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Prevalence, Level, and Types of *Salmonella* Isolated from North American In-Shell Pecans over Four Harvest Years

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ABSTRACT

In-shell pecan samples (500 g) were collected over four harvest seasons (2010 to 2014) from seven pecan shelling facilities located in five U.S. states. Four varieties of pecans were analyzed: Mexican Improved, Native Seedlings, Southern Improved, and Western Improved. Pecan samples (100 g) were sent to a third party laboratory for initial *Salmonella* screening. When a sample was positive for *Salmonella,* the pathogen level was determined by the most-probable-number (MPN) method (25, 2.5, and 0.25 g). Two sample preparation strategies were used for the MPN analysis, and both strategies were combined for the reported MPN values. Forty-four (0.95%) of 4,641 in-shell pecan samples were positive for *Salmonella* during initial screening; prevalence by year was 0.47 to 1.4%. Prevalence was not significantly different between varieties: Mexican Improved, 1.2%; Native/Seedling, 0.99%; Southern Improved, 0.97%; and Western Improved, 0.75%. *Salmonella* was not isolated from 31 of 44 samples upon retesting during MPN analysis (<0.47 MPN/100 g). When *Salmonella* was detected, the levels were 0.47 to 39 MPN/100 g, with a mean of 2.4 MPN/100 g. Thirty-one *Salmonella* serotypes were obtained from 42 *Salmonella*-positive pecan samples; Enteritidis was the most common (12% of samples) followed by Javiana (9%) and Braenderup (7%). All *Salmonella* Enteritidis isolates were phage type 8. Pulsed-field gel electrophoresis analysis (*Xba*I) revealed within-serotype diversity, indicating introduction of contamination from a variety of sources. Most (64%) of the isolates were resistant to streptomycin or tetracycline, and 13% were resistant to three or more antibiotics. *Salmonella* prevalence and level on in-shell pecans is comparable to that on other nuts.

Key words: Level; Pecans; Prevalence; *Salmonella*

Pecans (*Carya illinoinensis* L.) are the only tree nuts native to the United States and are produced in 14 U.S. states; Georgia, Texas, and New Mexico are the major producers *(13)*. Pecan harvesting is a mechanized process. Once mature, pecan trees are shaken to drop the mature nuts onto the orchard floor. Pecans are then removed from the ground and allowed to dry at ambient temperature under shade to reduce the moisture level before shelling *(20)*. Contact between almonds and the orchard floor was identified as a potential source of *Salmonella* contamination in the 2000 to 2001 almond-associated outbreak *(15)* and may be a risk factor for pecans. The hard, intact pecan shell may protect kernels from dust, discoloration, and mold *(13),* and the natural cracking of nuts in the orchard can increase the chances for contamination of the pecan kernel *(18)*.

The low moisture content and low water activity of pecans prevents the multiplication of most microorganisms on the pecan surface, but long-term survival of foodborne pathogens on both in-shell nuts and shelled kernels has been documented *(6, 9)*. The survival of *Salmonella* has been reported on pecan kernels stored at –24, 4, and 22°C for at least 1 year *(9)* and on in-shell pecans stored at –20, 4, 21, and 37°C for at least 1.5 years *(6)*. In both of these studies, *Salmonella* populations remained stable under frozen and refrigeration conditions but declined under ambient conditions (21 or 22°C) at the rate of 0.005 log CFU/g/day on both pecan kernels and in-shell pecans. During ambient storage at 37°C, *Salmonella* levels declined at a slightly higher rate of 0.006 log CFU/g/day on in-shell pecans *(6)*.

Pecans have been recalled because of potential *Salmonella* contamination, but no outbreaks of foodborne disease have been linked to pecans (*24–26*). Limited studies have been performed to determine the prevalence and levels of human pathogens naturally present on raw nuts. Published data on *Salmonella* prevalence and levels is available for raw almond kernels *(4, 11, 16),* raw shelled peanuts *(10, 19),* raw in-shell walnuts *(12),* and raw in-shell almonds *(4)*. *Escherichia coli* O157:H7 prevalence and contamination levels also have been studies on raw peanuts and walnuts *(9, 12)*.

