

Research Paper

Prevalence of *Salmonella* in Cashews, Hazelnuts, Macadamia Nuts, Pecans, Pine Nuts, and Walnuts in the United States

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ABSTRACT

Nuts have been identified as a vector for salmonellosis. The objective of this project was to estimate the prevalence and contamination level of *Salmonella* in raw tree nuts (cashews, pecans, hazelnuts, macadamia nuts, pine nuts, and walnuts) at retail markets in the United States. A total of 3,656 samples of six types of tree nuts were collected from different types of retail stores and markets nationwide between October 2014 and October 2015. These samples were analyzed using a modified version of the *Salmonella* culture method from the U.S. Food and Drug Administration's *Bacteriological Analytical Manual*. Of the 3,656 samples collected and tested, 32 were culturally confirmed as containing *Salmonella*. These isolates represented 25 serotypes. *Salmonella* was not detected in pecans and in-shell hazelnuts. *Salmonella* prevalence estimates (and 95% confidence intervals) in cashews, shelled hazelnuts, pine nuts, walnuts, and macadamia nuts were 0.55% [0.15, 1.40], 0.35% [0.04, 1.20], 0.48% [0.10, 1.40], 1.20% [0.53, 2.40], and 4.20% [2.40, 6.90], respectively. The rates of *Salmonella* isolation from major or big chain supermarkets, small chain supermarkets, discount, variety, or drug stores, and online were 0.64% [0.38, 1.00], 1.60% [0.80, 2.90], 0.00% [0.00, 2.40], and 13.64% [2.90, 35.00], respectively (Cochran-Mantel-Haenszel test: $P = 0.02$). The rates of *Salmonella* isolation for conventional and organic nuts were not significantly different. Of the samples containing *Salmonella*, 60.7% had levels less than 0.003 most probable number (MPN)/g. The highest contamination level observed was 0.092 MPN/g. The prevalence and levels of *Salmonella* in these tree nut samples were comparable to those previously reported for similar foods.

Key words: Low moisture; Low water activity; Nutmeat; Serotype; Tree nuts

Nuts (tree nuts and peanuts) have low water activity and thus do not provide favorable conditions for bacterial growth. However, *Salmonella* has been detected in pecans, peanuts, almonds, pistachios, walnuts, and pine nuts during the last few years (5–7, 9, 10, 15, 16, 22, 24), *Escherichia coli* O157:H7 has been found in peanuts, pecans, and walnuts (8, 16, 24), and *Listeria monocytogenes* has been found in peanuts, pecans, and mixed nuts (8, 17). Bacteria such as *Pseudomonas*, *Clostridium* spp., and *Klebsiella* spp. with potential health risks especially for young and immunocompromised people have also occasionally been found in nuts (3, 4). Many species of *Aspergillus*, including about 40 that have been implicated in human or animal infections, can infect and cause decay in nuts. Some *Aspergillus* species produce aflatoxin, which is both a toxin and a carcinogen (4). The presence of such contaminants in nuts could cause human health problems.

At least 25 recalls were issued in 2015 in the United States due to *Salmonella* contamination of walnuts, pecans, macadamia nuts, pine nuts, almonds, and hazelnuts (26).

Worldwide, numerous outbreaks of foodborne illness in recent years have been associated with almonds, cashews, hazelnuts, pine nuts, pistachios, and walnuts; a majority of these outbreaks were caused by *Salmonella* infection. For example, raw almonds contaminated with *Salmonella* Enteritidis caused 29 illnesses in Canada and 12 states in the United States during 2003 and 2004 (11). Recently, an outbreak of *Salmonella* Montevideo infection involving nine states was associated with pistachios (13). These events have raised concerns about the public health risk associated with consumption of tree nuts. Basic information, such as the extent of contamination and type of nuts contaminated, could provide important information to inform the development of mitigation strategies to reduce public health risks associated with consumption of nuts.

