Survey of *Salmonella* in raw tree nuts at retail in the United States

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Abstract: The objective of this survey was to estimate the prevalence, contamination level, and genetic diversity of *Salmonella* in selected raw, shelled tree nuts (Brazil nuts, cashews, hazelnuts, macadamia nuts, pecans, pine nuts, pistachios, and walnuts) at retail markets in the United States. A total of 3,374 samples of eight tree nuts were collected from different types of retail stores and markets nationwide between September 2015 and March 2017. These samples (375 g) were analyzed using a modified FDA's BAM *Salmonella* culture method. Of the 3,374 samples, 15 (0.44%) (95% confidence interval [CI] [0.25, 0.73]) were culturally confirmed as containing *Salmonella*; 17 isolates were obtained. Among these isolates, there were 11 serotypes. *Salmonella* was not detected in Brazil nuts (296), hazelnuts (487), pecans (510), pine nuts (500), and walnuts (498). *Salmonella* prevalence estimates in cashews (510), macadamia (278), and pistachios (295) were 0.20% (95% CI [<0.01, 1.09]), 2.52% (95% CI [1.02, 5.12]), and 2.37% (95% CI [0.96, 4.83]), respectively. The rates of *Salmonella* isolation from major/big-chain supermarkets (1381), small-chain supermarkets (328), discount/variety/drug stores (1329), and online (336) were 0.29% (95% CI [0.08, 0.74]), 0.30% (95% CI [0.01, 1.69]), 0.45% (95% CI [0.17, 0.98]), and 1.19% (95% CI [0.33, 3.02]), respectively. *Salmonella* prevalence in organic (530) and conventional (2,844) nuts was not different statistically ($P = 0.0601$). Of the enumerated samples (15), 80% had *Salmonella* levels ≤0.0092 most probable number (MPN)/g. The highest contamination level observed was 0.75 MPN/g. The prevalence and contamination levels of *Salmonella* in the tree nuts analyzed were generally comparable to previous reports. Pulsed-field gel electrophoresis, serotype, and sequencing data all demonstrated that *Salmonella* population in nuts is very diverse genetically.

Keywords: diversity, low moisture, low water activity, nuts, Salmonella, serotype, WGS

Practical Application: The prevalence, contamination level, and genetic diversity of *Salmonella* in eight types of tree nuts (3,374 samples collected nationwide) revealed in this survey could help the development of mitigation strategies to reduce public health risks associated with consumption of these nuts.

1. INTRODUCTION

Low-water-activity foods, such as tree nuts and peanuts, do not have the favorable characteristics for bacterial growth. However, *Salmonella* has been detected in almonds, cashews, hazelnuts, macadamia nuts, peanuts, pecans, pine nuts, pistachios, and walnuts in the past worldwide (Bansal, Jones, Abd, Danyluk, & Harris, 2010; Bedard, Kennedy, & Weimer, 2014; Blessington, Theofel, Mitcham, & Harris, 2013; Brar, Strawn, & Danyluk, 2016; Calhoun, Post, Warren, Thompson, & Bontempo, 2013; Danyluk et al., 2007; Davidson, Frelka, Yang, Jones, & Harris, 2015; Little, Jemmott, Surman-Lee, Hucklesby, & De Pinna, 2009; Miksch et al., 2013; Zhang, Hu, Melka, et al., 2017). *Salmonella* has been shown to survive for a long period of time on tree nuts at ambient temperature (Abd, McCarthy, & Harris, 2012; Frelka, Davidson, & Harris, 2016; Kimber, Kaur, Wang, Danyluk, & Harris, 2012; Santillana Farakos, Pouillot, & Keller, 2017). In addition to *Salmonella*, *Escherichia coli* O157:H7 has been found in hazelnuts, peanuts, and walnuts (Canadian Food Inspection Agency, 2011; Centers for Disease Control and Prevention, 2011a; Miksch et al., 2013; Yada, 2019), and was reported to survive on in-shell hazelnuts, almonds, pistachios, and walnuts (Blessington et al., 2013; Feng, Muyyarikkandy, Brown, & Amalaradjou, 2018; Kimber et al., 2012). *Listeria monocytogenes* has been found in cashews, almonds, walnuts, macadamia nuts, pine nuts, and mixed nuts (Eglezos, 2010; Ly, Parreira, & Farber, 2019; Yada, 2019). Bacteria with potential health risks especially for the young, old, and immunocompromised people, such as *Pseudomonas*, *Clostridium* spp., and *Klebsiella* spp., have also occasionally been found in nuts (Al-Moghazy, Boveri, & Pulvirenti, 2014; Atungulu & Pan, 2012). Approximately 40 species of *Aspergillus* have been implicated in human or animal infections that can infect and cause decay in nuts. Some *Aspergillus* species produce aflatoxin, which is both a toxin and a carcinogen (Atungulu & Pan, 2012). The presence of such biological contaminants on tree nuts has the potential to lead to foodborne illness.

There were at least 25 recalls in 2015 in the United States alone due to *Salmonella* contamination of walnuts, pecans, macadamia nuts, pine nuts, almonds, and hazelnuts (Yada, 2019). Worldwide, there have been outbreaks of foodborne illness associated with almonds, cashews, hazelnuts, pine nuts, pistachios, and walnuts, with a majority of them caused by *Salmonella*. For example, in 2000, an outbreak of *Salmonella* Enteritidis resulted in 157 cases of human illness in Canada and 11 cases in the United States. The outbreak strain was isolated from almond samples collected from home,

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Table 1–*Salmonella* prevalence in raw, shelled tree nut samples from the U.S. retail market, based on 375-g sample size.