A survey was conducted over four harvest years, the 2010 to 2011 through the 2013 to 2014 harvest seasons, to generate data on prevalence, levels, and types of *Salmonella* present on

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North American in-shell pecans. Pecans are an alternating-year crop, with significantly different production rates in alternating years *(1)*. The different volumes of nut samples analyzed each harvest year reflected production differences. Pecans grown in the United States and those grown in Mexico and shipped to the United States for shelling were included in the study because pecans from different sources are commonly comingled during processing. Contamination information obtained here could be used as baseline data for pecans in future quantitative microbial risk assessments.

MATERIALS AND METHODS

Sample collection. In-shell pecans were collected over four harvest years, from 2010 to 2011 through 2013 to 2014. During the low production years (2010 to 2011 and 2012 to 2013), approximately 1,000 samples were collected for *Salmonella* analysis, whereas during high production years (2011 to 2012 and 2013 to 2014), approximately 1,300 samples were collected. Seven pecan shellers in five U.S. states were recruited through the National Pecan Shellers Association. The seven shellers represent a cross-section of the pecan shelling industry, with annual shipping volumes of 1 to 80 million lb (36.3 million kg).

Shellers were asked to estimate the total amount of pecans that would be processed by their facility in a harvest season and the proportion of the processed pecans belonged to four broad varieties (Mexican Improved, Native/Seedling, Southeastern Improved, and Western Improved). Each participating sheller was given a total number of samples to collect annually, including how many samples of each variety, based on their proportion of the total estimated production volume of all seven shellers.

Sampling kits in zip-top bags (40.64 by 40.64 cm; Bitran, Compac International, Carbondale, IL) consisted of a pair of sterile disposable gloves, one large sterile disposable scoop, and one zip-top bag (30.48 by 30.48 cm; Bitran, Compac) with a label for the date of sampling and the variety of pecan. An instruction sheet detailing all steps needed to sample pecans was sent with the sampling kit to all shellers. Shellers were instructed to collect pecans with washed, gloved hands using the sterile disposable scoop provided and to collect samples at the same location every time. Shellers were asked to collect ca. 500-g samples of in-shell pecans entering the facility using methods similar to those for determining U.S. Department of Agriculture grades *(23)* and to return those samples to the University of Florida (UF) within 1 month. Samples were kept at room temperature at each sheller's facility. Upon arrival at UF, pecan samples were assigned a sample number; no information identifying the facility was kept. Samples were stored under refrigeration conditions (ca. 4°C) to keep pathogen populations stable for up to 5 months *(6)*.

Initial screening of pecan samples. A portion of each pecan sample (100 g) was weighed and sent to an outside laboratory for initial screening for *Salmonella*: DFA Laboratory (Modesto, CA) in harvest years 1 and 2 and Eurofins Microbiology Laboratory in years 3 (Jacksonville, FL) and 4 (Des Moines, IA). Preliminary screening by an outside laboratory has previously been used *(4, 11, 12)*; however, the laboratory used was changed after year 2 because of difficulties encountered when shipping pecan samples to California because of the potential for introduction of the pecan weevil in August 2011 *(2)*. In year 4, the same staff that processed pecans the previous year at Eurofins in Jacksonville moved to Des Moines, so pecan samples were sent there. Preliminary screening was conducted using AOAC official method 2001.09 for *Salmonella* with the mini-VIDAS assay system (bioMérieux, Hazelwood, MO) *(3)*.

In-shell pecan subsamples (100 g) were soaked in 900 ml of 0.1% buffered peptone water (pH 7.2 ± 0.2) in a sterile 946-ml plastic jar and blended at 4,500 rpm for 2 min with an Omni Mixer Homogenizer (Omni International, Kennesaw, GA). The blended samples were then loosely capped and incubated at 35 ± 2°C for 18 to 24 h. This overnight preenrichment culture was subjected to immunoconcentration with the automated mini-VIDAS system. Preenrichment broth (800 μl) was processed on an immunoconcentration test strip (bioMérieux), and the resulting concentrate was used to inoculate a 2-ml vial of ICS broth (bioMérieux); vials were incubated at 41 ± 2°C for 5 h. After incubation, 1 ml of the ICS broth culture was boiled for 15 min and then cooled to room temperature. To screen for *Salmonella,* 500 μl of the boiled ICS culture was added to an SLM (*Salmonella*) test strip (bioMérieux) and tested for *Salmonella* with the mini-VIDAS system based on an enzyme-linked fluorescent assay. A relative fluorescence value greater than 0.23 was considered a positive *Salmonella* result.

