). https://doi.org/10.3390/foods6120106

Podolak, R., Enache, E., Stone, W., Black, D. G., & Elliott, P. H. (2010). Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. *Journal of Food Protection*, *73*(10), 1919–1936. https://doi.org/10.4315/0362-028X-73.10.1919

Poret-Peterson, A. T., Sayed, N., Glyzewski, N., Forbes, H., González-Orta, E. T., & Kluepfel, D. A. (2020). Temporal responses of microbial communities to anaerobic soil disinfestation. *Microbial Ecology*, *80*(1), 191–201. https://doi.org/10.1007/s00248-019-01477-6

Sheng, L., & Zhu, M.-J. (2014). Inhibitory effect of cinnamomum cassia oil on non-O157 shiga toxin-producing *Escherichia coli*. *Food Control*, *46*, 374–381. https://doi.org/10.1016/j.foodcont.2014.05.050

Silva, P. L. A. P. A., Goulart, L. R., Reis, T. F. M., Mendonça, E. P., Melo, R. T., Penha, V. A. S., Peres, P. A. B. M., Hoepers, P. G., Beletti, M. E., & Fonseca, B. B. (2019). Biofilm formation in different *Salmonella* serotypes isolated from poultry. *Current Microbiology*, *76*(1), 124–129. https://doi.org/10.1007/s00284-018-1599-5

Srey, S., Jahid, I. K., & Ha, S.-D. (2013). Biofilm formation in food industries: a food safety concern. *Food Control*, *31*(2), 572–585. https://doi.org/10.1016/j.foodcont.2012.12.001

Staehlin, B. M., Gibbons, J. G., Rokas, A., O'Halloran, T. V., & Slot, J. C. (2016). Evolution of a heavy metal homeostasis/resistance island reflects increasing copper stress in enterobacteria. *Genome Biology and Evolution*, *8*(3), 811–826. https://doi.org/10.1093/gbe/evw031

Strauss, S. L., & Kluepfel, D. A. (2015). Anaerobic soil disinfestation: A chemical-independent approach to pre-plant control of plant pathogens. *Journal of Integrative Agriculture*, *14*(11), 2309–2318. https://doi.org/10.1016/S2095-3119(15)61118-2

U.S. Food and Drug Administration (2014). FDA investigation summary—multistate outbreak of *Salmonella* Senftenberg infections associated with pistachios from a California roaster. Available at: http://wayback.archive-it.org/7993/20171114154922/https://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm386377.htm. Accessed 1 October 20122

Uesugi, A. R., & Harris, L. J. (2006). Growth of *Salmonella* Enteritidis phage type 30 in almond hull and shell slurries and survival in drying almond hulls. *Journal of Food Protection*, *69*(4), 712–718. https://doi.org/10.4315/0362-028x-69.4.712

Vestby, L. K., Møretrø, T., Langsrud, S., Heir, E., & Nesse, L. L. (2009). Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal- and feed factories. *BMC Veterinary Research*, *5*, 20. https://doi.org/10.1186/1746-6148-5-20

Whitham, H. K., Sundararaman, P., Dewey-Mattia, D., Manikonda, K., Marshall, K. E., Griffin, P. M., Gleason, B. L., Subramhanya, S., & Crowe, S. J. (2021). Novel outbreak-associated food vehicles, United States. *Emerging Infectious Diseases*, *27*(10), 2554–2559. https://doi.org/10.3201/eid2710.204080

Yada, S., & Harris, L. J. (2022). Recalls of tree nuts and peanuts in the U.S., 2001 to present (version 2) [Table and references]. In U.S. recalls of nuts. Available at: https://ucfoodsafety.ucdavis.edu/low-moisture-foods/nuts-and-nut-pastes. Accessed April 2 2023

Yaghmour, M., Holtz, B., & Browne, G. (2019). Considering new orchard replacement options: whole orchard recycling and anaerobic soil disinfestation — SJV trees and vines. Available at: https://www.sjvtandv.com/blog/considering-new-orchard-replacement-options-whole-orchard-recycling-and-anaerobic-soil-disinfestation. Accessed 10 March 2023

Zaccaria, D. (2017). Efficient irrigation of pistachio: system selection, maintenance, and evaluation. Advances in pistachio production short course. Available at: https://ucanr.edu/sites/PistachioShortCourse/files/274437.pdf. Accessed: 10 March 2023

Zhang, G., Hu, L., Luo, Y., Santillana Farakos, S. M., Johnson, R., Scott, V. N., Curry, P., Melka, D., Brown, E. W., Strain, E., Bunning, V. K., Musser, S. M., & Hammack, T. S. (2021). Survey of *Salmonella* in raw tree nuts at retail in the United States. *Journal of Food Science*, *86*(2), 495–504. https://doi.org/10.1111/1750-3841.15569

Chapter IV. Survival of *Salmonella* strains associated with the pistachio supply chain during desiccation and subsequent storage.

Abstract

Salmonella enterica subsp. *enterica* strain diversity in California pistachios is limited; some strains have persisted in the pistachio supply chain for at least 10 years. Sensitivity to desiccation (expression of the red, rough, and dry (rdar) morphotype), presence of genes linked to *Salmonella* survival, and survival during storage was evaluated for six persistent and five sporadic *Salmonella* pistachio isolates. The rdar+ and rdar- morphotypes were observed in both persistent and sporadic *Salmonella* strains. All *Salmonella* strains contained nine of the 10 genes associated with desiccation and storage. Lawn-collected cells were inoculated onto sterile filters to 7 log CFU, dried overnight, and then stored at 24 ± 1 °C and 35% relative humidity for 50 days. After the drying population reductions of 0.50–1.25 log were observed for eight of the nine *Salmonella* strains. The population reductions (3.98–5.12 log) of eight strains were not significantly different at day 50. Except for one, all the *Salmonella* strains isolated from California pistachios were able to survive desiccation and storage, irrespective of their multicellular morphology or the presence of genes thought to aid in their survival during these processes. None of these characteristics explained or predicted the fate of *Salmonella* strains during simulated desiccation and storage conditions.

Introduction

California pistachios have been associated with three multistate foodborne illness outbreaks, all of which were linked to *Salmonella*. Over 30 serovars have been isolated from U.S. almonds and pecans (Bansal et al., 2010; Brar et al., 2016; Danyluk et al., 2007; Lambertini et al., 2012; Moyne et al., 2023). In contrast, *Salmonella* diversity in California pistachios is limited; the majority of pistachio isolates obtained from 2008 to 2018 belonging to five *Salmonella* serovars; Agona, Liverpool, Montevideo, Senftenberg, and Worthington (Bakker et al., 2011; Centers for Disease Control and Prevention, 2009; Chapter II; Haendiges et al., 2021; Harris et al., 2016; Zhang et al., 2021). Further phylogenetic analysis of these *Salmonella* isolates indicated the presence of six persistent strains within the California pistachio supply chain; two strains of *Salmonella* Montevideo and two of *Salmonella* Senftenberg, along with single strains of *Salmonella* Agona, *Salmonella* Liverpool, and *Salmonella* Worthington (Chapter II; Haendiges et al., 2021).

Salmonella contamination of pistachios may occur at any point in the production chain. It was proposed that the limited diversity and persistence of some *Salmonella* serovars in the pistachio production environment was driven by a gene island that conferred copper resistance (Haendiges et al., 2021). However, not all persistent strains contained the copper resistance gene island (Chapter II, 2023; Haendiges et al., 2021) and the phenotypical advantage of this gene island was only observed under anaerobic conditions (Chapter III). Representative persistent and sporadic strains of *Salmonella* grew equally well in pistachio hull extracts and were moderate biofilm formers. None of the phenotypic characteristics tested could independently account for *Salmonella* persistence in the California pistachio production chain (Chapter III). The underlying

reasons leading to the establishment of specific persistent strains of *Salmonella* associated with California pistachios remains unknown.

The California pistachio harvest season lasts approximately 3 months, from August to October. After pistachios are mechanically harvested from the trees, they are transported to a hulling facility where they are pre-cleaned, hulled, and separated in water based on buoyancy. Potential for delays (>3 h) between hulling and drying may result in increases in *Salmonella* populations on pistachios by up to 3.3 log CFU/g (Moussavi et al., 2019). The inshell pistachios are dried at temperature of 80 and 105 °C to a moisture level of <15% then transferred to a silo where the remaining moisture content is reduced to <7% (Harris & Ferguson, 2013). Pistachios are subsequently stored for up to 18 months. Data on the expected reduction of *Salmonella* during post-hulling drying is not available. However, isolation of *Salmonella* from dried pistachios collected from storage silos suggests that the organism can survive this process (Harris et al., 2016). During drying, *Salmonella* cells on contaminated pistachios are exposed to desiccation, possibly leading to enhanced resistant to subsequent stressors (Gruzdev et al., 2011).

Salmonella population reductions of 1 to 2 log CFU/g were observed on wet inoculated pistachios during a 24- or 72-h drying period (Haendiges et al., 2021; Kimber et al., 2012). During subsequent storage at 24°C and 35-40% relative humidity the calculated linear decline in *Salmonella* populations was 0.08 (Haendiges et al., 2021) or 0.15 CFU/g per month (Kimber et al., 2012). The desiccation tolerance among *Salmonella* strains has been documented (Kimber et al., 2012; Moussavi et al., 2020; Norberto et al., 2022). However, most survival studies for *Salmonella* on pistachios or other nuts have either used single isolates or cocktails of different strains (Blessington et al., 2012; Harris et al., 2012; Jayeola et al., 2020; Kimber et al., 2012; Limcharoenchat et al., 2019; Uesugi & Harris, 2006). Side-by-side comparisons of the survival

of individual *Salmonella* strains during desiccation and storage are scarce. The potential impact of desiccation and storage on the survival of individual *Salmonella* strains isolated from the California pistachio production chain has not previously been investigated; therefore, it is unclear whether these steps contribute to the persistence and diversity of strains found in later stages of the production chain.

The phenotypes and genotypes associated with survival of *Salmonella* during desiccation or on low-water-activity surfaces and foods have been investigated (Finn et al., 2013; Jayeola et al., 2020; Kieboom et al., 2006; Mandal & Kwon, 2017; White et al., 2006); however, the exact mechanisms of desiccation resistance have not been established. Exposure to low water activity changes the morphology of *Salmonella* by increasing the number of filaments on the cell (Eriksson de Rezende et al., 2001; Kieboom et al., 2006). Thin aggregative fimbria mediate cell aggregation in *Salmonella*, which produces a matrix that has been termed as a red, dry, and rough (rdar) morphotype (White & Surette, 2006). The rdar morphotype has been linked to enhanced survival of *Salmonella* in nutrient-limited environments and external stresses, and enhances the survival potential of *Salmonella* species to long-term desiccation (Gibson et al., 2006; U Römling, 2005; White & Surette, 2006). In addition to phenotypic changes, the presence and expression of specific genes during *Salmonella* responses to environmental stresses has been linked the *Salmonella* responses to such stresses. Ten genes mediating the survival of *Salmonella* on pistachios during industry-relevant storage temperatures were identified (Jayeola et al., 2020).