Nut type(Raw, Shelled)	No. of samples tested[a]			No. of samples positive for *Salmonella*[b]	*Salmonella* prevalence (%) [95% CI]
	Conventional	Organic	Total		
Brazil	239	57	296	0	0 [0, 1.24]
Cashews	343	167	510	1	0.20 [<0.01, 1.09]
Hazelnuts	426	61	487	0	0 [0, 0.75]
Macadamia	221	57	278	7	2.52 [1.02, 5.12]
Pecans	481	29	510	0	0 [0, 0.72]
Pine Nuts	438	62	500	0	0 [0, 0.74]
Pistachios	241	54	295	7	2.37 [0.96, 4.83]
Walnuts	455	43	498	0	0 [0, 0.74]
Total	2,844	530	3,374	15	0.44 [0.25, 0.73]

[a]Organic nuts accounted for 15.71% (530/3,374) of the total number of samples analyzed.
[b]Four isolates were from organic macadamia nuts; one isolate was from organic pistachios. *Salmonella* prevalence was 0.94% (5/530) in organic nuts and 0.35% (10/2,844) in conventional nuts. Chi-square test: $P = 0.0601$.
Abbreviation: CI, Clopper–Pearson's 95% confidence interval.

retail, distribution, and warehouse sources and from environmental swabs of processing equipment and associated farmers' orchards (Foodborne Illness Outbreak Database, 2000). In 2011, Turkish pine nuts from bulk bins contaminated with *Salmonella* Enteritidis caused 43 human illnesses in 5 states (Centers for Disease Control and Prevention, 2011b). During 2013–2014, raw cashew cheese contaminated with *Salmonella* Stanley caused 17 illnesses and 3 hospitalizations in 3 states (Centers for Disease Control and Prevention, 2014b). In 2014, 6 people in 5 states were infected with *Salmonella* Braenderup. Collaborative investigation indicated that almond and peanut butter was the likely source of this outbreak (Centers for Disease Control and Prevention, 2014a). In 2016, there was an outbreak of *Salmonella* Montevideo and *Salmonella* Senftenberg linked to pistachios involving 9 U.S. states, sickening 11 people (Centers for Disease Control and Prevention, 2016b). These events are examples of the potential public health risk associated with consumption of tree nuts contaminated with *Salmonella*. Information on the extent of *Salmonella* contamination, level of contamination, genetic diversity of *Salmonella* in tree nuts, and type of tree nuts contaminated could provide valuable information to help the development of mitigation strategies to reduce public health risks associated with consumption of nuts.

Pulsed-field gel electrophoresis (PFGE), multiple-locus variable-number tandem-repeat analysis (MLVA), and DNA microarray-based comparative genomic indexing (CGI) were used to evaluate the genetic relatedness of *Salmonella* Enteritidis isolates from three outbreaks associated with raw almonds. All three methods differentiated these *Salmonella* Enteritidis strains in a manner that correlated with phage type (PT). PFGE and plasmid profiling were capable of differentiating isolates of *Salmonella* Enteritidis PT1 and PT4. However, different PTs showed the same PFGE pattern; and different PFGE types were determined to be the same PT (Lukinmaa, Schildt, Rinttilä, & Siitonen, 1999). Besides, PFGE, MLVA, and CGI failed to discriminate between *Salmonella* Enteritidis PT30 strains related to outbreaks from unrelated clinical strains or between strains separated by up to 5 years (Parker, Huynh, Quiñones, Harris, & Mandrell, 2010). In a survey to determine the prevalence of *Salmonella enterica* in the environment of a major produce region in California, PFGE analysis indicated that some of the *Salmonella* isolates are indistinguishable and/or highly related (Gorski et al., 2011). High-resolution subtyping may provide more insight into the genetic makeup of the bacterial population. Whole genome sequencing (WGS) technology is advancing in an unprecedented pace in the last decade. It might be the ultimate tool to differentiate genetically closely related bacterial isolates. The U.S. Food and Drug Administration (FDA) and other public health agencies in the world are routinely using WGS in recent years allowing for significantly greater detailed analysis of isolates and outbreak investigations (Allard et al., 2016; Davis et al., 2015).

The objectives of this study were (1) to estimate the prevalence and contamination level of *Salmonella* in eight types of raw, shelled tree nuts in the U.S. retail markets, including products derived from conventional and organic production systems; (2) to investigate the diversity of the isolates obtained from these samples by means of PFGE, serotyping, and WGS; and (3) to characterize virulence genes and pathogenicity islands of the isolates using WGS data. The results will provide information about temporal variability in *Salmonella* prevalence, level of contamination, serotype, and genetic diversity in raw, shelled tree nuts and will assist the U.S. FDA in the development of a quantitative assessment of the risk of human salmonellosis associated with the consumption of tree nuts in the United States (U.S. Food and Drug Administration, 2013). This work was performed in the context of an ongoing national survey conducted by U.S. FDA on the prevalence of foodborne pathogens in produce, spices, and nuts (Zhang et al., 2018; Zhang, Hu, Melka, et al., 2017; Zhang, Hu, Pouillot, et al., 2017).

2. MATERIALS AND METHODS

2.1 Tree nuts

From September 30, 2015 to March 29, 2017, a total of 3,374 raw, shelled tree nut samples, including Brazil nuts, cashews, pecans, hazelnuts, macadamia nuts, pistachios, pine nuts, and walnuts, were collected and analyzed (Table 1). Samples were consisted of whole nuts, halves, pieces, and diced or chopped nuts. Tree nuts that had been roasted or coated with candy or chocolate seasonings, nut butters, nut pastes, nut meals, nut flours, or mixed nuts were not included in the sampling.

To be as representative as possible, the collection sites were selected using the U.S. Census Bureau map (https://www.census.gov/geo/maps-data/maps/reference.html): California, West Region, Pacific Division; Colorado, West Region, Mountain Division; Georgia, South Region, South Atlantic Division; Maryland, South Region, South Atlantic Division; Minnesota, Midwest Region, West North Central Division; Texas, South Region, West South Central Division; Washington, West Region, Pacific Division; Illinois, Midwest Region, East North Central Division; North Carolina, South Region, South Atlantic Division; and Vermont, Northeast region, New England Division.

Tree nut samples were collected from different types of retail venues categorized as (1) major chain/big-chain supermarkets,

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Table 2–*Salmonella* prevalence in raw, shelled tree nut samples according to retail source and serotypes of isolates recovered from positive samples, based on 375-g sample size.