When a sample was positive for *Salmonella* with the mini-VIDAS system, the remaining (unboiled) portion of the ICS broth culture from the vial was streaked onto selective agar media: bismuth sulfite agar (BSA), xylose lysine deoxycholate agar (XLD), and Hektoen enteric agar (HE) (Difco, BD, Franklin Lakes, NJ). Plates were examined for typical colonies after incubation at 35 ± 2°C for 24 to 48 h. Presumptive colonies were restreaked for isolation. Once an isolate was confirmed as *Salmonella,* information on the sample and the recovered isolates ($n = 3$) were shipped to UF for subsequent analysis.

Confirmation of negative samples. Positive results obtained with the mini-VIDAS system were confirmed using standard methods during initial testing before a sample was reported as positive for *Salmonella*. However, some samples were positive in the initial screening (mini-VIDAS), but further testing by culture on selective agars did not confirm the results. In the last sampling year (2013 to 2014), five samples that were positive with the mini-VIDAS but negative after further confirmation testing were retested at UF. These five samples were enriched using the standard U.S. Food and Drug Administration *Bacteriological Analytical Manual* (BAM) method *(8)*. Based on the remaining sample weight, samples were divided into 100-g subsamples prior to enrichment; one sample was split into four 100-g subsamples, and the other four samples were each split into three 100-g subsamples. Each 100-g subsample was blended with 900 ml of lactose broth (Difco, BD) in a sterile blender jar at low speed for 2 min and then incubated at 35 ± 2°C for 24 h. One hundred microliters of this blended mixture was transferred to 9.9-ml tubes of Rappaport-Vassiliadis R10 broth (RV; BD Difco), and 1 ml of the same mixture was transferred to 9.9-ml tubes of tetrathionate broth (TT; Difco, BD). Test tubes were incubated for 48 h at 42 ± 2°C for the RV culture and 24 h at 37 ± 2°C for the TT culture. A 10-μl loopful of each broth culture was streaked onto BSA, XLD, and HE and incubated at 37 ± 2°C for 24 h. Plates were then examined for typical *Salmonella* colonies. All presumptive-positive colonies were streaked and stabbed onto triple sugar iron agar and lysine iron agar slants (Difco, BD) and incubated at 35 ± 2°C for 24 ± 2 h. Isolates from samples with positive slant results were further confirmed using a latex agglutination test (Oxoid, Thermo Fisher Scientific, Waltham, MA) for *Salmonella*.

MPN analysis of positive pecan samples. After the initial screening, the level of *Salmonella* in positive pecans samples was determined with a three-tube MPN analysis. Two methods were used for MPN analysis. For method A, pecans were divided into 25-g MPN tube subsamples before blending and enrichment; for method B, 100 g of in-shell pecans was blended in an enrichment

TABLE 1. *Prevalence of* Salmonella *on in-shell pecans, 2010 to 2014*

Harvest year	No. of positive samples/total samples	Prevalence (%)	No. of positive samples		*Salmonella* level (MPN/100 g)	
			MPN method A	MPN method B	Range	Mean[a]
2010–2011	13/974	1.3	3	3	<0.47–3.4	0.89
2011–2012	7/1,489	0.47	3	2	<0.47–39	6.2
2012–2013	7/943	0.70	0	0	<0.47	0.47
2013–2014	17/1,235	1.4	5	3	<0.47–11	1.9
Total	44/4,641	0.95	11	8	<0.47–39	2.4

[a] Mean values were calculated using 0.47 MPN/100 g for samples for which no *Salmonella* was detected with the MPN methods.

broth before preparation of MPN tube subsamples. The purpose of comparing the methods was to determine whether all pecans in a sample were contaminated with a low level of *Salmonella* or a few pecans were contaminated with higher *Salmonella* levels.

For method A, the four 25-g subsamples from each 100-g sample were blended individually with 225 ml of lactose broth at low speed for 2 min in a sterile blender jar. Three of these subsamples (250 ml each) were each transferred to a 532-ml Whirl-Pak bag (Nasco, Ft. Atkinson, WI) for use in the three 25-g MPN tubes, and the fourth 250-ml subsample was divided into three 25-ml portions in 50-ml conical polypropylene tubes (three 2.5-g MPN tubes) and three 2.5-ml portions in 10-ml of conical polypropylene tubes with an additional 7.5 ml of lactose broth (three 0.25-ml MPN tubes). For method B, a 100-g pecan sample was blended with 900 ml of lactose broth for 2 min at low speed and divided into three 250-ml subsamples, three 25-ml portions in 50-ml conical polypropylene tubes and three 2.5-ml portions in 10-ml conical polypropylene tubes with an additional 7.5 ml of lactose broth. Subsequent enrichment followed the standard BAM method. All MPN tubes from a single pecan sample (2011-0123) were positive with the three 25-g, three 2.5-g, and three 0.25-g tubes. This sample was processed again using three 10-g, three 1-g, and three 0.1-g aliquots to determine the MPN level.