To date, the impact of phenotypic or genotypic characteristics related to desiccation tolerance and storage survival in individual *Salmonella* strains isolated from California pistachios has not been investigated. Therefore, the significance of these characteristics in *Salmonella* persistence during the drying and storage steps are still unknown. The objectives of

this study were to evaluate *Salmonella* survival, expression of the rdar morphotype, and the presence of genes linked to *Salmonella* survival on pistachios during desiccation and storage of both persistent and sporadic strains isolated from California pistachios, and to determine the possible correlation between these phenotypic and genetic factors and *Salmonella* persistence in the California production chain.

Materials and Methods

Bacterial strains and growth conditions

All isolates were isolated from raw California pistachios during a 2010 to 2013 survey (Harris et al., 2016) and genetically characterized to identify unique strains (Chapter II). To determine the rdar morphology of *Salmonella* strains, one representative isolate per strain per year was chosen (n = 23). For all other experiments, a single isolate per *Salmonella* strain with ≥2 isolates was selected: Agona (LJH1308), Enteritidis strain A (LJH1297), Enteritidis strain B (LJH1275-1), Liverpool strain A (LJH1350-1), Liverpool strain B (LJH1509), Montevideo strain A (LJH1347-1), Montevideo strain B (LJH1352-1), Senftenberg (LJH1310), and Worthington (LJH1288). All strains, with the exception of *Salmonella* Enteritidis (strains A and B) and Liverpool strain B, were considered persistent strains (isolated for ≥3 years from more than one avenue [e.g., surveys, outbreak investigations, or routine sampling]) (Chapter II).

The same culturing procedure was used for all phenotypical tests. *Salmonella* stock cultures (stored at −80°C) were streaked onto tryptic soy agar (TSA; tryptic soy broth plus 1.5% granulated agar) and incubated at 37°C for 24 h. A single isolated colony was transferred to a centrifuge tube (Corning) containing 10 or 3 mL of tryptic soy broth (TSB) and incubated at 37°C for 24 h. After incubation, the 10 mL culture was used for rdar morphotype testing, and 10

µL of the 3-mL culture was transferred into 10 mL of fresh TSB and then incubated for an additional 24 h at 37°C. The *Salmonella* suspension from the second overnight culture was used for inoculum preparation for the drying and storage experiments. Unless otherwise specified, culture media were Difco brand (BD).

Rdar cell morphology

The overnight culture (from the 10-mL TSB) was diluted 1:10 using 0.01% peptone water (seven times) to achieve a target final population of 2 log CFU/mL. Aliquots (100 µl) of the dilution were plated onto Luria-Bertani no salt agar (Fisher) supplemented with Congo red (Allied Chemicals) at 40 µg/mL and Coomassie brilliant blue (Fisher) at 20 µg/mL, and then incubated at 28°C for 7 days. After incubation, colony morphology on the plates was inspected and recorded (U Römling et al., 1998). This assay was performed in triplicate for each isolate.

Inoculum preparation

Liquid culture (1 mL) of the *Salmonella* suspension was spread on a 100 mm TSA plate and incubated at 37°C for 24 h. Cells were harvested by adding 3 mL of sterile ultrapure water (MilliQ Advantage A10, MilliporeSigma) to the agar surface, and then gently scrapping off the bacteria lawn with a sterile spreader. The cell suspension was collected into a 10-mL Falcon tube containing 8 mL of sterile ultrapure water. The cell suspension was diluted 1:10 using sterile ultrapure water to achieve a target population of 9 log CFU/mL (*Salmonella* inoculum).

Inoculation, drying and storage

Sterile cellulose filters (0.22-µm pore size; MF-Millipore mixed cellulose ester membranes, EMD Millipore) were used as the desiccation matrix (Suehr et al., 2020) to eliminate confounding variables (e.g., pistachios' heterogenic intrinsic properties [maturation

state, nutrient availability, shape]) that may affect the survival of *Salmonella* strains. Filters were inoculated with 100 µL of the *Salmonella* inoculum to obtain a concentration of 7 log CFU per filter prior drying. The inoculated filters were left to dry in a biosafety cabinet with the fan on for 18 h. Individual dried filters were then placed into separate Whirl-Pak bags (Nasco) and stored in a sealed container. Saturated magnesium chloride solutions in 250 mL glass beakers were placed inside to maintain a target 35% humidity in the sealed container. The sealed container was placed in an incubator at 25°C. The relative humidity and temperature inside the container were monitored and recorded using a data logger (TempTale 4, Sensitech Inc.).

Enumeration

To recover *Salmonella* on the inoculated filters, 5 mL of 0.1% peptone was added to individual Whirl-Pak bags (each containing one filter), and samples were homogenized for 120 s at the fast setting using a homogenizer (Smasher™, Biomerieux). The resuspended cells were plated onto TSA and CHROMagar™ Salmonella and incubated at 37°C for 24 h. *Salmonella* populations on filters were enumerated at the time of inoculation, at 0 h (18 h after inoculation), and at 10, 30, and 50 days of storage.

Gene identification

The sequences of the assembled draft genomes were compared against an annotated reference sequence of *Salmonella* Typhimurium (GenBank accession number CP019649.1) to identify the presence of 10 genes associated with survival of *Salmonella* in pistachios during storage; *sspA*, *barA*, *uvrB*, *damX*, *rfbD*, *uvrY*, *lrhA*, *yifE*, *rbsR*, and *ompR* (Jayeola et al., 2020). Using the "graphics" option on the National Center for Biotechnology Information (NCBI) database, the location of the 10 genes on the reference sequence was identified (Supplemental Table S2). The NCBI BLASTn tool was used to locate the sequences of genes of interest on the

draft genomes. The sequence of a gene was determined to be present when a contig of the draft sequence showed ≥98% coverage and identity against the annotated sequence.

Data analysis

For the drying and storage experiment, two inoculated samples were evaluated at each time point. The experiment was repeated three times to obtain a total of six analytical units for inoculated samples. Means and standard deviation were determined. Counts that were below the limit of detection (1 log CFU per filter) were not included in population mean calculations or statistical analyses (Garcés-Vega & Marks, 2014). Population levels were log transformed and analyzed using the Tukey–Kramer test, and differences between media types were analyzed using a Matched Pairs test, performed with JMP Pro 16 software (SAS Institute).

Results

Rdar morphotype

Three phenotypic morphological classes among 23 *Salmonella* isolates were observed after incubation at 25°C for 7 days: complete rdar, incomplete rdar patterns (partial), and smooth or no patterns (rdar negative) (Fig 1). *Salmonella* Enteritidis strain B, *Salmonella* Sandiego, *Salmonella* Liverpool strains A and B, and *Salmonella* Worthington displayed complete rdar morphotype (Table 1). *Salmonella* Senftenberg and *Salmonella* Tennessee formed partial rdar patterns while *Salmonella* Agona, *Salmonella* Enteritidis strain A, *Salmonella* Montevideo strains A and B had a smooth colony morphology (negative) (Table 1).

Salmonella population declines during drying and storage

Populations of *Salmonella* determined on TSA were consistently, but not always significantly, 0.37 log CFU/mL higher than those on the corresponding CHROMagar Salmonella (Table 2; statistics not shown). For this reason, only TSA results are described in the text. *Salmonella* populations of all strains were 7.70–8.05 log CFU per filter at time of inoculation. After 18 h of drying under ambient conditions in the biosafety cabinet (22°C and 30% RH), all *Salmonella* populations were significantly ($P < 0.05$) lower than the corresponding initial pre-drying level; the populations of eight of the nine *Salmonella* strains were not significantly ($P > 0.05$) different from each other, with populations of 6.62–7.02 log CFU per filter (Table 2). A significant ($P < 0.05$) population reduction of 4.9 log CFU per filter was observed for *Salmonella* Enteritidis strain A after drying for 18 h (to 2.88 log CFU per filter). Populations of S*almonella* Enteritidis strain A could not be quantified after the 18-h drying period, as they fell below the limit of detection (1 log CFU per filter). Therefore, the results discussed below are only for the remaining eight strains. After 10 days of storage at 25°C and 35% relative humidity, populations of *Salmonella* Agona, *Salmonella* Enteritidis strain B, *Salmonella* Montevideo strain B, and *Salmonella* Senftenberg were not significantly different from their respective populations after 18 h of drying (Table 1). In contrast, populations of *Salmonella* Montevideo strain A, *Salmonella* Liverpool strain A, *Salmonella* Liverpool strain B, and *Salmonella* Worthington decreased significantly after 10 days of storage, with reductions of 2.11, 2.94, 2.52, and 2.13 log CFU per filter, respectively (Table 2).

After 30 days of storage, populations of all strains, except for *Salmonella* Liverpool strain B, decreased significantly ($P < 0.05$) from the levels observed at 10 days of storage. All *Salmonella* populations at day 30 were between 4.00–4.81 log CFU per filter and were not significantly different from each other. Similarly, at day 50 no significant differences among

populations of all *Salmonella* strains were observed (Table 2); final *Salmonella* populations were between 2.93–3.73 log CFU per filter, with total reductions of 3.98–5.12 log CFU per filter between 0 and 50 days of storage.

Mean recorded temperatures of the three experiments during the storage period were 25.50 ± 0.10, 25.50 ± 0.10, and 25.40 ± 0.10°C (noted herein 25°C, respectively). The corresponding average recorded relative humidity was 35.0 ± 0.20, 35.70 ± 0.30, and 35.30% ± 0.30%, respectively.

Gene identification

All *Salmonella* strains contained nine of the 10 genes assessed (Supplemental Table S2). Sequences of *sspA*, *barA*, *uvrB*, *damX*, *uvrY*, *lrhA*, *yifE*, *rbsR*, and *ompR* genes were found in all *Salmonella* strains. The *rfbD* gene sequence was found in all strains except *Salmonella* Montevideo strains A and B.

Discussion

A low prevalence (0.61%) of *Salmonella* has been documented in raw California inshell pistachios, with predicted levels of <0.7 MPN/100 g (Harris et al., 2016). Three multistate salmonellosis outbreaks have been linked to this commodity (Harris et al., 2022). Although the original contamination source in each outbreak was not determined, it is possible for *Salmonella* to contaminate pistachios at any time in the production chain. The same strains of *Salmonella* Montevideo, *Salmonella* Senftenberg, *Salmonella* Liverpool and *Salmonella* Worthington were isolated from inshell pistachios during a 2010-2012 pistachio silo survey (Harris et al., 2016) and from retail pistachios in 2016 (Zhang et al., 2021). Additionally, a strain of *Salmonella* Montevideo which was isolated from both the pistachio silo survey and the retail survey was

linked to the 2009 and 2016 salmonellosis outbreaks associated with pistachios (Chapter II). Similarly, the *Salmonella* Senftenberg strain isolated from the silo survey was also isolated from the retail survey and linked to the 2016 salmonellosis pistachio outbreak (Chapter II). The limited diversity and the repeated isolation of certain strains indicate that *Salmonella* is established in the pistachio production chain (Chapter II; Haendiges et al., 2021). Different phenotypic characteristics contributing to *Salmonella* persistence during production, harvest, and post-harvest handling have been investigated (Chapter III), However, the mechanisms of persistence have yet to be elucidate as no clear distinction in phenotype of has been observed between persistent and sporadic *Salmonella* strains.