Retailer type	No. (%) of samples	No. of points of sale	No. of samples positive for *Salmonella*	*Salmonella* prevalence (%)[95% CI]	*Salmonella* serotype[a] (No. of isolates)
Major or big chain supermarkets	1,381 (40.93)	168	4	0.29 [0.08, 0.74]	Muenchen (1), Liverpool (1), Senftenberg (2)
Small chain supermarkets	328 (9.72)	57	1	0.30 [0.01, 1.69]	*diarizonae* O61(1)
Discount, variety, or drug stores	1329 (39.39)	148	6	0.45 [0.17, 0.98]	Mbandaka (1), Urbana (1), *diarizonae* N.N. (1), Give (3)
Online	336 (9.96)	120	4	1.19 [0.33, 3.02]	Senftenberg (1), Worthington (1), Montevideo (2), Mbandaka (1), Duisburg (1)
Total	3,374	493	15	0.44 [0.25, 0.73]	

Abbreviation: CI, Clopper–Pearson's 95% confidence interval.
[a]Two pistachios samples obtained from online retailers had two different serotypes each.

including national and regional supermarkets; (2) small-chain/independent organic and specialty supermarkets, including retail outlets; and (3) discount/variety/drug stores: discount stores, including large discount clubs such as BJ's, Costco, and Sam's Club, and smaller discount stores such as Dollar stores; other significant points of sale for which foods are just a fraction of their business, including national and regional drug stores, gas stations, and so on; (4) online retailers. The number of samples collected from each market category and the number of unique addresses visited when collecting samples are listed in Table 2.

In most cases, the minimum sample size was 800 g. For a few samples of pine nuts, where it was difficult to obtain the required amount from a single lot, 500 g per sample was purchased. The lesser amount was sufficient to ensure that 375 g would be analyzed for *Salmonella*. When a sample was positive and enumeration by MPN was needed, this lesser amount would not be enough for the 3 × 100 g subsequent dilution series. However, all pine nut samples were negative for *Salmonella* and thus the smaller sample size did not affect the survey results.

Only prepackaged (e.g., bags, cans, or jars) tree nuts labeled raw were collected. Samples were not repackaged at the time of purchasing to avoid cross-contamination. Collected samples remained sealed before microbiological analysis. Nuts in open bins, where consumers self-serve, were not sampled, nor were nuts from displays in the shops. Whenever multiple retail sale units were required to attain a sufficient sample size, all units were from the same lot and were placed in one plastic zip-style bag. All samples were assigned a unique identifier that identified the producer, or distributor, as well as a "use by" or "sell by" date and/or lot number. Collected nuts were held at 4 °C before microbiological analysis and analyzed in 2 weeks.

2.2 Microbiological analyses

The sample size used for analyzing the presence/absence of *Salmonella* in tree nuts was 375 g. Nut to pre-enrichment broth ratio was 1:9 (w/v). Detection, isolation, and confirmation of *Salmonella* from nuts were performed with a modified method described in the FDA *Bacteriological Analytical Method* (BAM) Chapter 5, *Salmonella* (U.S. Food and Drug Administration, 2018). Lactose broth used in the BAM *Salmonella* method was replaced with buffered peptone water.

Enumeration of *Salmonella* in samples that tested positive was conducted by a three-tube, five-dilution (100, 10, 1.0, 0.1, and 0.01 g) most probable number (MPN) method and FDA BAM Chapter 5, *Salmonella* (U. S. Food and Drug Administration, 2017; U.S. Food and Drug Administration, 2018). Lactose broth was replaced with buffered peptone water as above.

2.3 *Salmonella* serotyping

Serotyping of *Salmonella* isolates was conducted using the Luminex xMAP® *Salmonella* serotyping assay (Luminex, Austin, TX). Untypeable isolates by Luminex xMAP® *Salmonella* serotyping assay were serotyped using the conventional Kauffman–White antigenic formulae scheme (Grimont & Weill, 2007; U.S. Food and Drug Administration, 2018).

2.4 PFGE analysis

PFGE laboratory analysis of the *Salmonella* isolates followed official Centers for Disease Control and Prevention (CDC) PulseNet protocols. *XbaI* was utilized as the primary restriction enzyme and *BlnI* as the secondary restriction enzyme (Centers for Disease Control and Prevention, 2016c). PFGE patterns were analyzed with the BioNumerics v.7.6 software (Applied Maths, Austin, TX).

2.5 WGS and analysis

Salmonella isolates were grown in tryptic soy broth (TSB, Difco, Franklin Lakes, NJ) overnight at 37 °C. Genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA). DNA concentrations were measured with a Qubit fluorometer (Life Technologies, Invitrogen, CA, USA), standardized to 0.2 ng/μL, and stored at −20 °C prior to library preparation. Libraries were prepared with the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. All genomes were sequenced using the Illumina MiSeq sequencing technology with 500 (2 × 250) cycles (Illumina), pair-end library with coverage depth of 30 to 90× at the Center for Food Safety and Applied Nutrition (CFSAN), FDA. Assembled sequenced reads were submitted to the Sequence Read Archive (SRA) of National Center for Biotechnology Information (NCBI) (Allard et al., 2016).

A k-mer-based approach, kSNP3.0, was used to generate the pan single nucleotide polymorphism (SNP) matrix for the 17 draft genomes, with the optimum k-mer size of 19, which was determined by Kchooser provided in kSNP 3.0 (Gardner, Slezak, & Hall, 2015). The phylogenetic tree was then inferred on the pan SNP matrix using FastTree 2.1.7 with generalized time-reversible models of nucleotide evolution, two rate categories of sites, and Gamma likelihoods (Price, Dehal, & Arkin, 2010). We also checked the results by CFSAN SNP pipeline, using the complete genome of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2

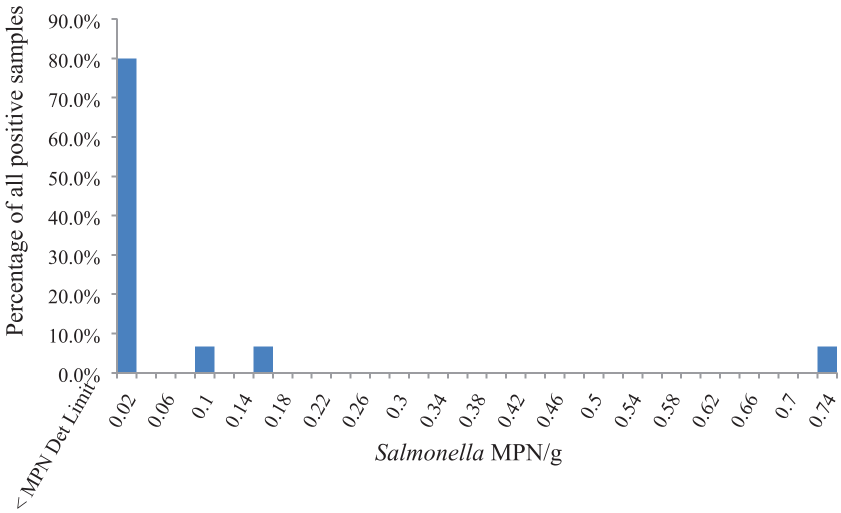

Figure 1–Estimates of *Salmonella* populations of contaminated raw, shelled tree nut samples, by a three-tube, five-dilution (100, 10, 1.0, 0.1, and 0.01 g) most probable number (MPN) assay.