The objective of this study was to estimate the prevalence and level of *Salmonella* in tree nuts at retail markets in the United States. This information will assist the U.S. Food and Drug Administration (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 (29). The tree nuts included in the study were selected to provide information on *Salmonella*

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TABLE 1. *Tree nut sample characterization and Salmonella prevalence*

Nut type	No. of samples tested ^a			No. of samples positive for <i>Salmonella</i> ^b	<i>Salmonella</i> prevalence (%) [95% CI] ^c
	Conventional	Organic	Total		
Cashews, shelled	552	181	733	4	0.55 [0.15, 1.40]
Hazelnuts, in shell	80	0	80	0	0 [0, 4.50]
Hazelnuts, shelled	557	20	577	2	0.35 [0.04, 1.20]
Macadamia nuts, shelled	338	17	355	15	4.20 [2.40, 6.90]
Pecans, shelled	597	26	623	0	0 [0, 0.59]
Pine nuts, shelled	589	41	630	3	0.48 [0.10, 1.40]
Walnuts, shelled	617	41	658	8	1.22 [0.53, 2.40]
Total	3,330	326	3,656	32	

^a Organic nuts accounted for 8.92% of the total number of samples analyzed.

^b Two isolates were from organic macadamia nuts and walnuts.

^c CI, confidence interval. *Salmonella* prevalence was 0.61% in organic nuts and 0.90% in conventional nuts. The number of *Salmonella* isolates recovered from organic nuts accounted for 6.25% of the total number of isolates.

prevalence and level for nuts where limited information was available, such as for cashews, pecans, hazelnuts, macadamia nuts, pine nuts, and walnuts. This study will help to fill some of the data gaps in the risk assessment of salmonellosis from consumption of tree nuts in the United States.

MATERIALS AND METHODS

Sample collection. A commercial testing laboratory under contract with the FDA between 6 October 2014 and 21 October 2015 collected and analyzed 3,656 samples of tree nuts: raw cashews (shelled), pecans (shelled), hazelnuts (shelled and in shell), macadamia nuts (shelled), pine nuts (shelled), and walnuts (shelled) (Table 1). To be as representative as possible, the collection sites were selected using U.S. Census Bureau maps (28) for California (West Region, Pacific Division), Colorado (West Region, Mountain Division), Connecticut (Northeast Region, New England 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).

Samples were selected from different types of retail markets categorized as (i) major chain or big chain supermarkets, both national and regional; (ii) small chain or independent organic and specialty supermarkets, including retail outlets; (iii) discount, variety, or drug stores and discount stores, including large discount clubs such as Costco, BJ's, and Sam's Club and smaller discount stores such as Dollar stores and other retail outlets for which foods

are just a fraction of their business, including national and regional drug stores and gas stations; and (iv) online Internet retail sellers (for a very limited number of samples of macadamia nuts that were difficult to obtain in regular retail stores). Numbers of samples collected from each category of market and numbers 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 for which it was difficult to obtain the required amount from a single lot (i.e., available packages with same lot or date), 500 g of nuts were purchased as one sample. Only prepacked (e.g., jars, bags, or cans) tree nuts were collected. Samples were not repackaged when purchasing to avoid cross-contamination. Bagged samples remained sealed before microbiological analysis was conducted. Nuts in open bins for self-serve by consumers and nuts in displays were excluded. 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. Valid samples consisted of raw whole nuts, halves, or pieces or nuts that were diced or chopped. Nuts that had been roasted or were coated with seasonings, chocolate, or other candy were excluded. Nut butters, nut pastes, nut meals, nut flours, and mixed nuts were also excluded. Valid samples required a unique identifier for the producer, grower, or distributor and a "use-by" or "sell-by" date and/or lot number. Collected samples were held at 4°C until used for microbiological analysis.

Microbiological assay. The sample size used for testing for the presence of *Salmonella* in nuts was 375 g. The nut to preenrichment broth ratio was kept at 1:9 (w/v). *Salmonella* detection, isolation, and confirmation from nuts were accomplished using a modification of the method described in the FDA's

TABLE 2. *Retailer collection sites and Salmonella prevalence by retailer type*

Retailer type	No. (%) of samples	No. of unique addresses visited	No. of samples positive for <i>Salmonella</i>	<i>Salmonella</i> prevalence (%) [95% CI] ^a
Major or big chain supermarkets	2,800 (76.6)	437	18	0.64 [0.38, 1.00]
Small chain supermarkets	685 (18.7)	136	11	1.61 [0.80, 2.90]
Discount, variety, or drug stores	149 (4.08)	44	0	0 [0, 2.40]
Online	22 (0.60)	7	3	13.64 [2.90, 35]
				CMH: <i>P</i> = 0.01

^a CI, confidence interval; CMH, Cochran-Mantel-Haenszel test.