In the present study, the phenotypes and genotypes that may contribute to persistence of *Salmonella* strains during pistachio drying and storage were evaluated. *Salmonella* multicellular behavior, which involves the formation of matrix components such as fimbriae and cellulose, has been suggested to play a significant role in *Salmonella* survival in the environment (White et al., 2006). This behavior can be categorized by colony morphology. The *Salmonella* strains associated with California pistachios had one of three morphotypes: rdar positive, rdar partial and rdar negative (smooth). These morphotypes have been observed in *Salmonella* from all phylogenic lineages (including *S. enterica* subspecies I, II, IIIa, IIIb, VI, and V) (White & Surette, 2006). In the present study, the rdar morphotype, which is formed by the expression of both cellulose and curli fimbriae in the colony's matrix (Nesse et al., 2020), was predominant (seven strains with partial or complete rdar morphotype) while the partial rdar patterns was only observed in *Salmonella* Senftenberg and *Salmonella* Tennessee.

Other morphotypes, such as pdar (pink, dry, and rough) and bdar (brown, dry, and rough), have also been identified in *Salmonella* strains (U Römling et al., 1998, 2000). The pdar

morphology is characterized by delayed roughness formation without the dry appearance caused by the production of only cellulose. In contrast, bdar colonies remain dry and rough due to the expression of curli, but their color changes to brown (U Römling et al., 2000). These morphotypes were not observed in the present study. Previous studies have indicated that all these morphotypes are conserved throughout *Salmonella*, with the rdar morphotype being the most prevalent (Karaca et al., 2013; Ute Römling et al., 2003; White & Surette, 2006). The rdar morphotype has been associated with contributing to the long-term survival of *Salmonella* in the environment by enhancing desiccation tolerance (Mattick et al., 2000; White et al., 2006).

The genetic mechanism underlying *Salmonella* survival during desiccation and in low-moisture, low-nutrient environments is not fully understood. Previous studies aimed at identifying the genes involved in this adaptation have been primarily focused on the survival of *Salmonella* on abiotic surfaces (Finn et al., 2013; Maserati et al., 2017; Vestby et al., 2009). Ten genes enhancing the survival of individual *Salmonella* strains, specifically on pistachios during desiccation and storage, were identified via transposon sequence analysis (Jayeola et al., 2020). The present study indicated that all *Salmonella* strains isolated from California pistachios contained at least nine of the 10 genes. The *rfbD* gene was absent in both *Salmonella* Montevideo persistent strains. This gene, along with several other genes, is involved with the biosynthesis of the O antigen of lipopolysaccharide (Tsukioka et al., 1997), which contributes to biofilm formation and, ultimately, enhances survival of bacteria in hostile environments (Wicaksono et al., 2023).

To assess the impact of cell morphology and the presence of the 10 genes associated with *Salmonella* survival during desiccation and storage, the fate of individual *Salmonella* strains was evaluated. The results indicated that all *Salmonella* strains, regardless of their morphotype or

genotype, exhibited comparable desiccation tolerance, except for *Salmonella* Enteritidis strain A. Three of the six persistent strains, namely *Salmonella* Agona and both *Salmonella* Montevideo strains (A and B), displayed smooth morphology, however survival during storage was not significantly different from rdar-positive strains. Additionally, the survival of both *Salmonella* Montevideo strains, which lacked one of the 10 genes associated with desiccation and storage survival, was not significantly different from those strains possessing all 10 genes. These findings suggest that at least some persistent strains may employ mechanisms of survival that are not related to the formation of extracellular matrix or pathways involving the *rfbD* gene. Future studies that investigate the expression of these genes under comparable desiccation and storage conditions can offer valuable insights into the significance of these genes. Moreover, such studies can shed light on the mechanisms employed by *Salmonella* to endure these environmental stresses encountered during pistachio processing.

A previous study investigating the persistence of *Salmonella* on different material types of conveyor belts indicated that the rdar morphotype was not a significant factor, but the type of conveyor belt affected *Salmonella* persistence (Stocki et al., 2007). In the current study, *Salmonella* strains were inoculated on filters to eliminate confounding variables that may affect *Salmonella* survival. Others have assessed the survival of two strains obtained from California pistachios, Montevideo strain A and Senftenberg (also classified as a multilocus sequence type 316 and 14, and other strains associated with low-moisture foods or produce on raw inshell pistachios during storage (Haendiges et al., 2021). During the desiccation period following inoculation (24 h in a biosafety cabinet) *Salmonella* populations declined by about 1 log CFU/g (Haendiges et al., 2021). Similarly, population reductions of about 1 log CFU/g were observed when inshell pistachios were inoculated with a six-strain *Salmonella* cocktail at 6 and 4 log

CFU/g levels and left to dry for 3 days at ambient temperature (Kimber et al., 2012). These desiccation-period reductions are consistent with the findings of the present study, except for *Salmonella* Enteritidis strain A, for which the population reduction during drying was about 5 log CFU/filter. Haendiges et al. (2021) observed no significant differences in bacterial counts among *Salmonella* strains when individual strains of *Salmonella*, including *Salmonella* Montevideo strain A and *Salmonella* Senftenberg, were inoculated, and stored at 25°C with 35% RH for 3 months. This result is comparable to the findings presented here for eight of the strains isolated from California pistachios, indicating no significant difference in populations after 50 days of storage at 25°C with 35% RH.

The long-term (over 300 days) survival of *Salmonella* on pistachios has been previously documented. Kimber et al. (2012) used a cocktail containing six *Salmonella* strains, including an isolate belonging to *Salmonella* Montevideo strain A and a *Salmonella* Enteritidis PT 9c isolate (corresponding to strain B), to inoculate inshell pistachios; after 12 months of storage at 24°C, a decline of 2 log CFU/g was observed. *Salmonella* Enteritidis PT9c and *Salmonella* Montevideo strain A cells were recovered from pistachios samples after 14 months of storage at 24°C. Similarly, in the present study, *Salmonella* Montevideo A and *Salmonella* Enteritidis PT 9c were recovered from inoculated filters after 50 days of storage, further indicating the resistance of these strains to survive in low-moisture environments. A reduction of 2 log CFU/g was observed when raw inshell pistachios were inoculated with five *Salmonella* strains including two persistent *Salmonella* strains associated with California pistachios (*Salmonella* Montevideo strain A and Senftenberg) when stored at 25°C for 365 days (Haendiges et al., 2021). In the present study, reductions of >2 log were observed after 30 days under similar storage conditions. Additionally, previous research has shown that a slower rate of decline of foodborne pathogens, including

Salmonella, occurs when pathogens are inoculated on pistachios than when inoculated on almonds dried and stored at the same conditions (Kimber et al., 2012). These results may suggest that some properties of pistachios may enhance the survival of *Salmonella* cells. To gain a more comprehensive understanding of the behavior of persistent and sporadic *Salmonella* strains associated with pistachios, further studies investigating gene expression and the fate of Salmonella strains on pistachios, and other surfaces (e.g., almonds and stainless steel) are needed. Such studies may provide a more accurate depiction of the fate of these strains as well as the different genetic mechanism employed by *Salmonella* strains in the pistachio environment during drying and storage.

The findings of this study indicate that, except for one, all the *Salmonella* strains isolated from California pistachios can survive desiccation and storage, irrespective of their multicellular morphology or the presence of genes thought to aid in their survival during these processes. These results highlight the significance of preventing or reducing the levels of *Salmonella* contamination in pistachios prior to desiccation or implementing an appropriate kill step in the pistachios during final processing. Neither rdar morphology nor the presence of the presence of genes identified in the present study were able to explain or predict the fate of *Salmonella* strains during simulated desiccation and storage conditions. Further research is needed to elucidate the impact of the roasting process on desiccated persistent and sporadic strains to determine whether the desiccation and storage process confers cross-protection against thermal resistance.

Tables and Figures

Table 1. Rdar (red, dry, and rough) cell morphology of *Salmonella* strains associated with California pistachios.

Rdar morphology	*Salmonella* serovar	Strain [a]	Persistent	Isolate ID	Collection year
Complete	Enteritidis	B	-	LJH1275-1	2010
				LJH1349-1	2011
	Liverpool	A	+	LJH1350-1	2011
				LJH1500	2012
		B	-	LJH1509	2012
	Sandiego	N/A[b]	-	LJH1292	2010
	Worthington	N/A	+	LJH1288	2010
				LJH1406	2011
				LJH1589	2012
Partial	Senftenberg	N/A	+	LJH1310	2010
				LJH1501	2012
	Tennessee	N/A	-	LJH1280-1	2012
Negative	Agona	N/A	+	LJH1308	2010
				LJH1429	2012
	Enteritidis	A	-	LJH1297	2010
				LJH1351-1	2011
				LJH1438-1	2012
	Montevideo	A	+	LJH1289-1	2010
				LJH1347-1	2011
				LJH1453	2012
		B	+	LJH1287-1	2010
				LJH1352-1	2011
				LJH1548	2012

[a] Strain designation as described in Chapter II.
[b] N/A, not applicable.

Table 2. Average *Salmonella* counts on filters dried under a safety cabinet, incubated at 25°C and 35% relative humidity, and then plated on tryptic soy agar (TSA) and CHROMagar Salmonella (CHROMSal)