(NC_003197.2) as the reference (Davis et al., 2015). Thirteen pathogenicity islands in *Salmonella* (SPIs; including SPI-1 to SPI-10, HPI, SGI-1, and CS54 island) and 45 selected virulence genes were used to characterize and profile the *Salmonella* isolates from the eight types of tree nuts studied (Borges et al., 2017; Levin, 2009; Schmidt & Hensel, 2004). The presence and absence of the genes and SPIs were analyzed by blasting WGS data of the isolates against the NCBI database (Camacho et al., 2009).

2.6 Statistical analysis

Confidence intervals (CIs) for prevalence rates were derived using the Clopper and Pearson's procedure; prevalence rates were compared using Fisher's exact test; and the Cochran–Mantel–Haenszel chi-square test was used to test the conditional independence of a factor (conventional vs. organically grown, types of retail) for each tree nut commodity. Statistical analysis was performed using GraphPad Prism v7.0 (GraphPad Software, La Jolla, CA, USA).

3. RESULTS AND DISCUSSION

3.1 *Salmonella* prevalence in tree nuts

The prevalence of *Salmonella* in raw, shelled nut samples collected at retail in the United States is shown in Table 1. It is important to point out that our analytical sample size is 375 g, instead of the regular 25 g. Theoretically, our method is 15 times more sensitive than the regular 25 g method. Overall, *Salmonella* was isolated from 15 of 3,374 samples analyzed over the course of the survey. *Salmonella* was not detected in Brazil nuts (n = 296), hazelnuts (n = 487), pecans (n = 510), pine nuts (n = 500), and walnuts (n = 498). *Salmonella* prevalence estimates for cashews (n = 510), macadamia nuts (n = 278), and pistachios (n = 295) were 0.20% ([95% CI [<0.01, 1.09]), 2.52% ([95% CI [1.02, 5.12]), and 2.37% ([95% CI [0.96, 4.83]), respectively. Macadamia nuts had the highest *Salmonella* prevalence nominally among all eight types of nuts. The overall prevalence of *Salmonella* among all 3,374 samples collected (eight types of tree nuts) was 0.44% (95% CI [0.25, 0.73]). *Salmonella* was not detected in pecans (n = 623) or in-shell hazelnuts (n = 80) during a previous nationwide survey (sample size = 375 g) we carried out between October 2014 and October 2015 (Zhang, Hu, Melka, et al., 2017). Moreover, prevalence in raw, shelled cashews (n = 733), hazelnuts (n = 577), pine nuts (n = 630), walnuts (n = 658), and macadamia nuts (n = 355) were 0.55%, 0.35%, 0.48%, 1.22%, and 4.20%, respectively, and overall prevalence in 3,656 samples analyzed was 0.88% (Zhang, Hu, Melka, et al., 2017). In both surveys (n = 7,030), the highest number of positive *Salmonella* samples was observed in macadamia nuts. Moreover, *Salmonella* was not detected on pecans in either survey. However, the present results differ from the results in the 2014 to 2015 survey where *Salmonella* prevalence rates in walnuts, hazelnuts (shelled), and pine nuts were 1.22, 0.35, and 0.48%, respectively (Zhang, Hu, Melka, et al., 2017). A recently published risk assessment of *Salmonella* on walnuts by the FDA suggested that the higher prevalence of *Salmonella* observed in the 2014 to 2015 retail survey is a reflection of variability in *Salmonella* contamination across the supply or an atypical event affecting a portion of the supply (Santillana Farakos et al., 2018). This might also indicate that the industry is working effectively to reduce pathogens in their products under the influence of Food Safety Modernization Act (FSMA). The FDA prevalence data were similar to limited published reports (Brar et al., 2016; Danyluk et al., 2007; Davidson et al., 2015; Eglezos, Huang, & Stuttard, 2008; Harris et al., 2016; Little et al., 2009) on *Salmonella* contamination of different varieties of tree nuts as summarized in our recent publication (Zhang, Hu, Melka, et al., 2017). When comparing the prevalence of *Salmonella* among different surveys, readers need to take consideration of differences in collection points, sample sizes, and detection methodologies used in different studies. Information on the production, harvest methods, and processing steps for tree nuts as well as the prevalence and levels of *Salmonella* in tree nuts at harvest, upstream from retail samples may support the development of mitigation strategies to control *Salmonella*.

Only 530 organic raw, shelled nut samples (15.71% of the total) were analyzed due to the limited availability of such products in the U.S. retail market. Four of the five *Salmonella* positive organic samples were macadamia nuts and one was pistachio. *Salmonella* prevalence rate for organic and conventional nuts overall was 0.94 and 0.35%, respectively, although the difference was not statistically significant (Chi-square test: $P = 0.0601$) (Table 1). Organic nuts had high *Salmonella* prevalence rate in this survey mainly due to the higher numbers of positive macadamia nuts samples. In our previous survey (Zhang, Hu, Melka, et al., 2017), macadamia nuts also had higher rate of *Salmonella* contamination than other nuts surveyed; there was no significant difference for prevalence

Table 3–Serotypes and NCBI accession numbers of *Salmonella* isolates recovered from raw, shelled tree nut samples.