Estimation of bacterial population in pecan samples. As previously described by Danyluk et al. *(11)* and Bansal et al. *(4)* for almonds, the *Salmonella* population was estimated using the Thomas approximation of MPN per gram for the three-tube MPN analysis:

$$\mathrm{MPN/g} = P/(NT)^{1/2}$$

where P is the number of positive tubes obtained during the analysis, N is the total sample amount (in grams) in all negative tubes, and T is total samples amount (in grams) in all tubes. The *Salmonella* population (MPN per 100 g) was calculated for both method A and method B, with the positive 100-g initial screening used as a positive tube. Bansal et al. *(4)* evaluated the same two MPN methods and found no significant differences in mean MPN per 100 g. Because similar trends were found in the present study, MPN results from methods A and B were combined. Including the results of both MPN methods and the initial 100-g positive sample, the limit of detection was calculated at 0.47 MPN/100 g. A 95% confidence interval (CI) was calculated for the MPN values:

$$\mathrm{CI} = \log(\mathrm{MPN/g}) \pm (1.96)(0.55)[\log(a/n)]^{1/2}$$

where a is the dilution ratio and n is the number of tubes per dilution.

Serotyping *Salmonella* isolates. The original isolates and the isolates recovered during MPN analysis were stored at −80°C in tryptic soy broth (Difco, BD) containing 15% (vol/vol) glycerol on glass beads. Isolates were streaked onto tryptic soy agar (TSA; Difco, BD) slants, and isolated colonies were sent to the National Veterinary Science Lab (NVSL; Ames, IA) for serotyping. When *Salmonella* Enteritidis or *Salmonella* Typhimurium were identified, phage typing was performed.

Antimicrobial susceptibility testing of isolates. *Salmonella* isolates were assayed for susceptibility to 14 antibiotics: ampicillin, amoxicillin–clavulanic acid, ampicillin, cefoxitin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, imipenem, kanamycin, nalidixic acid, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline. Antimicrobial susceptibility was examined using the calibrated dichotomous sensitivity method, followed by interpretation of results based on test standards *(5)*. *Salmonella* isolates were grown at 35 ± 2°C for 18 to 24 h on TSA plates, then one colony was transferred to 1 ml of sterile saline (0.85% NaCl), 300 μl was spread plated onto Sensitest agar (Oxoid, Thermo Fisher Scientific), the plate was dried for 30 min, and antibiotic disks were applied (BD). Four or five discs were applied to each plate, plates were incubated at 35 ± 2°C for 18 h, and zones of inhibition were measured with a ruler.

PFGE. DNA fingerprinting of original and MPN isolates were conducted using pulsed-field gel electrophoresis (PFGE) *(22)*. The universal standard strain *Salmonella* Braenderup H9812 from PulseNet (Centers for Disease Control and Prevention, Atlanta, GA) was used as the reference marker. Bacterial DNA restriction fragments in plugs were separated by electrophoresis using the CHEF-DRII system (BioRad, Hercules, CA) in 0.5× Tris-borate-EDTA buffer. Buffer was recirculated in the system at 14°C and 70 rpm. The initial switch time, final switch time, and run time were set at 2.2 s, 63.8 s, and 16.5 h, respectively. Voltage was set at 200 V. After electrophoresis, the gel was stained with 40 μl of ethidium bromide (10 mg/ml) in 400 ml of deionized water and then destained in deionized water. Bands were visualized under UV light, photographed, and analyzed with Bio-Numerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium) with a space tolerance of 1.5%. The similarity among strains was evaluated using the unweighted pair group method with arithmetic mean algorithm based on Dice coefficients.