Bacteriological Analytical Manual (BAM) (30) by replacing lactose broth with buffered peptone water.

Salmonella levels in nut samples that tested positive were estimated with a three-tube five-dilution (100, 10, 1.0, 0.1, and 0.01 g) most-probable-number (MPN) method (31) and the BAM *Salmonella* method. For a few samples for which it was difficult to obtain the required amount of nuts from a single lot, the three-tube 100-g dilution series was not analyzed. This exception applied to a few pine nut samples where the quantities available for retail purchase were so small that 800 g could not be obtained from a single lot.

Serotyping *Salmonella*. *Salmonella* isolates were serotyped using the Luminex xMAP *Salmonella* serotyping assay (23). Isolates that were untypeable with this assay were serotyped using the conventional Kauffman-White antigenic formulae scheme (19, 30).

PFGE. Pulsed-field gel electrophoresis (PFGE) laboratory analysis followed the official PulseNet protocol (14). *Xba*I was utilized as the primary restriction enzyme, and *Bln*I was used as the secondary restriction enzyme.

Genome sequencing. Isolates obtained were grown in tryptic soy broth (Difco, BD, Franklin Lakes, NJ) overnight at 37°C, and genomic DNA was extracted using the DNeasy blood & tissue kit (Qiagen, Valencia, CA). DNA concentrations were measured with a Qubit fluorometer (Life Technologies, Carlsbad, CA), standardized to 0.2 ng/μL, and stored at -20°C until library preparation. Libraries were prepared with the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Genomes were sequenced using the MiSeq sequencing technology (Illumina) with 500 (2 × 250) cycles and the pair-end library with coverage depth of 30 to 90× at the FDA Center for Food Safety and Applied Nutrition genomics laboratory. All genomes were submitted as assembled reads to the National Center for Biotechnology Information (NCBI) (2).

Statistical analysis. Confidence intervals for prevalence were derived using the Clopper and Pearson procedure, prevalences were compared using Fisher's exact test, and the Cochran-Mantel-Haenszel (CMH) chi-square test was used to test the conditional independence of a factor (conventional versus organically grown, types of retail) for each tree nut type (1). Statistical analyses were performed using R software (27).

RESULTS AND DISCUSSION

***Salmonella* prevalence.** Of the 3,656 nut samples collected and tested, 32 were confirmed by culture as containing *Salmonella*. Information about these samples is provided in Table 1. *Salmonella* was not detected in pecans (623 samples) or in-shell hazelnuts (80 samples). *Salmonella* prevalence in cashews, shelled hazelnuts, and pine nuts was 0.55% (4 of 733 samples), 0.35% (2 of 577 samples), and 0.48% (3 of 630 samples), respectively. *Salmonella* prevalence in walnuts was 1.22% (8 of 658 samples). Macadamia nuts had the highest *Salmonella* prevalence among all six types of nuts tested, at 4.23% (15 of 355 samples). Of the 18 macadamia samples purchased online, 3 were positive for *Salmonella*. Even without the 18 online samples, the prevalence in macadamia nuts was 3.60%, still the highest among the six nut types studied. These data were similar to

limited published reports on *Salmonella* contamination in various varieties of tree nuts in the United States, including almonds, pecans, walnuts, and pistachios. Brar et al. (9) tested *Salmonella* in in-shell pecans over four harvest years. *Salmonella* prevalence ranged across years from 0.47 to 1.40%, with an average of 0.95%. Danyluk et al. (15) reported a *Salmonella* prevalence in raw California almonds of 0.87%. In-shell California walnuts had an average annual *Salmonella* prevalence of 0.14% (16). Harris et al. (20) reported 0.61% *Salmonella* prevalence in 3,966 in-shell pistachio samples collected during 2010, 2011, and 2012 in California. Internationally, a 3-year survey of 921 samples of pre-roasted peanuts, almonds, cashews, hazelnuts, and Brazil nut kernels received at three Australian nut-processing facilities revealed one *Salmonella*-positive raw almond sample. *Salmonella* prevalence among almonds and all nuts was 1.67 and 0.11%, respectively (18). In England, one pistachio sample was positive for *Salmonella* among a total of 727 edible roasted nut kernels (almonds, Brazil nuts, cashews, hazelnuts, macadamia nuts, peanuts, pecans, pine nuts, pistachios, walnuts, tiger nuts, and mixed nuts) on retail sale, for a *Salmonella* prevalence of 4 and 0.14% for pistachios and for all nuts, respectively (22). These findings of *Salmonella*-positive nut samples after roasting treatments indicates the importance of validating *Salmonella*-reduction treatments and implementing controls to minimize recontamination after such treatments to adequately reduce the risk from *Salmonella* in nuts.