Sample code	NCBI accession number[a]	Sampling date	Nut	Store type	*Salmonella* serotype	MPN/g,[95% CI]
1108208-28	SAMN04396122	12/28/2015	Cashews	Discount, variety, or drug stores	Mbandaka	<0.003 [N/A, N/A]
1108793-11	SAMN04500976	2/6/2016	Macadamia Nuts	Small Chain	*diarizonae* O61	0.0036 [0.00, 0.03]
1110945-01	SAMN05359991	6/9/2016	Macadamia Nuts	Discount, variety, or drug stores	Urbana	<0.003 [N/A, N/A]
1112207-05	SAMN05784536	8/13/2016	Macadamia Nuts	Major or big Chain	Muenchen	<0.003 [N/A, N/A]
1108248-25	SAMN04413829	12/31/2015	Macadamia Nuts, organic	Discount, variety, or drug stores	*diarizonae* N.N.	<0.003 [N/A, N/A]
1108248-26	SAMN04413830	12/31/2015	Macadamia Nuts, organic	Discount, variety, or drug stores	Give	0.15 [0.01, 0.52]
1108261-12	SAMN04413828	1/2/2016	Macadamia Nuts, organic	Discount, variety, or drug stores	Give	0.75 [0.18, 3.20]
1108447-08	SAMN04451260	1/16/2016	Macadamia Nuts, organic	Discount, variety, or drug stores	Give	<0.003 [N/A, N/A]
1108448-10	SAMN04451261	1/16/2016	Pistachios	Major or big Chain	Liverpool	<0.003 [N/A, N/A]
1108480-16	SAMN04451263, SAMN04451264	1/19/2016	Pistachios	Online	Senftenberg Montevideo	0.092 [0.02, 0.37]
1109149-08	SAMN04549535	2/27/2016	Pistachios	Major or big Chain	Senftenberg	0.0036 [0.00, 0.03]
1110485-14	SAMN05181543, SAMN05181544	5/17/2016	Pistachios	Online	Worthington Montevideo	0.0092 [0.00, 0.04]
1114245-01	SAMN06125464	11/30/2016	Pistachios	Major or big Chain	Senftenberg	<0.003 [N/A, N/A]
1114461-01	SAMN06213901	12/9/2016	Pistachios	Online	Mbandaka	<0.003 [N/A, N/A]
1108480-10	SAMN04451262	1/19/2016	Pistachios, organic	Online	Duisburg	<0.003 [N/A, N/A]

Note. MPN was estimated by a three-tube, five-dilution (100, 10, 1.0, 0.1, and 0.01 g) assay.
[a]NCBI accession number is isolate identification number assigned by the National Center for Biotechnology Information (NCBI) in their database.
Abbreviation: CI, Clopper–Pearson's 95% confidence interval.

of *Salmonella* between organic and conventional tree nuts, which were 0.61% (326) and 0.90% (3330), respectively.

Samples were obtained from 493 sources including 1,381 major/big-chain supermarkets, 328 small-chain supermarkets, 1,329 discount/variety/drug stores, and 336 online retailers. Corresponding *Salmonella* prevalence rates of 0.29, 0.30, 0.45, and 1.19%, respectively, were not statistically different (Chi-square test: $P = 0.1633$) (Table 2).

3.2 Estimates of *Salmonella* populations in contaminated tree nuts

Salmonella populations in the 15 contaminated samples were estimated by a three-tube, five-dilution (100, 10, 1.0, 0.1, and 0.01 g) MPN method. The total sample size for MPN analysis was 333.33 g of nuts for each sample. *Salmonella* populations ranged from <0.003 to 0.75 MPN/g among these nut samples. The highest estimate was 0.75 MPN/g and 80% of the samples contained ≤0.0092 MPN/g (Figure 1). Similar *Salmonella* population estimates in positive tree nut samples were observed in the previous similarly structured survey, where 60.7% of *Salmonella*-positive tree nuts samples had *Salmonella* below the limit of detection of 0.003 MPN/g, 25% had 0.003 to 0.005 MPN/g, and the highest was 0.092 MPN/g (Zhang, Hu, Melka, et al., 2017). Similar results have been reported for surveys of nut commodities including almonds, peanuts, pecans, walnuts, and pistachios (Brar et al., 2016; Calhoun et al., 2013; Danyluk et al., 2007; Davidson et al., 2015; Harris et al., 2016). An estimate of 8.5 MPN/100 g has been reported for recalled raw almonds associated with a *Salmonella* outbreak (Danyluk et al., 2007). In another investigation, raw almonds that arrived at processors were positive for *Salmonella* at a prevalence rate of 0.87% with contamination levels at 1.2 to 2.9 MPN/100 g (Danyluk et al., 2007). In a survey on prevalence of *Salmonella* on in-shell California walnuts, an average prevalence rate of 0.14% was determined and contamination levels for positive samples were estimated to be 0.32 to 0.42 MPN/100 g (Davidson et al., 2015). In another study on raw California in-shell pistachios, prevalence rates of *Salmonella* for floaters (nuts that float in a float tank containing water during processing) and sinkers (nuts that sink) were 2.0% and 0.37%, respectively, with a weighted average of 0.61% (Harris et al., 2016). In-shell pecans were tested for *Salmonella* and a prevalence rate of 0.95% was reported with *Salmonella* levels at 0.47 to 39 MPN/100 g, averaging at 2.4 MPN/100 g (Brar et al., 2016). In another survey on *Salmonella* contamination of raw, shelled peanuts, 2.33% of the samples were determined to be positive for *Salmonella* with concentration levels ranging from <0.03 to 2.4 MPN/g (Calhoun et al., 2013).

3.3 Diversity of serotypes and PFGE profiles of *Salmonella* isolated from tree nuts

We obtained 17 *Salmonella* isolates out of the 15 *Salmonella*-positive tree nut samples with two pistachio samples containing two different isolates each (Table 3). Among the 17 isolates, there were 11 different serotypes. Except for one isolate from cashews (*Salmonella* Mbandaka), all others were isolated from macadamia nuts and pistachios. In macadamia nuts, five different serotypes were found (*Salmonella* serotypes *diarizonae* O61, *diarizonae* N.N., Urbana, Muenchen, and Give) and six different serotypes were isolated in pistachios (*Salmonella* serotypes Liverpool, Senftenberg, Montevideo, Worthington, Mbandaka, and Duisburg). *Salmonella* Give and *Salmonella* Senftenberg were each isolated three times from macadamia and pistachios, respectively; all *Salmonella* Give isolates were found in macadamia nuts and all