Media	*Salmonella* serovar	Strain [a]	Isolate ID	*Salmonella* population (average log CFU per filter) [b]				
				Initial (h)	After drying (h)	Storage (days)		
				0 [c]	18 [d]	10	30	50
TSA	Agona	N/A [e]	LJH1308	7.86 ± 0.30Aa	6.62 ± 0.36Bb	6.22 ± 0.26ABb	4.85 ± 0.38Ac	3.69 ± 0.80Ad
	Enteritidis	A	LJH1297	7.84 ± 0.37Aa	2.88 ± 0.68Cb	<1[f]	<1	<1
		B	LJH1275-1	8.03 ± 0.17Aa	7.02 ± 0.33ABb	6.24 ± 0.32ABb	4.47 ± 1.12Ac	3.21 ± 1.24Ad
	Liverpool	A	LJH1350	8.05 ± 0.21Aa	6.73 ± 0.36Bb	5.11 ± 0.59Dc	4.00 ± 0.81Ad	2.93 ± 0.95Ae
		B	LJH1509	7.70 ± 0.44Aa	6.68 ± 0.28Bb	5.18 ± 0.69CDc	4.74 ± 0.39Ac	2.83 ± 0.78Ad
	Montevideo	A	LJH1347	7.83 ± 0.23Aa	6.91 ± 0.18ABb	5.73 ± 0.50BCc	4.33 ± 0.73Ad	2.80 ± 0.58Ae
		B	LJH1352-1	7.85 ± 0.24Aa	6.75 ± 0.32Bb	5.97 ± 0.33ABb	4.29 ± 0.74Ac	3.27 ± 1.46Ad
	Senftenberg	N/A	LJH1310	7.99 ± 0.10Aa	6.78 ± 0.49Bb	5.85 ± 0.43Bb	4.06 ± 0.60Ac	3.44 ± 1.88Ac
	Worthington	N/A	LJH1288	8.01 ± 0.15Aa	7.01 ± 0.39ABb	5.88 ± 0.55Bc	4.81 ±0.38Ad	3.73 ± 0.86Ae
CHROMSal	Agona	N/A	LJH1308	7.74 ± 0.10ABCa	6.23 ± 0.14Cb	5.76 ± 0.14ABb	4.38 ± 0.10Ac	2.95 ± 0.31Ad
	Enteritidis	A	LJH1297	7.57 ± 0.07Ca	2.89 ± 0.19Db	<1	<1	<1
		B	LJH1275-1	7.96 ± 0.03Aa	7.09 ± 0.08ABa	5.82 ± 0.19ABb	4.55 ± 0.22Ac	2.87 ± 0.33Ad
	Liverpool	A	LJH1350	7.96 ± 0.04Aa	6.29 ± 0.15Cb	4.70 ± 0.15Cc	3.30 ± 0.36Cd	2.94 ± 0.22Ad
		B	LJH1509	7.58 ± 0.15BCa	6.51 ± 0.13BCb	4.91 ± 0.15Cc	4.23 ± 0.16ABCc	2.40 ± 0.24Ad
	Montevideo	A	LJH1347	7.75 ± 0.09ABCa	6.79 ± 0.09ABCb	5.35 ± 0.12BCc	3.85 ± 0.24ABCd	2.30 ± 0.20Ae
		B	LJH1352-1	7.72 ± 0.07ABCa	6.45 ± 0.16Cb	5.35 ± 0.16BCc	3.74 ± 0.20ABCd	3.08 ± 0.46Ad
	Senftenberg	N/A	LJH1310	7.92 ± 0.03ABa	6.71 ± 0.08ABCb	5.23 ± 0.20BCc	3.43 ± 0.24BCd	3.68 ± 0.60Ad
	Worthington	N/A	LJH1288	7.84 ± 0.06ABCa	7.08 ± 0.10ABb	5.24 ± 0.24BCc	4.18 ± 0.12ABCd	3.46 ± 0.14Ae

[a] Strain designation as presented in Chapter II.
[b] Values are means ± standard deviation ($n = 6$, or $n = 4$ for counts on CHROMSal at 18 h). Within media type and within columns, mean values with different uppercase letters are significantly different ($P < 0.05$); within rows, mean values with different lowercase letters are significantly different ($P < 0.05$).
[c] Values at 0 h represent populations immediately after inoculation.
[d] After drying for 18 h is considered day 0 of storage.
[e] NA, not applicable.
[f] LOD, limit of detection; 1 log CFU per filter

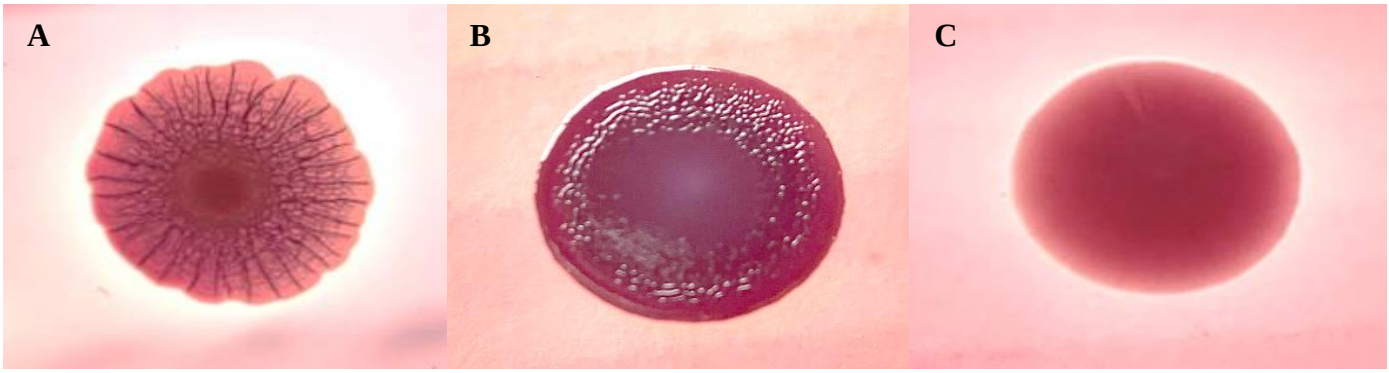

Figure 1. Phenotypic classification of rough, dry, and red (rdar) morphology displayed by *Salmonella* strains isolated from California pistachios; (A) Complete rdar morphotype. (B) Partial rdar morphotype. (C) Negative rdar morphotype, i.e., "smooth."

References

Bakker, H. C. den, Switt, A. I. M., Cummings, C. A., Hoelzer, K., Degoricija, L., Rodriguez-Rivera, L. D., Wright, E. M., Fang, R., Davis, M., Root, T., Schoonmaker-Bopp, D., Musser, K. A., Villamil, E., Waechter, H., Kornstein, L., Furtado, M. R., & Wiedmann, M. (2011). A whole-genome single nucleotide polymorphism-based approach to trace and identify outbreaks linked to a common *Salmonella enterica* subsp. *enterica* serovar Montevideo pulsed-field gel electrophoresis type. *Applied and Environmental Microbiology*, *77*(24), 8648–8655. https://doi.org/10.1128/AEM.06538-11

Bansal, A., Jones, T. M., Abd, S. J., Danyluk, M. D., & Harris, L. J. (2010). Most-probable-number determination of *Salmonella* levels in naturally contaminated raw almonds using two sample preparation methods. *Journal of Food Protection*, *73*(11), 1986–1992. https://doi.org/10.4315/0362-028x-73.11.1986

Blessington, T., Mitcham, E. J., & Harris, L. J. (2012). Survival of *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on inoculated walnut kernels during storage. *Journal of Food Protection*, *75*(2), 245–254. https://doi.org/10.4315/0362-028X.JFP-11-278

Brar, P. K., Strawn, L. K., & Danyluk, M. D. (2016). Prevalence, level, and types of *Salmonella* isolated from North American in-shell pecans over four harvest years. *Journal of Food Protection*, *79*(3), 352–360. https://doi.org/10.4315/0362-028X.JFP-15-365

Centers for Disease Control and Prevention. (2009). Multistate Outbreak of *Salmonella* Infections Linked to Pistachio Nuts (final update). Available

at: https://www.cdc.gov/salmonella/2009/pistachio-nuts-4-14-2009.html. Accessed 10 February 2023

Danyluk, M. D., Jones, T. M., Abd, S. J., Schlitt-Dittrich, F., Jacobs, M., & Harris, L. J. (2007). Prevalence and amounts of *Salmonella* found on raw California almonds. *Journal of Food Protection, 70*(4), 820–827. https://doi.org/10.4315/0362-028x-70.4.820

Eriksson de Rezende, C. L., Mallinson, E. T., Gupte, A., & Joseph, S. W. (2001). *Salmonella* spp. are affected by different levels of water activity in closed microcosms. *Journal of Industrial Microbiology & Biotechnology*, *26*(4), 222–225. https://doi.org/10.1038/sj.jim.7000116

Finn, S, Hinton, J. C., McClure, P., Amézquita, A., Martins, M., & Fanning, S. (2013). Phenotypic characterization of *Salmonella* isolated from food production environments associated with low-water activity Foods. *Journal of Food Protection*, *76*(9), 1488–1499. https://doi.org/10.4315/0362-028X.JFP-13-088

Finn, Sarah, Händler, K., Condell, O., Colgan, A., Cooney, S., McClure, P., Amézquita, A., Hinton, J. C. D., & Fanning, S. (2013). ProP is required for the survival of desiccated *Salmonella enterica* serovar typhimurium cells on a stainless steel surface. *Applied and Environmental Microbiology*, *79*(14), 4376–4384. https://doi.org/10.1128/AEM.00515-13

Garcés-Vega, F., & Marks, B. P. (2014). Use of simulation tools to illustrate the effect of data management practices for low and negative plate counts on the estimated parameters of microbial reduction models. *Journal of Food Protection*, *77*(8), 1372–1379. https://doi.org/10.4315/0362-028X.JFP-13-462

Gibson, D. L., White, A. P., Snyder, S. D., Martin, S., Heiss, C., Azadi, P., Surette, M., & Kay, W. W. (2006). *Salmonella* produces an O-antigen capsule regulated by AgfD and important for environmental persistence. *Journal of Bacteriology*, *188*(22), 7722–7730. https://doi.org/10.1128/JB.00809-06

Gruzdev, N., Pinto, R., & Sela, S. (2011). Effect of desiccation on tolerance of *Salmonella enterica* to multiple stresses. *Applied and Environmental Microbiology*, *77*(5), 1667–1673. https://doi.org/10.1128/AEM.02156-10

Haendiges, J., Davidson, G. R., Pettengill, J. B., Reed, E., Ramachandran, P., Blessington, T., Miller, J. D., Anderson, N., Myoda, S., Brown, E. W., Zheng, J., Tikekar, R., & Hoffmann, M. (2021). Genomic evidence of environmental and resident *Salmonella* Senftenberg and Montevideo contamination in the pistachio supply-chain. *Plos One*, *16*(11), e0259471. https://doi.org/10.1371/journal.pone.0259471

Harris, & Ferguson. (2013). Improving the safety of almonds and pistachios, p. 350–378. In L. J. Harris (ed.), Improving the safety and quality of nuts. Woodhead Publishing Ltd., Cambridge.

Harris, L., Lieberman, V., Mashiana, R. P., Atwill, E., Yang, M., Chandler, J. C., Bisha, B., & Jones, T. (2016). Prevalence and amounts of *Salmonella* found on raw California inshell pistachios. *Journal of Food Protection*, *79*(8), 1304–1315. https://doi.org/10.4315/0362-028X.JFP-16-054

Harris, Linda J., Uesugi, A. R., Abd, S. J., & McCarthy, K. L. (2012). Survival of *Salmonella* Enteritidis PT 30 on inoculated almond kernels in hot water treatments. *Food Research International*, *45*(2), 1093–1098. https://doi.org/10.1016/j.foodres.2011.03.048

Harris, L J, Yada, S., Beuchat, L. R., & Danyluk, M. D. (2022). Outbreaks of foodborne illness associated with the consumption of tree nuts, peanuts, and sesame seeds (version 2) [Table and references]. In Outbreaks from tree nuts, peanuts, and sesame seeds. Available at: https://ucfoodsafety.ucdavis.edu/low-moisture-foods/nuts-and-nut-pastes

Jayeola, V., McClelland, M., Porwollik, S., Chu, W., Farber, J., & Kathariou, S. (2020). Identification of novel genes mediating survival of *Salmonella* on low-moisture foods via transposon sequencing analysis. *Frontiers in Microbiology*, *11*, 726. https://doi.org/10.3389/fmicb.2020.00726

Karaca, B., Akcelik, N., & Akcelik, M. (2013). Biofilm-producing abilities of *Salmonella* strains isolated from Turkey. *Biologia*, *68*(1), 1–10. https://doi.org/10.2478/s11756-012-0138-2