Efforts were made to collect organically grown samples. Except for cashews (181 samples tested), fewer than 50 organic samples were obtained for each of hazelnuts, macadamia nuts, pecans, pine nuts, and walnuts because of their limited availability in the markets. However, 326 organic nut samples from the six types of nuts combined were collected and analyzed for *Salmonella*, accounting for 8.92% of the total number of samples collected. Two isolates were recovered from organic nuts; thus, the prevalence of *Salmonella* in organic nuts was 0.61% (Table 1). Overall, the difference in *Salmonella* prevalence between conventional and organically grown tree nuts was not significantly different (CMH test, $P = 0.74$). We are unaware of any published data on *Salmonella* prevalence and levels in organically grown nuts.

Numbers of samples collected from major or big chain supermarkets, small chain supermarkets, discount, variety, or drug stores, and online retailers were 2,800, 685, 149, and 22, respectively (Table 2). A total of 624 unique location addresses were visited during the sample collection process in an effort to sample as many sites as possible to make the result more representative of nuts across the United States. Among the 32 *Salmonella* strains isolated from the 3,656 nut samples, 18, 11, 0, and 3 were from major or big chain supermarkets, small chain supermarkets, discount, variety, or drug stores, and online retailers, respectively. Corresponding prevalences were 0.64, 1.60, 0, and 13.64, respectively (Table 2). Overall, the proportion of positive samples per type of retail outlet was significantly different (CMH, $P = 0.01$). The *Salmonella* prevalence in online samples was higher than that for samples from other retailers

TABLE 3. Serotypes and PFGE patterns of *Salmonella* isolates from tree nuts and their NCBI accession numbers

Isolate code	NCBI accession no. ^a	Sampling date	Nut type	Store type	<i>Salmonella</i> serotype
1102944-01	SAMN03285117	8 Oct. 2014	Cashews	Major chain	Weltevreden
1103130-15	SAMN03285121	31 Oct. 2014	Cashews	Small chain	Nima
1104883-09	SAMN04461351	27 Apr. 2015	Cashews	Major chain	Give
1105439-22	SAMN03795480	8 June 2015	Cashews	Major chain	Brunei
1103469-14	SAMN03285122	12 Dec. 2014	Hazelnuts	Major chain	Typhimurium
1104031-31	SAMN03354387	5 Feb. 2015	Hazelnuts	Small chain	Escanaba
1103009-04	SAMN03285118	14 Oct. 2014	Macadamia nuts	Small chain	Florida
1103854-27	SAMN03329748	19 Jan. 2015	Macadamia nuts	Small chain	Shamba
1104328-07	SAMN03464588	9 Mar. 2015	Macadamia nuts	Major chain	IIIb ^b
1104630-03	NA ^c	7 Apr. 2015	Macadamia nuts	Major chain	Diarizonae
1104803-18	SAMN03580885	20 Apr. 2015	Macadamia nuts	Major chain	Orientalis
1104919-35	SAMN03658272	29 Apr. 2015	Macadamia nuts	Major chain	Mbandaka
1105046-08	SAMN03736683	12 May 2015	Macadamia nuts	Small chain	Plymouth
1105398-26	SAMN03795481	9 June 2015	Macadamia nuts	Major chain	Gaminara
1105862-03	SAMN03965300	13 July 2015	Macadamia nuts, organic	Small chain	Worthington
1106205-04	SAMN04102311	12 Aug. 2015	Macadamia nuts	Online	Uzaramo
1106205-05	SAMN04102312	12 Aug. 2015	Macadamia nuts	Online	Heidelberg
1106205-08	SAMN04102313	12 Aug. 2015	Macadamia nuts	Online	II 42:r:–
1106225-01	NA	11 Aug. 2015	Macadamia nuts	Major chain	Plymouth
1106587-09	NA	2 Sep. 2015	Macadamia nuts	Small chain	II 42:r:–
1106818-10	SAMN04156853	21 Sep. 2015	Macadamia nuts	Major chain	Orientalis
1104554-15	NA	21 Mar. 2015	Pine nuts	Major chain	Thompson
1104727-07	SAMN03580883	17 Apr. 2015	Pine nuts	Small chain	Derby
1107070-03	SAMN04273147	14 Oct. 2015	Pine nuts	Major chain	Baildon
1103516-06	SAMN03285123	18 Dec. 2014	Walnuts	Major chain	Montevideo
1103547-04	SAMN03285124	23 Dec. 2014	Walnuts	Small chain	Irumu
1103911-16	SAMN03354385	27 Jan. 2015	Walnuts	Major chain	Thompson
1104268-07	SAMN03418481	3 Mar. 2015	Walnuts, organic	Small chain	Thompson
1104268-08	SAMN03418482	3 Mar. 2015	Walnuts	Small chain	Muenchen
1104277-06	NA	3 Mar. 2015	Walnuts	Major chain	Thompson
1104630-14	SAMN03497614	7 Apr. 2015	Walnuts	Major chain	Muenchen
1104790-13	NA	20 Apr. 2015	Walnuts	Major chain	Oranienburg