RESULTS

Prevalence of *Salmonella* on in-shell pecans. Over the four harvest years, a total of 4,641 in-shell pecan samples were analyzed, and 44 (0.95%) samples were positive for *Salmonella* upon initial screening (Table 1). The highest annual prevalence occurred in the last sampling year (2013 to 2014), where 17 (1.4%) of 1,235 samples were positive for *Salmonella*; a similar prevalence was found in

TABLE 2. *Summary of survey results for* Salmonella *on four varieties of in-shell pecans over four harvest years*

	No. of positive samples/total samples tested				
Pecan variety	2010–2011	2011–2012	2012–2013	2013–2014	Total
Mexican Improved	4/187	1/290	2/372	7/307	14/1,156
Native/Seedling	1/149	2/251	1/112	2/96	6/608
Southern Improved	4/336	1/502	2/199	4/432	11/1,469
Western Improved	4/302	3/433	2/260	4/418	13/1,413
Southern/Native		0/3			0/3
Total	13/974	7/1,479	7/943	17/1,253	44/4,649

the first harvest year (2010 to 2011), where 13 (1.3%) of 974 samples were positive for *Salmonella*. Lower *Salmonella* prevalence was observed in 2011 to 2012 and 2012 to 2013, where 7 (0.47%) of 1,489 and 7 (0.70%) of 1,235 samples were positive for *Salmonella,* respectively. *Salmonella* prevalence in a given year was not significantly associated with the low or high production alternating year bearing cycle.

More positive samples were obtained with MPN method A (11 samples) than with method B (8 samples) (Table 1). In 2010 to 2011, three samples were positive for *Salmonella*: one sample was positive with method A, one was positive with method B, and one was positive with both methods. In 2011 to 2012, three samples were positive with method A, and two of these samples also were positive with method B. None of samples for 2012 to 2013 were positive for *Salmonella* based on the MPN analysis. In the last year of sampling (2013 to 2014), five samples were positive with method A, and three of these samples also were positive with method B. Because Bansal et al. *(4)* found no significant differences in the average MPN per 100 g calculated for the 2006 and 2007 almond crops using either MPN method, no attempt was made to determine *Salmonella* levels based on results from the individual methods; instead, we combined the results of both MPN methods to improve the limit of detection of the analysis (0.47 MPN/100 g).

The mean level calculated for the four pecan harvest years was 2.4 MPN/100 g (Table 1). For individual years, means were 0.89 MPN/100 g for harvest year 2010 to 2011, 6.2 MPN/100 g for 2011 to 2012, 0.47 MPN/100 g for 2012 to 2013, and 1.9 MPN/100 g for 2013 to 2014 (Table 1). A higher mean was obtained for 2011 to 2012, which was likely due to the high level in one positive sample. When that sample was excluded, the mean for the four pecan sampling years was 1.0 MPN/100 g.

Among the four varieties of pecan investigated, the *Salmonella* prevalence was not significantly different ($P > 0.05$) based on Student's t test. In general, the highest prevalence was observed in Mexican Improved (14 of 1,156 samples; 1.2%), followed by Native/Seedling (6 of 608 samples; 0.99%), Western Improved (13 of 1,413 samples; 0.92%), and Southern Improved (11 of 1,469 samples; 0.75%). During the 2011 to 2012 harvest year, three samples of mixed Southern Improved and Native/Seedling pecans were tested, and all were negative for *Salmonella* (Table 2).

***Salmonella* levels on in-shell pecans.** Of the 44 *Salmonella*-positive pecan samples obtained upon initial screening over the 4 years of the survey, *Salmonella* was not recovered (values < 0.47 MPN/100 g) from 31 of these samples. Levels in the positive pecan samples were <0.47 to 39 MPN/100 g. The highest levels in the 2010 to 2011, 2011 to 2012, 2012 to 2013, and 2013 to 2014 were 3.4 MPN/100 g (95% CI = 0.56 and 20 MPN/100 g), 39 MPN/100 g (6.5 and 235 MPN/100 g), <0.47 MPN/100 g, and 11 MPN/100 g (1.8 and 66 MPN/100 g), respectively (Table 3). In nearly 50% (6 of 13) of these samples, only the 25-g tubes (either one or several tubes) were positive with the MPN analysis.

Confirmation of positive mini-VIDAS results and enrichment of negative samples during initial screening. During the study, pecan samples occasionally were positive for *Salmonella* with the initial rapid screening test but were negative based on the subsequent enrichment culture. During the 2013 to 2014 harvest year, we reevaluated the remaining five samples for *Salmonella* using the BAM enrichment culture method. None of these samples were positive based on the culture results.