Kieboom, J., Kusumaningrum, H. D., Tempelaars, M. H., Hazeleger, W. C., Abee, T., & Beumer, R. R. (2006). Survival, elongation, and elevated tolerance of *Salmonella* enterica serovar Enteritidis at reduced water activity. *Journal of Food Protection*, *69*(11), 2681–2686. https://doi.org/10.4315/0362-028X-69.11.2681

Kimber, M. A., Kaur, H., Wang, L., Danyluk, M. D., & Harris, L. J. (2012). Survival of *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on inoculated almonds and pistachios stored at -19, 4, and 24° C. *Journal of Food Protection*, *75*(8), 1394–1403. https://doi.org/10.4315/0362-028X.JFP-12-023

Lambertini, E., Danyluk, M. D., Schaffner, D. W., Winter, C. K., & Harris, L. J. (2012). Risk of salmonellosis from consumption of almonds in the North American market. *Food Research International*, *45*(2), 1166–1174. https://doi.org/10.1016/j.foodres.2011.05.039

Limcharoenchat, P., James, M. K., & Marks, B. P. (2019). Survival and thermal resistance of *Salmonella* Enteritidis PT 30 on almonds after long-term storage. *Journal of Food Protection*, *82*(2), 194–199. https://doi.org/10.4315/0362-028X.JFP-18-152

Mandal, R. K., & Kwon, Y. M. (2017). Global screening of *Salmonella* enterica Serovar Typhimurium genes for desiccation Survival. *Frontiers in Microbiology*, *8*, 1723. https://doi.org/10.3389/fmicb.2017.01723

Maserati, A., Fink, R. C., Lourenco, A., Julius, M. L., & Diez-Gonzalez, F. (2017). General response of *Salmonella enterica* serovar Typhimurium to desiccation: A new role for the virulence factors sopD and sseD in survival. *Plos One*, *12*(11), e0187692. https://doi.org/10.1371/journal.pone.0187692

Mattick, K. L., Jørgensen, F., Legan, J. D., Cole, M. B., Porter, J., Lappin-Scott, H. M., & Humphrey, T. J. (2000). Survival and filamentation of *Salmonella enterica* serovar enteritidis PT4 and *Salmonella enterica* serovar typhimurium DT104 at low water activity. *Applied and Environmental Microbiology*, *66*(4), 1274–1279. https://doi.org/10.1128/AEM.66.4.1274-1279.2000

Moussavi, M., Frelka, J. C., Hildebrandt, I. M., Marks, B. P., & Harris, L. J. (2020). Thermal resistance of foodborne pathogens and *Enterococcus faecium* NRRL B-2354 on inoculated pistachios. *Journal of Food Protection*, *83*(7), 1125–1136. https://doi.org/10.4315/JFP-19-561

Moussavi, M., Lieberman, V., Theofel, C., Barouei, J., & Harris, L. J. (2019). Growth of *Salmonella* and other foodborne pathogens on inoculated inshell pistachios during simulated

delays between hulling and drying. *Journal of Food Protection*, *82*(5), 815–825. https://doi.org/10.4315/0362-028X.JFP-18-450

Moyne, A. M., Lawal, O., Goodridge, L., & Harris, L. J. (2023). Genetic diversity of *Salmonella enterica* isolated from raw almonds and an almond orchard. Submitted to *PLOSOne* 12 May 2023

Nesse, L. L., Osland, A. M., Mo, S. S., Sekse, C., Slettemeås, J. S., Bruvoll, A. E. E., Urdahl, A. M., & Vestby, L. K. (2020). Biofilm forming properties of quinolone resistant *Escherichia coli* from the broiler production chain and their dynamics in mixed biofilms. *BMC Microbiology*, *20*(1), 46. https://doi.org/10.1186/s12866-020-01730-w

Norberto, A. P., Alvarenga, V. O., Hungaro, H. M., & Sant'Ana, A. S. (2022). Desiccation resistance of a large set of *Salmonella enterica* strains and survival on dry- and wet-inoculated soybean meal through storage. *LWT*, *158*, 113153. https://doi.org/10.1016/j.lwt.2022.113153

Römling, U, Rohde, M., Olsén, A., Normark, S., & Reinköster, J. (2000). AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella* typhimurium regulates at least two independent pathways. *Molecular Microbiology*, *36*(1), 10–23. https://doi.org/10.1046/j.1365-2958.2000.01822.x

Römling, U, Sierralta, W. D., Eriksson, K., & Normark, S. (1998). Multicellular and aggregative behaviour of *Salmonella* typhimurium strains is controlled by mutations in the agfD promoter. *Molecular Microbiology*, *28*(2), 249–264. https://doi.org/10.1046/j.1365-2958.1998.00791.x

Römling, U. (2005). Characterization of the rdar morphotype, a multicellular behaviour in *Enterobacteriaceae*. *Cellular and Molecular Life Sciences*, *62*(11), 1234–1246. https://doi.org/10.1007/s00018-005-4557-x

Römling, Ute, Bokranz, W., Rabsch, W., Zogaj, X., Nimtz, M., & Tschäpe, H. (2003). Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. *International Journal of Medical Microbiology*, *293*(4), 273–285. https://doi.org/10.1078/1438-4221-00268

Stocki, S. L., Annett, C. B., Sibley, C. D., McLaws, M., Checkley, S. L., Singh, N., Surette, M. G., & White, A. P. (2007). Persistence of *Salmonella* on egg conveyor belts is dependent on the belt type but not on the rdar morphotype. *Poultry Science*, *86*(11), 2375–2383. https://doi.org/10.3382/ps.2007-00121

Suehr, Q. J., Chen, F., Anderson, N. M., & Keller, S. E. (2020). Effect of pH on Survival of *Escherichia coli* O157, *Escherichia coli* O121, and *Salmonella enterica* during desiccation and short-term storage. *Journal of Food Protection*, 211–220. https://doi.org/10.4315/0362-028X.JFP-19-195

Tsukioka, Y., Yamashita, Y., Oho, T., Nakano, Y., & Koga, T. (1997). Biological function of the dTDP-rhamnose synthesis pathway in *Streptococcus* mutans. *Journal of Bacteriology*, *179*(4), 1126–1134. https://doi.org/10.1128/jb.179.4.1126-1134.1997

Uesugi, A. R., & Harris, L. J. (2006). Growth of *Salmonella* Enteritidis phage type 30 in almond hull and shell slurries and survival in drying almond hulls. *Journal of Food Protection*, *69*(4), 712–718. https://doi.org/10.4315/0362-028x-69.4.712

Vestby, L. K., Møretrø, T., Ballance, S., Langsrud, S., & Nesse, L. L. (2009). Survival potential of wild type cellulose deficient *Salmonella* from the feed industry. *BMC Veterinary Research*, *5*, 43. https://doi.org/10.1186/1746-6148-5-43

White, A. P., Gibson, D. L., Kim, W., Kay, W. W., & Surette, M. G. (2006). Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of *Salmonella*. *Journal of Bacteriology*, *188*(9), 3219–3227. https://doi.org/10.1128/JB.188.9.3219-3227.2006

White, A. P., & Surette, M. G. (2006). Comparative genetics of the rdar morphotype in *Salmonella*. *Journal of Bacteriology*, *188*(24), 8395–8406. https://doi.org/10.1128/JB.00798-06

Wicaksono, W. A., Egamberdieva, D., Cernava, T., & Berg, G. (2023). Viral community structure and potential functions in the dried-out aral sea basin change along a desiccation Gradient. *MSystems*, *8*(1), e0099422. https://doi.org/10.1128/msystems.00994-22

Zhang, G., Hu, L., Luo, Y., Santillana Farakos, S. M., Johnson, R., Scott, V. N., Curry, P., Melka, D., Brown, E. W., Strain, E., Bunning, V. K., Musser, S. M., & Hammack, T. S. (2021). Survey of *Salmonella* in raw tree nuts at retail in the United States. *Journal of Food Science*, *86*(2), 495–504. https://doi.org/10.1111/1750-3841.15569

Chapter V. Conclusions, limitations, and recommendations for further research

Phylogenetic analysis of *Salmonella* isolates isolated from California pistachios.

Salmonella diversity on pistachios.

A phylogenetic SNP analysis was used to determine the diversity of *Salmonella* isolates associated with pistachios grown in California (Chapter II). The results of this analysis indicated that in the decade between 2008 and 2018 a relatively small number of strains (13) were associated with California pistachios (Table 1). The analysis also provided further evidence that two strains of *Salmonella* Montevideo (strains A and B) and a *Salmonella* Senftenberg strain have persisted in the California pistachio supply chain over this time frame. In addition, this study identified, for the first time, additional persistent strains of *Salmonella* serovars Agona, Worthington, and Liverpool (strains A).

Distribution of Salmonella strains associated with pistachios.

Isolates of *Salmonella* serovars Agona, Enteritidis, Montevideo, and Senftenberg have been associated with both California pistachios and almonds. SNP analyses of these isolates indicated that *Salmonella* Enteritidis strains A and B and *Salmonella* Senftenberg sequence type (ST) 14, the only strain isolated from the pistachio silo survey, were recovered from both tree nuts. However, five of the strains that have been persistent in pistachios (*Salmonella* Agona, *Salmonella* Liverpool A, *Salmonella* Montevideo strains A and B, and *Salmonella* Worthington) have not been isolated from more than 15,000 almond samples evaluated over a similar time frame despite the geographic proximity of these two tree nut production areas. Moreover, using the NCBI (National Center for Biotechnology Information) Pathogen Database, we identified that all the closely related food isolates (≤15 SNPs) of *Salmonella* Agona, *Salmonella*

Montevideo, *Salmonella* Liverpool, and *Salmonella* Worthington strains found in California pistachios have been exclusively associated with U.S. pistachios and their environment. Overall, the genomic analysis in this study indicates that the diversity of *Salmonella* strains in the pistachio production chain is limited and a subset of those strains has persisted in and is unique to this environment.

Limitations and future research

The CFSAN SNP Pipeline was used in the present study for phylogenetic analysis, however, there are other genetic approaches that can be used to determine phylogeny based on SNP differences. Utilizing other approaches can lead to slightly different numbers of SNPs, consequently affecting the strain categories and the SNP threshold used. To our knowledge, there is no universal threshold for SNP differences to categorize isolates into strains. Additionally, there is no formal definition to differentiate persistent from sporadic strains. Therefore, consensus guidance is needed to develop phylogenies and classify food isolates into persistent or sporadic strains using whole-genome sequencing and metadata. In this study, we utilized a threshold of ≤14 SNPs to separate isolates into different strains. However, most of the isolates (exceptions *Salmonella* Enteritidis and *Salmonella* Senftenberg) clustered with seven or less SNP differences. As more *Salmonella enterica* isolate sequences populate the NCBI database, further SNP analyses are necessary to validate the hypothesis that the diversity of *Salmonella* strains in the pistachio production chain is limited and that a subset of those strains has persisted in and is unique to this environment.

Strain characteristics explaining *Salmonella* persistence in California pistachios.

Copper resistance in the production environment.