^a Accession number assigned by the National Center for Biotechnology Information (NCBI) in their database (25).

^b Specific serotype formula is not available.

^c NA, NCBI accession number and sequencing data are not available.

(Fisher's exact test, $P < 0.01$). Two of the three positive samples from online purchases of macadamia nuts were bought on the same day and originated from the same producer (but the lot numbers were different).

Tree nut samples were collected all year, and *Salmonella*-positive nuts were detected throughout the year (Table 3). However, because typically only one tree nut production cycle occurs per year and information was not available as to the production year or length of storage for each tree nut sample, the finding of *Salmonella*-positive samples throughout the sampling period provided no additional insights into *Salmonella* contamination of tree nuts. Collection of data over multiple years would provide a better understanding of *Salmonella* prevalence and levels in these tree nuts by elucidating yearly variability.

***Salmonella* levels.** *Salmonella* levels were determined for 28 of the 32 confirmed positive samples. Because of the very low *Salmonella* levels, enumeration was conducted using the MPN method, which requires a relatively large sample size. For 4 of the 32 samples, too few nuts were available for MPN enumeration. The values for the

remaining 28 samples ranged from <0.003 to 0.092 MPN/g; 60.7% had below the limit of detection of 0.003 MPN/g; 25% had 0.003 to 0.005 MPN/g; and the remaining 14.3% had 0.005 to <0.095 MPN/g (Fig. 1). The highest level observed was 0.092 MPN/g in one sample of macadamia nuts ordered online, corresponding to 2.3 MPN/25 g of sample. The two *Salmonella*-positive organically grown nut samples were macadamia nuts and walnuts, with levels of 0.0092 and <0.003 MPN/g, respectively (data not shown).

Kirk et al. (21) tested 142 unopened packets of flavored peanuts and roasted peanuts in Australia, Canada, England, and Wales for the presence of *Salmonella* and concluded that the *Salmonella* levels was generally very low, <0.03 to ~2 cells per gram of peanuts in the shell. Calhoun et al. (10) determined that the *Salmonella* level in raw shelled peanuts was <0.03 to 2.4 MPN/g. Danyluk et al. (15) reported *Salmonella* levels in raw California almonds of 1.2 to 2.9 MPN/100 g. The levels of *Salmonella* in contaminated in-shell California walnuts were estimated at 0.32 to 0.42 MPN/100 g (16). In raw California in-shell pistachios, Harris et al. (20) observed *Salmonella* levels of 0.10 to 5.3 MPN/100 g.