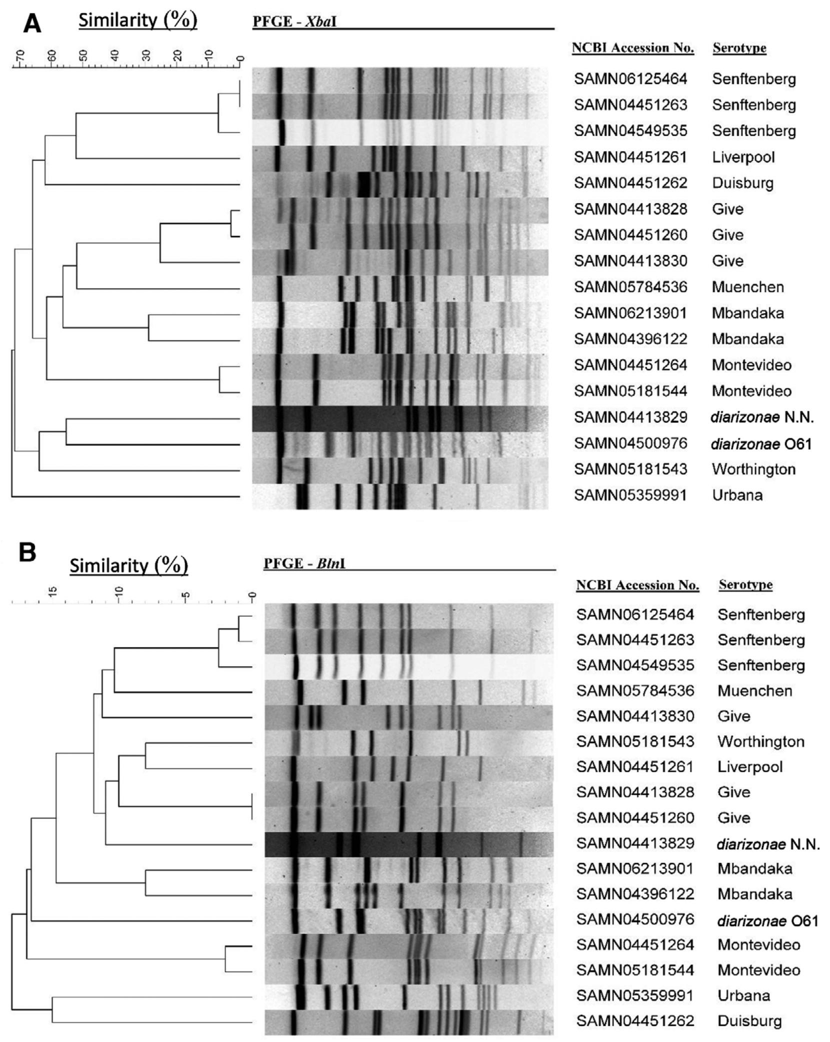

Figure 2–PFGE profiles of *Salmonella* isolates recovered from raw, shelled tree nuts. Profiles (A) was obtained using restriction enzyme *Xba*I and (B) restriction enzyme *Bln*I.

Salmonella Senftenberg isolates were found in pistachios. PFGE patterns of *Salmonella* Give isolates SAMN04413828 and SAMN04451260 were the same (by restriction enzyme Bln*I*) or similar (by restriction enzyme Xba*I*) (Figure 2). PFGE profiles of *Salmonella* Senftenberg isolates SAMN06125464 and SAMN04451263 were the same (by restriction enzyme Xba*I*) or similar (by restriction enzyme Bln*I*); they also formed a small cluster with *Salmonella* Senftenberg isolate SAMN04549535 by both enzymes (Figure 2). The PFGE profile similarities indicate that they were from the same environmental source.

In our previous survey of *Salmonella* prevalence on tree nuts in the United States (Zhang, Hu, Melka, et al., 2017), a total of 12 serotypes (15 isolates) were isolated from macadamia nuts. In the current survey, there were five serotypes (seven isolates) isolated. Only *Salmonella enterica* subsp. *diarizonae* was observed in both surveys. These results showed there is wide genetic variation of *Salmonella* isolated from macadamia nuts, in both the 2014 to 2015 survey and the 2015 to 2016 survey. Among the 11 serotypes of *Salmonella* obtained in the current nationwide survey from various nuts (Table 3), only *Salmonella* Muenchen was on the CDC's top 10 culture-confirmed *Salmonella* infections list (Centers for Disease Control and Prevention, 2016a). More information on contamination at different points in the supply chain among producers is needed to interpret these data. We hypothesize that environments where macadamia nuts were produced may be favorable for *Salmonella* survival. In-depth investigation of the environment to figure out the factors beneficial for *Salmonella* survival might help the industry develop better mitigation strategies to control *Salmonella*.

There were reports on diverse *Salmonella* populations of isolates recovered from tree nuts and peanuts revealed by serotype and PFGE data, which are similar to our current observations of the isolates obtained in this project (Calhoun et al., 2013; Danyluk et al., 2007; Harris et al., 2016; Zhang, Hu, Melka, et al., 2017). This phenomenon might be caused by the diverse nut production environments, soil contamination during harvesting, and subsequent contamination during processing and in storage facilities. Soil harbors an extremely diverse and complicated microbiota. Soil and dust are heavily involved in harvesting and processing of tree nuts, creating more chances of contamination of nuts.

3.4 Genomics of *Salmonella* isolates from tree nuts

Sequencing data and metadata of the 17 isolates recovered from tree nuts are available from the NCBI database (https://www.ncbi.nlm.nih.gov/) with Accession Numbers listed in Table 3. The overall tree topology of *Salmonella* isolates constructed by kSNP3.0

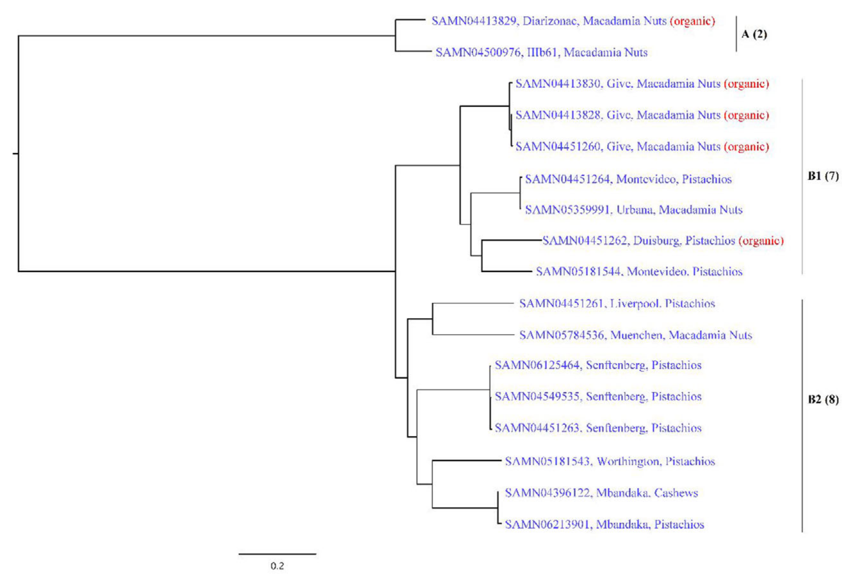

Figure 3–Phylogenetic analysis of *Salmonella* isolates recovered from contaminated raw, shelled tree nut samples. Diarizonae represents *S. enterica* subsp. *diarizonae* N.N.; IIIb61 represents *S. enterica* subsp. *diarizonae* O61.

was concordant with what is obtained when inferring the unfiltered SNP matrix generated by CFSAN SNP pipeline (Figure 3). Two major lineages (Clade A and B) were exhibited among these isolates. Clade A only contained two isolates, *S. enterica* subsp. *diarizonae* N.N. (organic macadamia nuts) and *S. enterica* subsp. *diarizonae* O61 (macadamia nuts), which was an early diverging lineage to the remaining isolates in Clade B. Clade B is consisted of B1 (seven isolates) and B2 (eight isolates). Previous reports on phylogenetic analysis of *Salmonella* also revealed two sister lineages for the subspecies (den Bakker et al., 2011; Timme et al., 2013).