Chapter III provides further evidence that both *Salmonella* Montevideo strains A and B and a *Salmonella* Senftenberg strain (ST14), all previously described as persistent strains, contained the copper homeostasis and silver resistance island (CHASRI) (Table 2). Another persistent strain, *Salmonella* Worthington, also contained the CHASRI sequence. Phenotypic studies showed that the presence of CHASRI was associated with an increased minimal inhibitory concentration (MIC) to $CuSO_4$ under anaerobic conditions. However, no advantage was observed when cells were exposed to high concentrations of Cu-EDTA solution. Moreover, this study demonstrated that not all persistent strains contain the CHASRI sequence, suggesting that the presence of CHASRI alone cannot explain *Salmonella* persistence in the California pistachio environment. In the present study, *Salmonella* strains were inoculated into copper solutions in a 96-well plate. Future studies should evaluate the impact of copper in an environment that mimics the orchard environment, for example, by inoculating *Salmonella* in soil in the presence of competing microbiota and then spraying copper solutions, as soil components (chemical and microbial) may impact the effect of *Salmonella* MIC against copper.

Growth on pistachio hulls between harvest and hulling.

Chapter III demonstrated that both persistent and sporadic strains of *Salmonella* increased by 5 to 6 logs after 24 h of incubation at ambient by utilizing the nutrients from pistachio hulls (Table 2). A single sporadic strain, *Salmonella* Enteritidis strain A, showed a significantly lower population increase during this period; however, by 40 h, the mean population for this strain was not different from mean populations of the other strains. These results highlight the importance of reducing delays between harvest and hulling to prevent the growth of *Salmonella* on inhull pistachios. Future experiments should investigate the growth of individual *Salmonella* strains on inhull pistachios, as their fate may be affected by the presence of native bacteria and hull

characteristics. Additionally, studies should assess the potential for post-hulling cross-contamination in the float tank and explore the use of antimicrobials to reduce cross-contamination from occurring.

Biofilm-forming potential during post-harvest handling.

Seven of the nine *Salmonella* strains evaluated formed moderate biofilms; *Salmonella* Enteritidis strain A and *Salmonella* Liverpool strain A formed weak and strong biofilms, respectively (Chapter IV; Table 2). The biofilm-forming potential of each strain was evaluated by staining all components in the biofilm, including the biofilm matrix, live cells, and dead cells. However, this approach does not allow for the determination of the number of live cells in the biofilm, and thus the biofilm-forming potential presented here may not be correlated with the risk for *Salmonella* strain survival or cross-contamination potential. Furthermore, the biofilm-forming potential of *Salmonella* strains was investigated only on polystyrene. Future studies should assess *Salmonella* biofilm production on other surfaces commonly found in the postharvest handling environment such as stainless steel, conveyor belts, and plastic polymers. Additionally, different approaches to measure biofilm potential and biofilm components should be explored.

Resistance to desiccation and storage.

A total of four strains, two persistent and two sporadic, displayed a red, dry, and rough (rdar) morphology associated with enhancing desiccation resistance (Chapter IV, Table 2). All *Salmonella* strains contained the sequences of nine of the 10 genes that have previously been shown to be associated with the enhanced resistance of *Salmonella* on pistachios during drying and storage. However, persistent *Salmonella* Montevideo strains A and B were missing one of these genes (*rfbD*). Except for *Salmonella* Enteritidis strain A, the survival of the 10 *Salmonella*

strains evaluated, was comparable after drying and low relative humidity ambient storage for up 50 days. These studies suggest that neither the rdar morphotype nor the lack of the *rfbD* gene has an effect during desiccation and subsequent low humidity storage of these *Salmonella* strains. In the present study, filters were used as the matrix for the desiccation and storage; future research should use inshell pistachios, hull debris, or food contact surfaces as a matrix. Future studies should determine the impact that pistachio shell properties and native microbiota have on the survival of *Salmonella* strains. Additional studies should evaluate the role thermal resistance for each strain as this characteristic may play a role in *Salmonella* persistence during pistachio drying and roasting.

Overall conclusion

The present study sheds light on the diversity and persistence of *Salmonella* strains in the California pistachio production chain. While the number of strains is limited, some of them have been exclusive to this environment for at least a decade, indicating the presence of at least one reservoir of *Salmonella* that is in and specific to the pistachio supply chain. The location of such reservoirs is still unknown and thus further research should focus on investigating potential contamination patterns and routes. The present research has provided an understanding of the phenotypic and genotypic characteristics of *Salmonella* strains associated with California pistachios. However, no single characteristic studied here fully explained the association of specific *Salmonella* strains with pistachios. Instead, it appears that multiple factors, including phenotypic and genotypic features and environmental factors, may be involved in the persistence of this foodborne pathogen in this environment. Currently, the pistachio industry has implemented downstream process controls to manage the risk of *Salmonella* in pistachios. Knowledge regarding the original contamination source, the contamination routes, and the

determinants for *Salmonella* persistence may result in the development more effective and targeted strategies to mitigate *Salmonella* contamination risk in the pistachio production chain, and ultimately to protect public health.

Summary Table

Table 1. Occurrence of *Salmonella enterica* strains on U.S. pistachios and pistachio environments from 2008–2018

Occurrence in the pistachio environment [a]	***Salmonella* serovar**	**Strain** [a]	**Isolation year** [b]
Persistent	Agona	N/A [c]	2010, 2012, 2013
	Liverpool	A	2011, 2012, 2014, 2015, 2016
	Montevideo	A	2008, 2009, 2010, 2011, 2012, 2014, 2015, 2016, 2017, 2018
	Montevideo	B	2009, 2010, 2011, 2013, 2014, 2015 2017
	Senftenberg	ST14 [d]	2009, 2010, 2012, 2013, 2014, 2015 2016, 2017
	Senftenberg [f]	ST185 [d]	2005, 2011, 2013, 2014, 2015 2018
	Worthington	N/A	2010, 2011, 2012, 2014, 2016
Sporadic	Enteritidis	A	2010, 2011, 2012
	Enteritidis [g]	B	2010, 2011
	Liverpool	B	2012,
	Newport [f]	N/A	2009, 2016
	Tennessee	N/A	2012, 2014
	Sandiego	N/A	2010

[a] As described on Chapter II
[b] Data collected using isolates from Harris et al. 2016, Haendiges et al. 2020, and the NCBI Pathogen Detection Database.
[c] Not applicable; N/A
[d] Sequence type (ST) as described in Haendiges et al., 2020.
[f] *Salmonella* Senftenberg ST185 and *Salmonella* Newport isolates were not included in the genetic and phenotypic analysis presented
[g] Phage type 9c

Chapter VII. Appendix

Chapter II Supplemental Tables

Supplemental Table S1. *Salmonella* serovar information from outbreak investigation and current study (Estrada, Moyne, and Harris, Characterizing the genetic diversity of *Salmonella* isolated from U.S. raw inshell pistachios using whole genome sequencing). Sequences were submitted to NCBI under BioProject ID PRJNA976331.

Strain	Serovar	Collection year	Accession Number
LJH1274-1	Enteritidis	2010	SAMN35365209
LJH1275-1	Enteritidis	2010	SAMN35365210
LJH1279-2	Montevideo	2010	SAMN35365211
LJH1280-1	Tennessee	2010	SAMN35365212
LJH1281-1	Montevideo	2010	SAMN35365213
LJH1287-2	Montevideo	2010	SAMN35365214
LJH1288	Worthington	2010	SAMN35365215
LJH1289-1	Montevideo	2010	SAMN35365216
LJH1289-4	Montevideo	2010	SAMN35365217
LJH1290	Worthington	2010	SAMN35365218
LJH1291	Montevideo	2010	SAMN35365219
LJH1292-1	Sandiego	2010	SAMN35365220
LJH1293-1	Senftenberg	2010	SAMN35365221
LJH1295-1	Montevideo	2010	SAMN35365222
LJH1296-1	Montevideo	2010	SAMN35365223
LJH1297	Enteritidis	2010	SAMN35365224
LJH1298	Worthington	2010	SAMN35365225
LJH1299	Montevideo	2010	SAMN35365226
LJH1301	Montevideo	2010	SAMN35365227
LJH1302	Worthington	2010	SAMN35365228
LJH1303	Montevideo	2010	SAMN35365229
LJH1304	Worthington	2010	SAMN35365230
LJH1305-1	Worthington	2010	SAMN35365231
LJH1306	Senftenberg	2010	SAMN35365232
LJH1308	Agona	2010	SAMN35365233
LJH1309	Montevideo	2010	SAMN35365234
LJH1310	Senftenberg	2010	SAMN35365235
LJH1347-1	Montevideo	2011	SAMN35365236
LJH1348-1	Montevideo	2011	SAMN35365237
LJH1349-1	Enteritidis	2011	SAMN35365238

LJH1350-1	Liverpool	2011	SAMN35365239
LJH1351-1	Enteritidis	2011	SAMN35365240
LJH1352-1	Montevideo	2011	SAMN35365241
LJH1358	Montevideo	2011	SAMN35365242
LJH1359	Montevideo	2011	SAMN35365243
LJH1362	Montevideo	2011	SAMN35365244
LJH1363	Montevideo	2011	SAMN35365245
LJH1364	Montevideo	2011	SAMN35365246
LJH1373	Montevideo	2011	SAMN35365247
LJH1383	Montevideo	2011	SAMN35365248
LJH1384	Montevideo	2011	SAMN35365249
LJH1392	Montevideo	2011	SAMN35365250
LJH1393	Montevideo	2011	SAMN35365251
LJH1397	Montevideo	2011	SAMN35365252
LJH1398	Montevideo	2011	SAMN35365253
LJH1405	Montevideo	2011	SAMN35365254
LJH1406	Worthington	2011	SAMN35365255
LJH1407	Montevideo	2011	SAMN35365256
LJH1410	Montevideo	2011	SAMN35365257
LJH1411	Montevideo	2011	SAMN35365258
LJH1417	Montevideo	2011	SAMN35365259
LJH1418	Montevideo	2011	SAMN35365260
LJH1419	Montevideo	2011	SAMN35365261
LJH1428-1	Montevideo	2012	SAMN35365262
LJH1429	Agona	2012	SAMN35365263
LJH1430	Montevideo	2012	SAMN35365264
LJH1432-2	Montevideo	2012	SAMN35365265
LJH1433	Montevideo	2012	SAMN35365266
LJH1434	Montevideo	2012	SAMN35365267
LJH1435	Montevideo	2012	SAMN35365268
LJH1437-1	Senftenberg	2012	SAMN35365269
LJH1438-1	Enteritidis	2012	SAMN35365270
LJH1439-1	Montevideo	2012	SAMN35365271
LJH1440	Montevideo	2012	SAMN35365272
LJH1442	Montevideo	2012	SAMN35365273
LJH1443	Montevideo	2012	SAMN35365274
LJH1446	Montevideo	2012	SAMN35365275
LJH1447	Senftenberg	2012	SAMN35365276
LJH1448	Montevideo	2012	SAMN35365277
LJH1450	Montevideo	2012	SAMN35365278