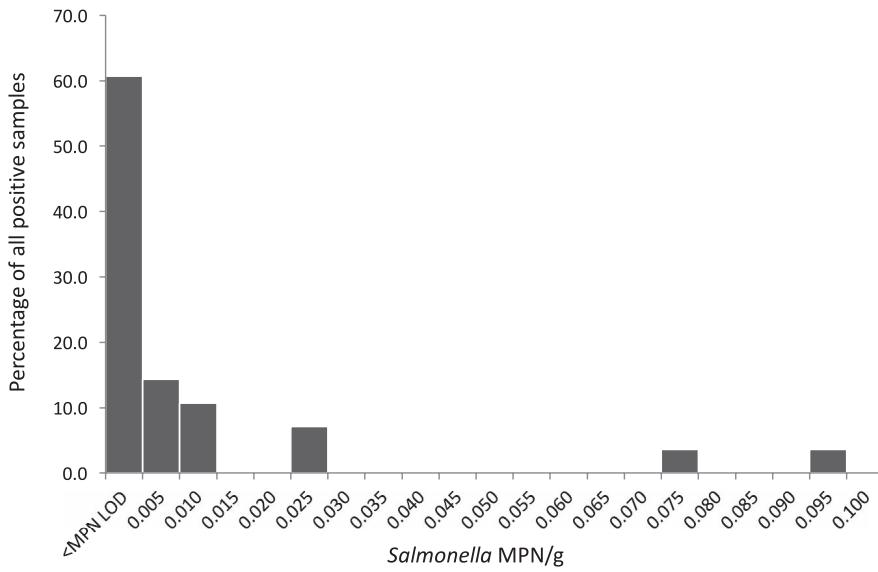


FIGURE 1. MPN results for *Salmonella* in contaminated nut samples.