Serotyping and phage typing of *Salmonella* isolates. Of the 44 pecan samples positive for *Salmonella* with the initial screening test, three isolates from each of 42 samples (126 total isolates) were shipped to the NVSL for serotyping and phage typing. In the 2012 to 2013 harvest year, isolates from two *Salmonella*-positive samples were not shipped to UF because of an error by the outside laboratory. An additionally 54 isolates were recovered during MPN analysis; isolates from three samples from the 2010 to 2011 harvest year could not be recovered from the frozen stock. In total, 180 *Salmonella* isolates from the initial isolation and MPN analysis were serotyped (Table 3).

A single *Salmonella* serotype was obtained from almost all samples during initial screening (41 of 42 samples) and subsequent analysis with MPN (40 of 42 samples). Two *Salmonella* serotypes, Cubana and Livingstone, were isolated from the initial screening from one sample, 2011-0932 in the 2011 to 2012 harvest year. Upon subsequent MPN screening, in two samples two *Salmonella* serotypes were recovered with the MPN analysis; 2010-0173 contained Braenderup and Michigan, and 2011-0123 contained Enteritidis RDNC and Tennessee. The *Salmonella* serotype recovered with the MPN analysis did not always match that of the initial isolate. When a single

TABLE 3. *Prevalence, level, and serotypes of* Salmonella *obtained from naturally contaminated in-shell pecans, 2010 to 2014*

Pecan variety	Sample no.[a]	Original *Salmonella* serotype[b]	*Salmonella*-positive MPN tube	*Salmonella* level (MPN/100 g)[c]		MPN *Salmonella* serotype(s)[d]
				Mean	CI	
Mexican Improved	2010-0227	Enteritidis PT 8		0.47		
	2010-0229	Enteritidis PT 8	Method B: one 2.5-g, one 0.25-g tube	1.4	0.23–8.5	Braenderup
	2010-0235	Enteritidis PT 8		0.47		
	2010-0239	Enteritidis PT 8		0.47		
	2011-0803	Tucson		0.47		
	2012-0744	Poona		0.47		
	2012-0823	Oranienburg		0.47		
	2013-0026	Bredeney		0.47		
	2013-0742	III-61:I,v:1,5,7		0.47		
	2013-0746	Tennessee		0.47		
	2013-0770	4,5,12:k:1,7		0.47		
	2013-0832	Oranienburg		0.47		
	2013-1194	Newport	Method A: three 25-g tubes; method B: three 25-g tubes	11	1.8–66	Newport
	2013-1218	III45:z_4,z_{23}:−		0.47		
Native/Seedling	2010-0038	Thompson		0.47		
	2011-0429	Mbandaka		0.47		
	2011-1463	Saintpaul		0.47		
	2012-0024	LE		0.47		
	2013-0213	6,7:−:1,6		0.47		
	2013-0323	Javiana	Method A: one 25-g, two 2.5-g tubes; method B: three 25-g, one 2.5-g, three 0.25-g tubes	8.8	1.5–53	Javiana
Southern Improved	2010-0173	Braenderup	Method A: two 25-g tubes; method B: three 0.25-g tubes	3.4	0.56–20	Braenderup, Michigan
	2010-0179	6,7:−:1,5		0.47		
	2010-0189	Newport	Method A: one 25-g tube	1.0	0.17–6.0	NS[e]
	2010-0515	Braenderup		0.47		
	2011-1412	Braenderup		0.47		
	2012-0053	LE		0.47		
	2012-0056	6,7:y:−		0.47		
	2013-0321	Hartford		0.47		
	2013-0486	Typhimurium var. 5−		0.47		
	2013-0490	Pensacola	Method A: two 25-g, one 0.25-g tubes	2.3	0.38–14	Javiana
	2013-0983	Rubislaw		0.47		
Western Improved	2010-0293	Drac		0.47		
	2010-0316	iii_48;g,z_{51}:−	Method A: one 25-g tube	1.0	0.17–6.0	NS
	2010-0319	iii_48;g,z_{51}:−	Method B: one 25-g tube	1.0	0.17–6.0	NS
	2010-0774	Sandiego		0.47		
	2011-0123	Enteritidis PT 8	Method A: two 10-g, three 1-g, three 0.1-g tubes; method B: two 10-g, two 1-g, two 0.1-g tubes	39	6.5–235	Enteritidis RDNC, Tennessee
	2011-0932	Cubana, Livingstone	Method A: one 25-g tube; method B: one 2.5-g tube	1.6	0.27–9.6	Tennessee
	2011-1012	Livingstone	Method A: one 25-g tube	1.0	0.17–6.0	Tennessee
	2012-0424	Javiana		0.47		
	2012-0713	Javiana		0.47		
	2013-0442	Rough O-d:e,n,z_{15}	Method A: one 25-g tube, one 2.5-g tube	1.6	0.27–9.6	Javiana
	2013-0699	Senftenberg	Method A: one 25-g tube; method B: two 25-g tubes	2.6	0.43–16	Javiana