Our data showed that Clade B1 included *Salmonella* serotypes Give, Montevideo, Urbana, and Duisburg, whereas Clade B2 contained serotypes Liverpool, Muenchen, Senftenberg, Worthington, and Mbandaka. All *Salmonella* isolates from organic tree nuts, except for SAMN04413829, belonged to Clade B1. The identified *Salmonella* Give isolates (SAMN04413828 and SAMN04451260) from organic macadamia nuts collected in 2015 were closely linked to *Salmonella* Give isolated from organic macadamia nuts collected in 2016 (Clade B1), with a distance of 541 to 614 SNPs (Table 4). Three isolates of *Salmonella* Senftenberg (SAMN06125464, SAMN04549535, and SAMN04451263) obtained from different times in 2016 were all shown in Clade B2 and might be recognized as the same one, due to the close distance between each other (<100 bp). The two *Salmonella* Mbandaka isolated from cashews in 2015 and pistachios in 2016 were genetically close (279 SNPs difference). In addition, the phylogenetic tree by WGS was similar to the obtained PFGE profiles with different restriction enzymes, *Xba*I and *Bln*I (Table 4 and Figure 2). Although it is able to discriminate among *Salmonella* isolates in a general manner, PFGE might be insufficient when very closely related strains are analyzed and lack the resolution necessary to reveal differences between isolates from the same outbreak; WGS is better fit to solve this problem with a higher level of resolution (Allard et al., 2012; Boxrud et al., 2007). WGS and bioinformatic analyses are revolutionizing the field of *Salmonella* genomics by rapidly mining sufficient information for the surveillance and investigation of *Salmonella* outbreaks. Increasing international use of WGS in parallel with other techniques such as PFGE and MLVA hints at their eventual displacement by WGS for typing *Salmonella* (Nadon et al., 2017; Rantsiou et al., 2017).

Figure 4 shows that all 17 *Salmonella* isolates contained many genes/gene clusters studied, such as 16s rRNA, agfBAC, bcfD, fhlA, fur, hilA, himA, hisJ, invA, invH, mgtB, misL, orgC, phoP, rpoS, sipB, sirA, slyA, ssaQRSTU, sseC, sspABCD, stn, and ttrRSBCA. Genes grvA, pefA, prot6E, sefA, sodC, spvDCBAR, tviA, and viaB were completely absent from all these isolates. Some genes only occurred in certain serotypes. For example, avrA gene had low existence (17%) in *Salmonella* Duisburg and Liverpool, although highly present in other serotypes. sinH gene was absent from the two *S. enterica* subsp. *diarizonae*, highly present in the rest of the isolates. ratB gene was present in *Salmonella* Duisburg, Liverpool, IIIb61, Urbana, Muenchen, Montevideo, and Give; it was absent from most other isolates. shdA gene was absent from *S. enterica* subsp. *diarizonae* isolates, and partially present in other serotypes (>72%). In addition, both gene clusters of fimAICDHF (>86%) and iroBC (>89%) were partially present in all *Salmonella* isolates.

For the 13 SPIs studied (Figure 4), SGI-1 was present at <8% among the 17 *Salmonella* isolates. SPI-1, SPI-2, SPI-3, SPI-4, and SPI-9 were present in all isolates. SPI-5 was prevalent at 73 to 100% among these isolates. SPI-6 was absent in *S. enterica* subsp. *diarizonae* (SAMN04413829 and SAMN04500976) isolates. SPI-8 was present at 4% and 99% in *Salmonella* Montevideo isolates SAMN04451264 and SAMN05181544, respectively; and all three *Salmonella* Senftenberg isolates contained SPI-8. SPI-7 was present at <20% in *S. enterica* subsp. *diarizonae*, Liverpool, Urbana, Mbandaka, Senftenberg, and Montevideo (SAMN05181544) and at 20 to 49% in other isolates. SPI-10 sequence was missing in all isolates except for *Salmonella* Montevideo isolate SAMN05181544, where it was present at 42%. HPI was completely absent from these isolates except for *Salmonella* Liverpool and IIIb61 (30 to 32%). SPIs 1 to 5 were widespread in genus *Salmonella*. SPI-6 was mainly present in *Salmonella* subspecies I, IIIb, IV, and VII. SPI-7, SPI-9, SPI-10, and SGI-1 were frequently observed in subspecies I. SPI-8 was common in *Salmonella* Typhi. HPI often occurred in *Salmonella* subspecies IIIa, IIIb, and IV (Hensel, 2004). The gain/loss/mutation of virulence determinant genes and SPIs among the different subspecies and serovars might have

Table 4–Pairwise SNP distances (number of nucleotide differences) between the *Salmonella* isolates from contaminated tree nut samples.