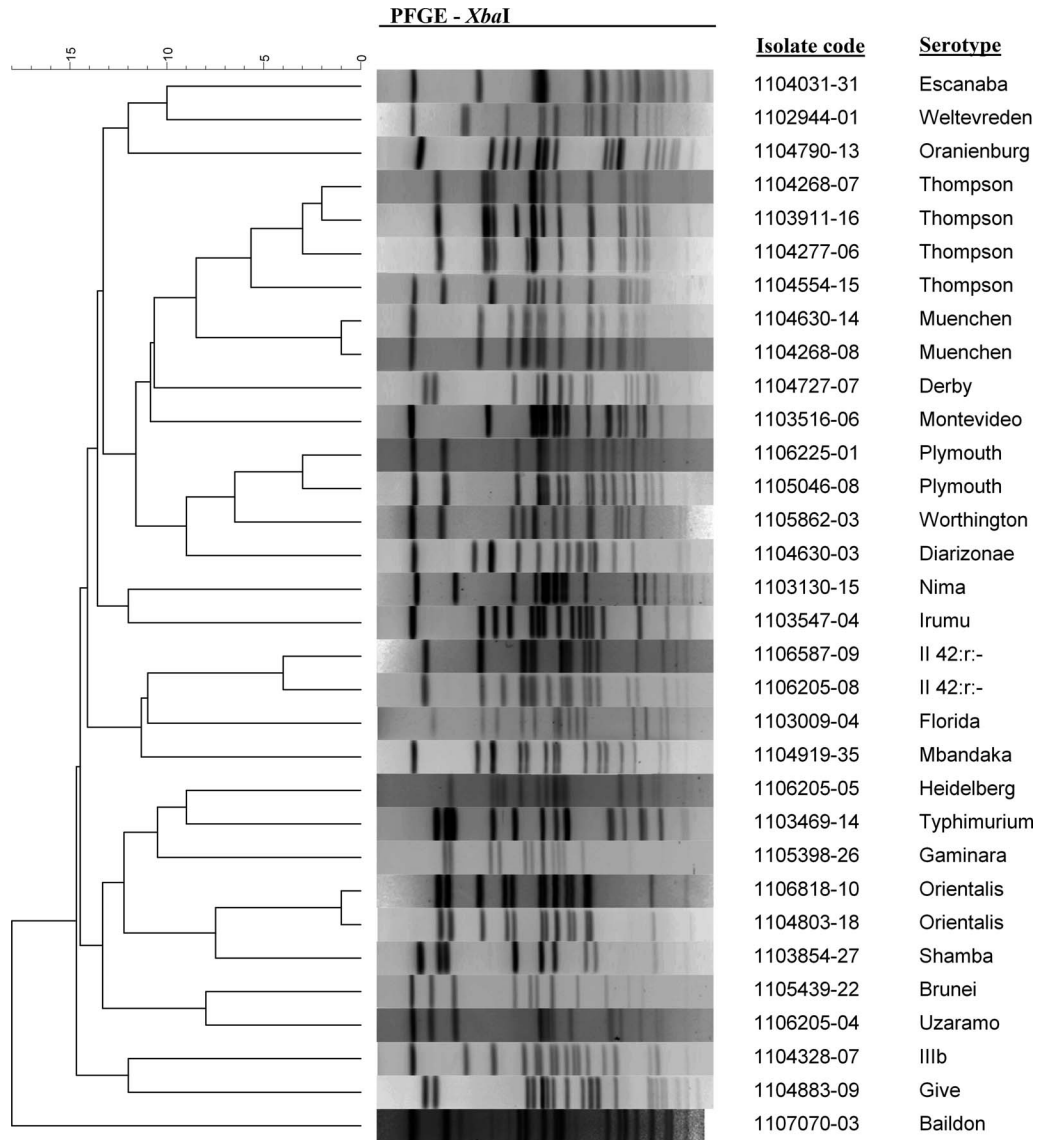


FIGURE 2. PFGE profiles of *Salmonella* isolates from tree nuts using the restriction enzyme *XbaI*.