TABLE 3. *Continued*

Pecan variety	Sample no.[a]	Original *Salmonella* serotype[b]	*Salmonella*-positive MPN tube	*Salmonella* level (MPN/100 g)[c]		MPN *Salmonella* serotype(s)[d]
				Mean	CI	
	2013-1010	Denver		0.47		
	2013-1093	Javiana		0.47		

[a] In-shell pecans samples obtained from the 2010 to 2011 through 2013 to 2014 harvest years.
[b] Isolated from the original samples during the initial screening test. PT, phage type. LE, laboratory error (outside laboratory conducting the initial testing did not save the isolates).
[c] Mean was calculated by combining results obtained with method A and method B. CI, 95% confidence interval.
[d] Isolated from *Salmonella*-positive MPN tubes.
[e] NS, no survivors (isolates did not survive in the freezer).

Salmonella serotype was recovered from a sample, only one isolate was selected for antimicrobial susceptibility testing and DNA fingerprinting for both original and MPN sample isolates.

Of the 42 samples submitted to further testing, 31 different *Salmonella* isolates (original and MPN) were obtained. Among the original *Salmonella* isolates, serotype Enteritidis (12%) was the most common followed by Javiana (9%) and Braenderup (7%). Serotypes Newport, iii_48;g,z_{51}:–, Livingstone, and Oranienburg were isolated with the same frequency of 5%. *Salmonella* Enteritidis obtained from the years 2010 to 2011 and 2011 to 2012 were further characterized and determined to be phage type 8 for all the original isolates. In the 2013 to 2014 samples, a *Salmonella* Typhimurium isolate was determined to be var. 5– after phage typing by the NVSL.

Antibiotic susceptibility of serotypes. Among all the 55 isolates (original and MPN) tested, 15 (27%) were susceptible to the 14 antibiotics tested during the experiment, 22 (40%) were resistant to one antibiotic, and 11 (20%) were resistant to two antibiotics. Sixteen isolates (29%) were resistant to streptomycin, 27 isolates (49%) were resistant to tetracycline, and 35 isolates (64%) were resistant to either streptomycin or tetracycline. Seven strains were resistant to three or more antibiotics tested (Table 4). All the multidrug-resistant strains were resistant to more than one class of antibiotics, including aminoglycosides, cephalosporins, penicillins, tetracyclines, and quinolones.

PFGE profiling of serotypes. Isolates were subjected to PFGE using the *Xba*I enzyme. The similarity analysis generated 11 genotype clusters with a Dice coefficient cutoff point of 80%, representing 47 of 55 isolates (Fig. 1).

Three isolates of *Salmonella* Enteritidis phage type 8 recovered in the harvest year 2010 to 2011 and one isolate obtained in 2011 to 2012 had identical *Xba*I profiles. Similarly, iii-48:g,z_{51}:– isolates obtained from two samples during the 2010 to 2011 year were 100% related based on their *Xba*I profiles. The profiles for *Salmonella* Javiana from isolates recovered from 2 years (2012 to 2013 and 2013 to 2014) were indistinguishable. An identical profile for *Salmonella* Javiana also was observed for isolates recovered during MPN analysis of three samples in the 2013 to 2014 harvest year. Similarly, *Salmonella* Tennessee isolates obtained from two samples in harvest year 2011 to 2012 had identical profiles.