LJH1452	Montevideo	2012	SAMN35365279
LJH1453	Montevideo	2012	SAMN35365280
LJH1455	Montevideo	2012	SAMN35365281
LJH1456	Montevideo	2012	SAMN35365282
LJH1457	Montevideo	2012	SAMN35365283
LJH1462-1	Worthington	2012	SAMN35365284
LJH1463-1	Worthington	2012	SAMN35365285
LJH1464-1	Montevideo	2012	SAMN35365286
LJH1465-1	Worthington	2012	SAMN35365287
LJH1466-1	Liverpool	2012	SAMN35365288
LJH1467	Liverpool	2012	SAMN35365289
LJH1468-1	Montevideo	2012	SAMN35365290
LJH1469	Worthington	2012	SAMN35365291
LJH1470	Senftenberg	2012	SAMN35365292
LJH1471	Montevideo	2012	SAMN35365293
LJH1473	Liverpool	2012	SAMN35365294
LJH1475	Liverpool	2012	SAMN35365295
LJH1477	Worthington	2012	SAMN35365296
LJH1478	Montevideo	2012	SAMN35365297
LJH1480	Montevideo	2012	SAMN35365298
LJH1481	Worthington	2012	SAMN35365299
LJH1483	Liverpool	2012	SAMN35365300
LJH1485	Senftenberg	2012	SAMN35365301
LJH1486	Montevideo	2012	SAMN35365302
LJH1489	Liverpool	2012	SAMN35365303
LJH1490	Worthington	2012	SAMN35365304
LJH1491	Montevideo	2012	SAMN35365305
LJH1494	Montevideo	2012	SAMN35365306
LJH1496	Liverpool	2012	SAMN35365307
LJH1497	Worthington	2012	SAMN35365308
LJH1498	Montevideo	2012	SAMN35365309
LJH1499	Worthington	2012	SAMN35365310
LJH1500	Liverpool	2012	SAMN35365311
LJH1501	Senftenberg	2012	SAMN35365312
LJH1503	Worthington	2012	SAMN35365313
LJH1504	Montevideo	2012	SAMN35365314
LJH1505	Senftenberg	2012	SAMN35365315
LJH1507	Worthington	2012	SAMN35365316
LJH1508	Worthington	2012	SAMN35365317
LJH1509	Liverpool	2012	SAMN35365318

LJH1510	Liverpool	2012	SAMN35365319
LJH1511	Montevideo	2012	SAMN35365320
LJH1512	Worthington	2012	SAMN35365321
LJH1514	Worthington	2012	SAMN35365322
LJH1515	Liverpool	2012	SAMN35365323
LJH1516	Montevideo	2012	SAMN35365324
LJH1517	Worthington	2012	SAMN35365325
LJH1519	Worthington	2012	SAMN35365326
LJH1520	Worthington	2012	SAMN35365327
LJH1522	Montevideo	2012	SAMN35365328
LJH1523	Liverpool	2012	SAMN35365329
LJH1525	Montevideo	2012	SAMN35365330
LJH1529	Worthington	2012	SAMN35365331
LJH1530	Montevideo	2012	SAMN35365332
LJH1534	Montevideo	2012	SAMN35365333
LJH1535	Worthington	2012	SAMN35365334
LJH1537-1	Montevideo	2012	SAMN35365335
LJH1538-1	Senftenberg	2012	SAMN35365336
LJH1539-1	Senftenberg	2012	SAMN35365337
LJH1547	Liverpool	2012	SAMN35365338
LJH1548	Montevideo	2012	SAMN35365339
LJH1552	Worthington	2012	SAMN35365340
LJH1553	Liverpool	2012	SAMN35365341
LJH1554	Senftenberg	2012	SAMN35365342
LJH1559	Liverpool	2012	SAMN35365343
LJH1560	Liverpool	2012	SAMN35365344
LJH1561	Senftenberg	2012	SAMN35365345
LJH1562	Worthington	2012	SAMN35365346
LJH1564	Senftenberg	2012	SAMN35365347
LJH1565	Worthington	2012	SAMN35365348
LJH1566	Liverpool	2012	SAMN35365349
LJH1567	Liverpool	2012	SAMN35365350
LJH1569	Worthington	2012	SAMN35365351
LJH1572	Montevideo	2012	SAMN35365352
LJH1576	Liverpool	2012	SAMN35365353
LJH1577	Montevideo	2012	SAMN35365354
LJH1578	Worthington	2012	SAMN35365355
LJH1579	Senftenberg	2012	SAMN35365356
LJH1580	Worthington	2012	SAMN35365357
LJH1583	Montevideo	2012	SAMN35365358

LJH1584	Worthington	2012	SAMN35365359
LJH1585	Montevideo	2012	SAMN35365360
LJH1586	Worthington	2012	SAMN35365361
LJH1587	Liverpool	2012	SAMN35365362
LJH1588	Montevideo	2012	SAMN35365363
LJH1589	Worthington	2012	SAMN35365364
LJH1590	Montevideo	2012	SAMN35365365
LJH1594	Worthington	2012	SAMN35365366
LJH1595	Liverpool	2012	SAMN35365367
LJH1596	Worthington	2012	SAMN35365368
LJH1597	Worthington	2012	SAMN35365369
LJH1598	Liverpool	2012	SAMN35365370
LJH1601	Liverpool	2012	SAMN35365371
LJH1602	Montevideo	2012	SAMN35365372
LJH1603	Liverpool	2012	SAMN35365373
LJH1606	Montevideo	2012	SAMN35365374
LJH1608	Worthington	2012	SAMN35365375
LJH1609	Liverpool	2012	SAMN35365376
LJH1610	Senftenberg	2012	SAMN35365377

Supplemental Table 2. *Salmonella enterica* isolates (from pistachios collected in U.S. states) from NCBI and selected for SNP analysis and comparison in Estrada et al. characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

Serovar	Strain	Isolate identifiers	U.S. state	Collection year	Isolation source	SNP cluster
Agona	CFSAN027942	CFSAN027942, SRS824013	California	2013	raw pistachio	PDS000106143.6
	CFSAN027941	CFSAN027941, SRS824055	California	2013	raw pistachio	PDS000106143.6
	CFSAN027940	CFSAN027940, SRS822693	California	2013	roasted salted pistachio	PDS000106143.6
Liverpool	CFSAN034798	CFSAN034798, SRS1157046	California	2014	pistachio	PDS000001378.254
	CFSAN069050	CFSAN069050, SRS2548212	California	2015	roasted light salt pistachio	PDS000001378.254
	CFSAN045761[a]	CFSAN045761, SRS1296264	Maryland	2016	raw pistachio kernel	PDS000001378.254
Montevideo	531954[b]	CFSAN000258, FDA_2010_149_Pistachio-2, SRS267423	N/A[c]	2009	pistachio	PDS000027237.224
	CFSAN051296[ad]	CFSAN051296, SRS1508280	N/A	2016	raw pistachio	PDS000027237.224
	CFSAN045764[ad]	CFSAN045764, SRS1296256	California	2016	raw pistachio	PDS000027237.224
	FCC0123	CFSAN010209, SRS621727	California	2016	pistachio	PDS000032600.9
Senftenberg	FSW0103[e]	CFSAN010507, SRS594877	California	2013	dried roasted pistachio	PDS000031739.10
	CFSAN045763[ad]	CFSAN045763, SRS1296255	California	2016	raw pistachio	PDS000031814.96
	CFSAN047866 [ad]	CFSAN047866, SRS1353149	California	2016	pistachio	PDS000031814.96
	CFSAN058295[a]	CFSAN058295, SRS1870973	Texas	2016	shelled pistachio	PDS000031814.96
Worthington	CFSAN057988	CFSAN057988, SRS1961867	California	2014	raw pistachio	PDS000031070.34
	CFSAN051295[a]	CFSAN051295, SRS1519260	N/A	2016	raw pistachio	PDS000031070.34
	CFSAN051201	CFSAN051201, SRS1473336	Washington	2016	pistachio	PDS000031070.34

[a] Isolate obtained from pistachios during a retail survey of raw tree nuts (Zhang et al. 2021).
[b] Isolate associated with the 2009 outbreak linked to pistachios (Whitham et al. 2021; Bakker et al. 2011)
[c] No available data; N/A
[d] Isolates associated with the 2016 outbreak linked to pistachios (Haendiges et al. 2018).
[e] Isolate associated with the 2013 outbreak linked to pistachios (Haendiges et al. 2021).

Supplemental Table 3. Culture collection identification of *Salmonella enterica* isolates from almond surveys (Moyne et al., 2023) selected for SNP analysis and comparison in Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

***Salmonella* serotype**	**Isolate identification (collection year)**
Agona	LJH1100 (2006), LJH1106 (2006)
Enteritidis	LJH0762 (2003), LJH1028 (2005), LJH1046 (2005), LJH1059 (2006**)**
Liverpool	LJH0783 (2004)
Montevideo	LJH0653 (2001), LJH0657 (2001), LJH0760 (2003), LJH1020 (2005), LJH1094 (2006), LJH1623-1 (2013)
Senftenberg	LJH0667 (2002), LJH0713 (2002), LJH1011 (2004), LJH1150 (2007), LJH1154 (2007)
Tennessee	LJH0715 (2002), LJH1330 (2005)
Worthington	LJH0692 (2002)

Supplemental Table 4. Click Here

Supplemental Table 5. SNP matrix generated by analyzing *Salmonella* Montevideo isolates in Cluster B with the CFSAN SNP Pipeline. In Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

	LJH1281	LJH1287-2	LJH1289-4	LJH1291	LJH1301	LJH1303	LJH1352-1	LJH1363	LJH1405	LJH1468	LJH1478	LJH1511	LJH1516	LJH1522	LJH1525	LJH1530	LJH1537-1	LJH1548	LJH1577	LJH1588
LJH1281	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1287-2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1
LJH1289-4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1291	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1301	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1303	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1352-1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LJH1363	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LJH1405	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1468	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1478	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1511	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1516	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1522	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LJH1525	1	2	1	1	1	1	0	0	1	1	1	1	1	0	0	1	1	0	1	1
LJH1530	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1537-1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1548	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LJH1577	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
LJH1588	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0

Supplemental Table 6. SNP matrix generated by analyzing *Salmonella* Agona isolates with the CFSAN SNP Pipeline. In Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

	LJH1308	LJH1429
LJH1308	0	3
LJH1429	3	0

Supplemental Table 7. SNP matrix generated by analyzing *Salmonella* Senftenberg isolates from the pistachio silo survey (Harris et al. 2016) and isolates associated with pistachio outbreaks with the CFSAN SNP Pipeline. In Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

	CFSAN045763	CFSAN047866	LJH1310	LJH1501	CFSAN058295
CFSAN045763	0	9	6	1	4
CFSAN047866	9	0	7	10	7
LJH1310	6	7	0	7	4
LJH1501	1	10	7	0	5
CFSAN058295	4	7	4	5	0

Supplemental Table 8. SNP matrix generated by analyzing *Salmonella* Enteritidis isolates isolated from pistachio (Harris et al. 2016) and almond surveys (Moyne et al. 2023) with the CFSAN SNP Pipeline. In Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