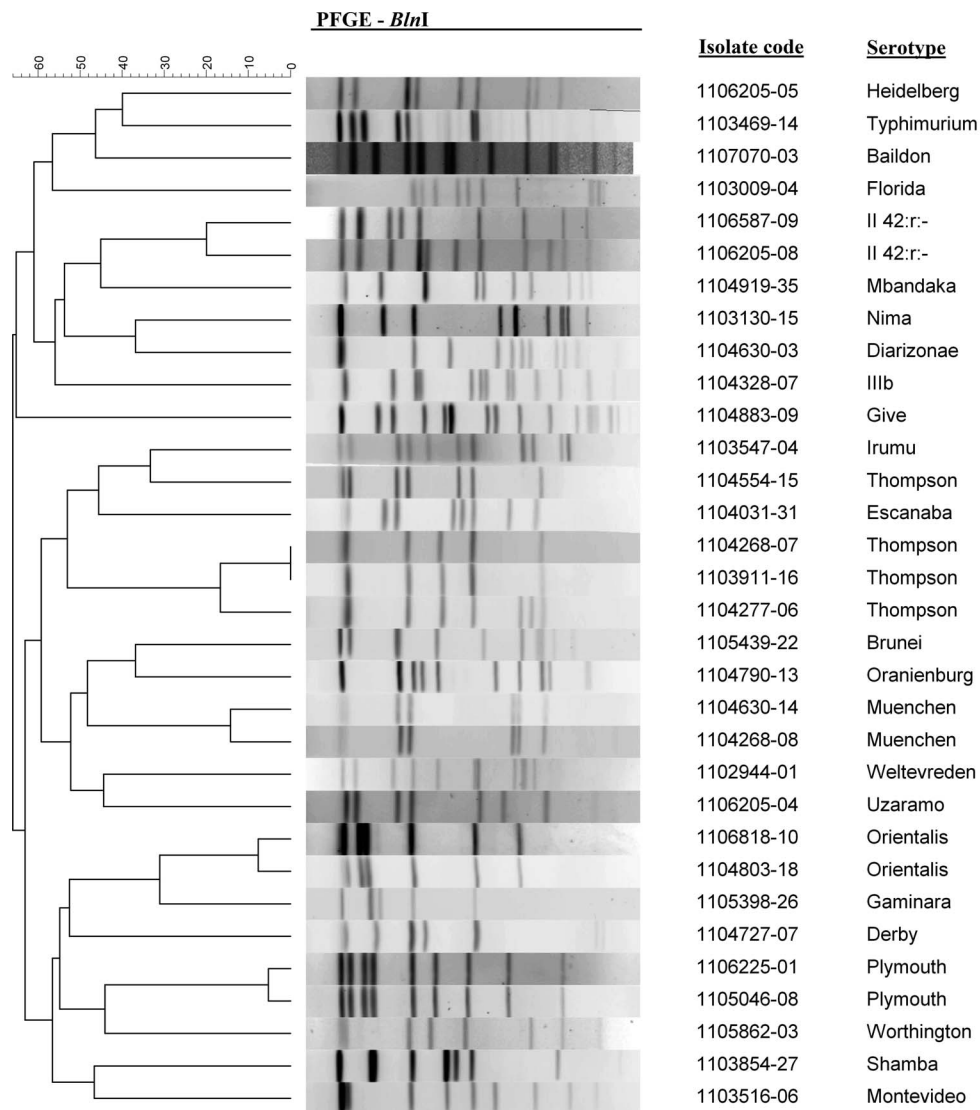


FIGURE 3. PFGE profiles of *Salmonella* isolates from tree nuts using the restriction enzyme BlnI.

Serotypes and PFGE profiles of *Salmonella* isolates from tree nuts. Serotypes of *Salmonella* isolates from these tree nuts were very diverse (Table 3); 25 serotypes were identified among the 32 isolates. The majority of the serotypes were isolated only once from the 3,656 samples tested. However, five serotypes were isolated more than once. *Salmonella* Thompson was isolated from four samples, three of which were walnuts. *Salmonella* Muenchen was isolated from two walnut samples. *Salmonella* Plymouth, *Salmonella* II 42:r:- (also called II Nairobi), and *Salmonella* Orientalis were each isolated from two different macadamia nuts samples. Among the 25 serotypes, *Salmonella* Typhimurium, *Salmonella* Heidelberg, and *Salmonella* Muenchen were on the Centers for Disease Control and Prevention 2015 top 10 culture-confirmed *Salmonella* infections list (12).

Calhoun et al. (10) recovered 22 *Salmonella* isolates belonging to 12 serotypes from raw shelled peanut samples. Kirk et al. (21) found *Salmonella* Stanley, *Salmonella* Newport, *Salmonella* Kottbus, *Salmonella* Lexington, and an unnamed serotype in 142 unopened packets of flavored

peanuts and roasted peanuts from Australia, Canada, England, and Wales. Danyluk et al. (15) obtained 81 *Salmonella* isolates belonging to 35 serotypes from raw California almonds over a 5-year period. Harris et al. (20) found *Salmonella* Montevideo in 44% of their contaminated in-shell pistachio samples. Compared with previous surveys, our results, with 25 *Salmonella* serotypes among 32 isolates (Table 3), were more diverse. The difference could be owing to the fact that previous studies focused on only one type of nut, whereas we evaluated six types. However, even within a single nut type we found a high diversity of serotypes, e.g., 12 serotypes in 15 contaminated macadamia nut samples and 5 serotypes in 8 contaminated walnut samples. We also collected samples at many different locations and in retail stores and markets nationwide, which may have contributed to the *Salmonella* diversity seen in this study. This diversity of *Salmonella* is also reflected in the PFGE patterns (Figs. 2 and 3).

Genomic sequences of *Salmonella* isolates from tree nuts. Sequencing data of most isolates from this project are available from the NCBI database (accession numbers listed

in Table 3). The closest relative for each isolate can be found in the subtree based on single nucleotide polymorphisms (25).

Salmonella was not detected on pecans and in-shell hazelnuts. The prevalence of *Salmonella* on cashews, shelled hazelnuts, pine nuts, and walnuts was similar at 0.35 to 1.22%. Higher prevalences of *Salmonella* were found on macadamia nuts (4.2% [2.40, 6.90]). In 61% of contaminated samples, the *Salmonella* levels were below 1 MPN/100 g. The highest level was found in one macadamia nut sample with an estimated 9 MPN/100 g. These results confirm that the presence of *Salmonella* on tree nuts continues to pose a challenge to the food industry. The 25 different *Salmonella* serotypes found among the 32 positive samples suggest different contamination sources. To our knowledge, this study is the first in which prevalence and contamination level data have been collected for *Salmonella* on tree nuts at the retail level in the United States. These data are important for the development of quantitative microbial risk assessments and more specifically will assist the FDA in estimating the risk of human salmonellosis arising from the consumption of tree nuts in the United States.

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Mention of trade names or commercial products in the paper is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the FDA.

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