SAMN04 413829	**SAMN04 500976**	**SAMN05 359991**	**SAMN04 451262**	**SAMN05 181543**	**SAMN04 451264**	**SAMN04 413830**	**SAMN04 451260**	**SAMN04 413828**	**SAMN04 451261**	**SAMN05 784536**	**SAMN05 181544**	**SAMN04 396122**	**SAMN06 213901**	**SAMN04 549535**	**SAMN04 451263**	**SAMN06 125464**
SAMN04 413829																
SAMN04 500976	11,941															
SAMN05 359991	140,578	139,559														
SAMN04 451262	139,107	137,990	30,754													
SAMN05 181543	139,457	138,565	31,101	31,180												
SAMN04 451264	139,494	138,608	31,140	31,226	102											
SAMN04 413830	141,373	140,259	32,102	31,605	31,101	31,081										
SAMN04 451260	141,273	140,163	32,010	31,494	30,945	30,957	614									
SAMN04 413828	141,352	140,239	32,042	31,564	31,021	31,007	541	101								
SAMN04 451261	139,527	138,320	53,924	53,559	53,735	53,845	55,174	54,971	55,144							
SAMN05 784536	140,289	139,405	53,879	53,558	53,434	53,638	55,147	55,016	55,183	42,137						
SAMN05 181544	135,986	134,965	44,677	44,396	44,388	44,500	45,674	45,575	45,689	41,309	40,987					
SAMN04 396122	139,842	139,003	50,282	50,072	50,298	50,363	51,554	51,445	51,505	47,097	46,844	32,360				
SAMN06 213901	139,628	138,793	49,980	49,626	49,954	50,014	51,216	51,115	51,175	46,817	46,437	32,092	279			
SAMN04 549535	140,492	139,465	51,139	50,969	50,673	50,738	52,465	52,410	52,492	46,273	45,742	35,321	41,818	41,491		
SAMN04 451263	140,514	139,468	51,155	50,925	50,633	50,725	52,502	52,295	52,438	46,287	45,722	35,317	41,872	41,546	92	
SAMN06 125464	140,556	139,489	51,243	51,027	50,724	50,810	52,602	52,427	52,550	46,375	45,825	35,378	41,957	41,622	69	53

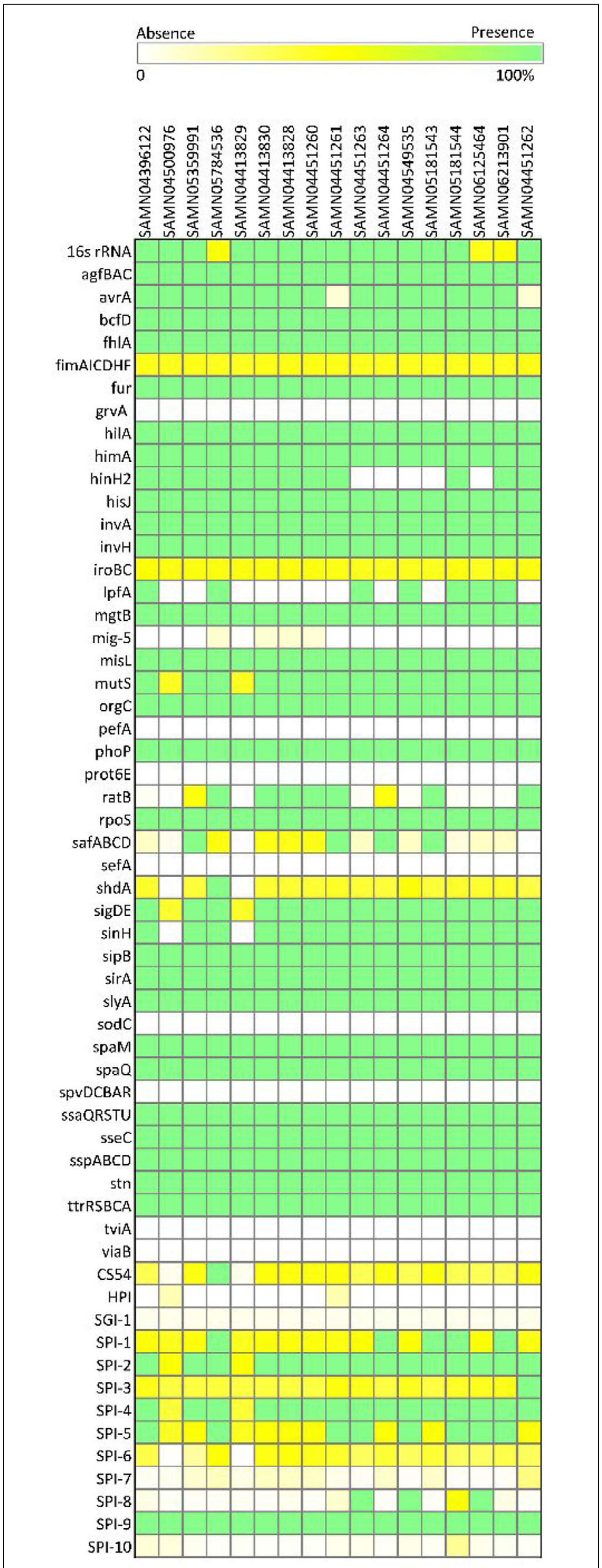

Figure 4–Presence or absence of virulence genes and gene clusters detected in *Salmonella* isolates from contaminated raw, shelled tree nut samples.

contributed to the elevated level of diversities in *Salmonella* genus. Additionally, the acquisition of SPIs by horizontal gene transfer is often reflected in the base composition of SPIs that are different from that of the core genome of the host, and the association with insertion sites such as tRNA genes (Lamas et al., 2017).

4. CONCLUSION

Salmonella was not detected in 2,291 samples of raw, shelled Brazil nuts, hazelnuts, pecans, pine nuts, and walnuts collected from U.S. retail outlets. In contrast, 17 isolates from 11 serotypes were recovered from 15 of 1,083 samples of contaminated raw, shelled cashews, macadamia nuts, or pistachios. The rate of *Salmonella* isolation from major/big chain-supermarkets, small-chain supermarkets, discount/variety/drug stores, and online was 0.29% (95% CI [0.08, 0.74]), 0.30% (95% CI [0.01, 1.69]), 0.45% (95% CI [0.17, 0.98]), and 1.19% (95% CI [0.33, 3.02]), respectively. The majority of contaminated samples had very low numbers of *Salmonella* cells ($\leq$0.0092 MPN/g nuts), with the highest contamination level observed being 0.75 MPN/g on a sample of macadamia nuts. More surveillances and surveys should be considered for macadamia and pistachios, including the environments where they are produced. The contamination levels of *Salmonella* in the tree nuts analyzed were generally comparable to previous reports. Prevalence estimates for walnuts, hazelnuts, and pine nuts were smaller than those observed in the similarly structured 2014 to 2015 survey. Detailed information on the contamination levels at harvest and production processes for these and other tree nuts may help reveal the source of this variability.

AUTHOR CONTRIBUTIONS

G. Zhang and T. Hammack designed the study and collected the data. L. Hu and G. Zhang analyzed the data. G. Zhang and L. Hu interpreted the results and wrote the manuscript. All other authors reviewed and revised the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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