	LJH0762 Almond	LJH1024 Almond	LJH1028 Almond	LJH1046 Almond	LJH1059 Almond	LJH1275 Pistachio	LJH1297 Pistachio	LJH1349 Pistachio	LJH1351 Pistachio	LJH1438 Pistachio
LJH0762 Almond	0	527	522	788	48	526	788	535	788	788
LJH1024 Almond	527	0	5	766	533	8	769	16	769	769
LJH1028 Almond	522	5	0	760	528	5	760	13	760	760
LJH1046 Almond	788	766	760	0	796	766	1	774	3	3
LJH1059 Almond	48	533	528	796	0	533	796	541	796	796
LJH1275 Pistachio	526	8	5	766	533	0	768	14	768	768
LJH1297 Pistachio	788	769	760	1	796	768	0	777	4	4
LJH1349 Pistachio	535	16	13	774	541	14	777	0	777	777
LJH1351 Pistachio	788	769	760	3	796	768	4	777	0	0
LJH1438 Pistachio	788	769	760	3	796	768	4	777	0	0

Supplemental Table 9. SNP matrix generated by analyzing *Salmonella* Montevideo isolates isolated from pistachio (Harris et al. 2016) and almond surveys (Moyne et al. 2023) with the CFSAN SNP Pipeline. In Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

	LJH1020 Almond	LJH1094 Almond	LJH12872 Pistachio	LJH1289-1 Pistachio	LJH1347-1 Pistachio	LJH1352-1 Pistachio	LJH1453 Pistachio	LJH1548 Pistachio	LJH16231 Almond	LJH653 Almond	LJH657 Almond	LJH760 Almond
LJH1020 Almond	0	25	39	314	309	36	315	38	299	316	36	297
LJH1094 Almond	25	0	28	323	319	25	325	27	306	326	43	305
LJH12872 Pistachio	39	28	0	335	329	1	337	1	319	338	58	317
LJH1289-1 Pistachio	314	323	335	0	3	325	2	338	330	3	347	330
LJH1347-1 Pistachio	309	319	329	3	0	325	1	331	325	4	342	325
LJH1352-1 Pistachio	36	25	1	325	325	0	325	0	313	326	53	311
LJH1453 Pistachio	315	325	337	2	1	325	0	340	331	3	350	331
LJH1548 Pistachio	38	27	1	338	331	0	340	0	318	341	59	316
LJH16231 Almond	299	306	319	330	325	313	331	318	0	332	313	20
LJH653 Almond	316	326	338	3	4	326	3	341	332	0	351	332
LJH657 Almond	36	43	58	347	342	53	350	59	313	351	0	311
LJH760 Almond	297	305	317	330	325	311	331	316	20	332	311	0

Supplemental Table 10. SNP matrix generated by analyzing *Salmonella* Liverpool isolates isolated from pistachio (Harris et al. 2016) and almond surveys (Moyne et al. 2023) with the CFSAN SNP Pipeline. In Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

	LJH0783 Almond	LJH1350-1 Pistachio	LJH1500 Pistachio	LJH1509 Pistachio
LJH0783 Almond	0	46	47	34
LJH1350-1 Pistachio	46	0	3	30
LJH1500 Pistachio	47	3	0	31
LJH1509 Pistachio	34	30	31	0

Supplemental Table 11. SNP matrix generated by analyzing *Salmonella* Tennessee isolates isolated from pistachio (Harris et al. 2016) and almond surveys (Moyne et al. 2023) with the CFSAN SNP Pipeline. In Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

	LJH0715 Almond	LJH1030 Almond	LJH1280 Pistachio
LJH0715 Almond	0	134	132
LJH1030 Almond	134	0	114
LJH1280 Pistachio	132	114	0

Supplemental Table 12. SNP matrix generated by analyzing *Salmonella* Worthington isolates isolated from pistachio (Harris et al. 2016) and almond surveys (Moyne et al. 2023) with the CFSAN SNP Pipeline. In Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

	LJH0692 Almond	LJH1288 Pistachio	LJH1406 Pistachio	LJH1589 Pistachio
LJH0692 Almond	0	66	63	63
LJH1288 Pistachio	66	0	3	3
LJH1406 Pistachio	63	3	0	0
LJH1589 Pistachio	63	3	0	0

Supplemental Table 13. *Salmonella* serotypes isolated from positive pistachio samples (Harris et al. 2016; Table 5). In Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

		Serovar[c]									
Harvest Yr (pistachio type)[a]	Sample no. (Table 5 Harris et al., 2016)[b]	Ag	En 9c	En 37	Lp A	Lp B	Mo A	Mo B	Se	Te	Wo
2010 (floater)	1 (1)						1	1			1
	2 (2)			1			1	1			1
	3 (3)										1
	4 (4)	1					1				
								1			1
2011 (floater)	1						1				
	2						1				
	3							1			
								1			
							1				
							1				
							1				
							1				
							1				
							1				
							1				
											1
							1				
2012 (floater)	1	1					1				
	2						1				
	3						1		1		
	4			1			1				
	5						1				
	6				1		1		1		1
	7										1
	8				1		1				1
	9				1		1	1			1
	10				1		1		1		1
	11				1		3				1
	12						1				
	13				1			1	1		1
	14				1				1		1
							1				
							1				

						1					
						1		1			
						1					
						1					
				1		1		1		1	
						1		1		1	
										1	
				1	1		1			1	
										1	
				1			1			1	
				1			1			1	
							1			1	
						1				1	
				1		1				1	
				1		1				1	
				1		1				1	
						1	1			1	
				1						1	
				1						1	
				1							
				1		1				1	
2010 (sinker)	1				1						
	2		1								
	3								1		
	4					1					
	5					1	1			1	
	6							1			
	7							1			
								1			
2011 (sinker)	1		1								
	2				1						
	3			1							
2012 (sinker)	1							1			
								1			
No. of samples (of 69)		2	2	4	19	1	43	13	13	1	30
% total samples with serovar		3%	3%	6%	28%	1%	62%	19%	19%	1%	43%

[a] F, floater; S, sinker

[b] Harris, L. J., V. Lieberman, R. P. Mashiana, E. Atwill, M. Yang, J. C. Chandler, B. Bisha, and T. Jones. 2016. Prevalence and amounts of *Salmonella* found on raw California inshell pistachios. J. Food Prot. 79(8):1304–1315. https://doi.org/10.4315/0362-028X.JFP-16-054. Initially positive samples are numbered; samples that were initially negative but positive on retesting have no number.

[c] Ag, Agona; En 9c, Enteritidis PT 9c; En 37, Enteritidis PT 37; Lp, Liverpool; Mo, Montevideo; Se, Senftenberg; Te, Tennessee; Wo, Worthington.

Supplemental Table 14. Isolates (as of March 2023) on the NCBI Pathogen Database neighboring (≤ 15 SNPs) NCBI *Salmonella* Agona, Worthington, and Liverpool isolates that clustered with isolates from the pistachio silo survey as shown in Estrada et al. Characterizing the genetic diversity of *Salmonella* isolated from raw inshell pistachios using whole genome sequencing.

Salmonella serotype	Strain	Strain	Location	Isolation source
Agona	N/A[a]	CFSAN027940	USA:CA	roasted salted pistachio
		CFSAN027942	USA:CA	raw pistachio
		CFSAN027941	USA:CA	raw pistachio
		95060	United Kingdom	human
		29239	United Kingdom	human
Liverpool	A	CFSAN045761	USA:MD	raw pistachio kernel
		CDPHFDLB-F1602021-001B	USA:CA	pistachio
		CDPHFDLB-F1602021-001A	USA:CA	pistachio
		FDA930361-15-XLD1	USA:CA	raw pistachio
		CFSAN048787	USA:CA	pistachio
		CFSAN049333	USA:CA	pistachio
		CFSAN049334	USA:CA	pistachio
		PNUSAS005061	USA	stool
		CFSAN059814	USA:CA	pistachio
		CFSAN059784	USA:CA	pistachio
		CFSAN059783	USA:CA	pistachio
		PNUSAS010320	USA	urine
		PNUSAS011919	USA	stool
		CFSAN069051	USA:CA	finished pistachio
		CFSAN069050	USA:CA	roasted light salt pistachio
		CFSAN078000	USA:CA	environmental swab sponge
		CFSAN088284	USA:CA	sponge
		CDPH_CSalm365	USA: CA	unknown[b]
	B	CFSAN034798	USA:CA	pistachio
Worthington	N/A	CFSAN051201	USA:WA	pistachio
		CFSAN057988	USA:CA	raw pistachio
		CFSAN069054	USA:CA	environmental swab
		13-0328	Canada	unknown
		CFSAN051295	USA	raw pistachio
		PNUSAS070514	USA	unknown
		2016AM-1038	USA	unknown
		PNUSAS293645	USA	unknown

[a] N/A, not applicable.

[b] Samples with isolation source missing in the NCBI Database.

Chapter IV Supplemental Tables

Supplemental Table 1. Presence of genes mediating the survival of *Salmonella* on pistachios during storage

Salmonella serovar	Strain [a]	Isolate ID	Presence of survival genes in *Salmonella* isolates obtained from California pistachios [b]									
			sspA	*barA*	*uvrB*	*damX*	*rfbD*	*uvrY*	*lrhA*	*yifE*	*rbsR*	*ompR*
Agona	NA [d]	LJH1308	+	+	+	+	+	+	+	+	+	+
Enteritidis	A	LJH1297	+	+	+	+	+	+	+	+	+	+
Enteritidis	B	LJH1275-1	+	+	+	+	+	+	+	+	+	+
Liverpool	A	LJH1350	+	+	+	+	+	+	+	+	+	+
Liverpool	B	LJH1509	+	+	+	+	+	+	+	+	+	+
Montevideo	A	LJH1347	+	+	+	+	-	+	+	+	+	+
Montevideo	B	LJH1352-1	+	+	+	+	-	+	+	+	+	+
Senftenberg	NA	LJH1310	+	+	+	+	+	+	+	+	+	+

[a] Strain designation as presented in Estrada et al. (2023).
[b] Selected genes were identified important for the survival of *Salmonella* on pistachios (Jayeola et al., 2020).
[c] NA, not applicable.

Supplemental Table. Genes mediating the survival of *Salmonella* on pistachios during desiccation and storage.

Gene	Gene location	Description [a]
sspA	3531817–3532455	Stringent starvation protein A
barA	3096636–3099392	Sensory histidine kinase of two-component regulatory system with UvrY
uvrB	884568–886589	Response regulator of two-component system along with BarA
damX	3660940–3662217	Cell division protein, septum
rfbD	2186732–2187631	dTDP-4-dehydrorhamnose reductase, LPS O-antigen biosynthesis
uvrY	2008299–2008955	Response regulator of two-component system along with BarA
lrhA	2450142–2451080	LysR family NADH dehydrogenase transcriptional repressor
yifE	4127740–4128078	Hypothetical protein
rbsR	4117784–4118782	Ribose operon repressor, D-ribose utilization
ompR	3680842–3681561	Transcriptional osmolarity response regulator for *ompF* and *ompC*

[a] As described in Supplementary Table 2; *Data for each individual survival experiment using single gene knockouts* by Jayeola et al., 2020.

Jayeola V, McClelland M, Porwollik S, Chu W, Farber J, Kathariou S. Identification of novel genes mediating survival of *Salmonella* on low-moisture foods via transposon sequencing analysis. Front Microbiol. 2020 May 15;11:726. doi: 10.3389/fmicb.2020.00726.