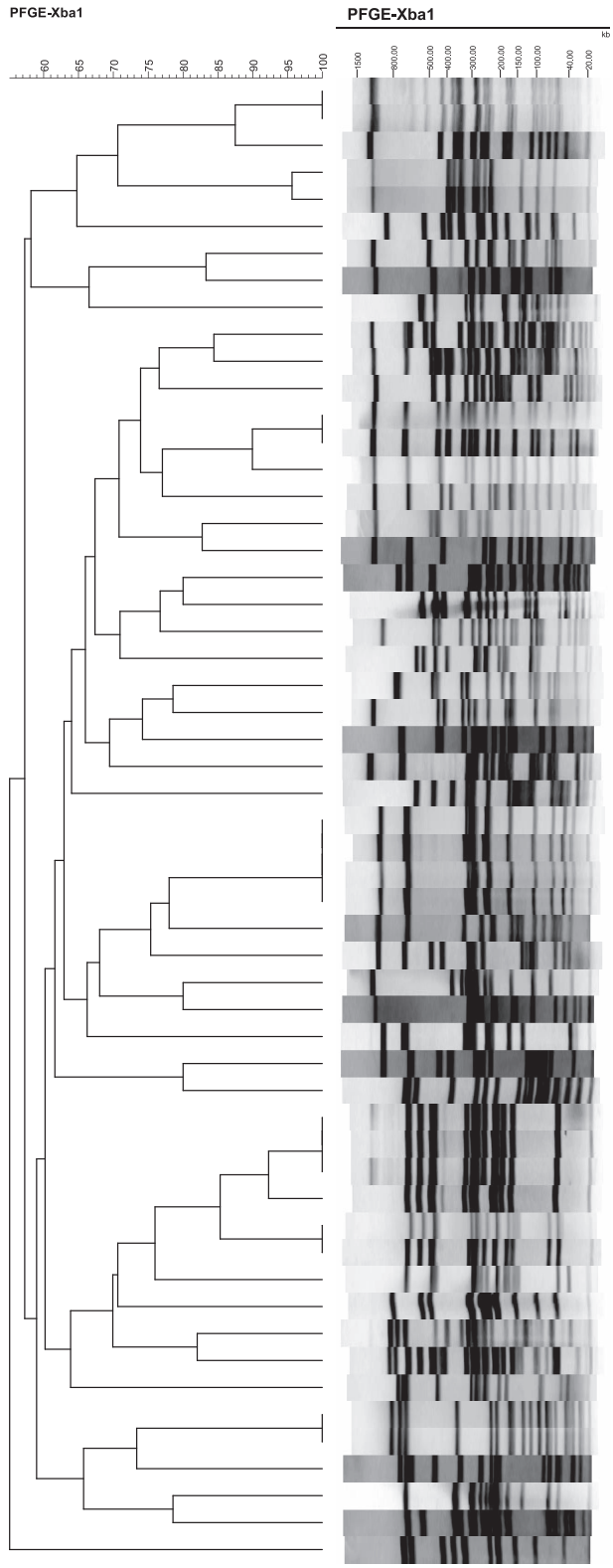
DISCUSSION

The prevalence of *Salmonella* observed over the 4-year survey period on in-shell pecans was 0.95%. The results from this study are in agreement with those of previous studies conducted on other nut types (e.g., almonds and peanuts). The prevalence of *Salmonella* observed during 7 years of sampling of raw almonds was 0.97% ± 0.34% *(4),* and on peanuts, initial screening of three crop years resulted in 2.33% *Salmonella*-positive samples *(10)*. In a similar study, *Salmonella* was found in 0.67% of shelled peanuts over a 3-year period *(19)*. In the United Kingdom retail market, 0.4% of 469 samples of Brazil nuts were positive for

TABLE 4. *Antibiotic resistance profiles of multidrug-resistant* Salmonella *isolates obtained from in-shell pecans over a 4-year period*[a]

Harvest year	Sample no.	*Salmonella* serotype	Susceptibility	Resistance
2010–2011	2010-0038	Thompson	An, Amc, Am, Fox, Cro, C, Cip, Gm, Ipm, Na, Sxt	K, S, Te
2010–2011	2010-0189	Newport	An, Am, Cro, C, Cip, Gm, Ipm, Na, Sxt, Te	Amc, Fox, K, S
2010–2011	2010-0173	Michigan	An, Cro, C, Cip, Gm, Ipm, K, Na, Sxt, Te	Amc, Am, Fox
2010–2011	2010-0229	Braenderup	An, Cro, C, Cip, Gm, Ipm, K, Na, Sxt, Te	Amc, Am, Fox
2011–2012	2011-0932	Tennessee	An, Amc, Am, Fox, Cro, C, Cip, Gm, Ipm, K, Sxt	Na, S, Te
2011–2012	2011-1012	Tennessee	An, Amc, Cro, C, Cip, Gm, Ipm, K, Sxt, Te	Am, Fox, Na, S
2013–2014	2013-1194	Newport	An, Amc, Am, Fox, Cro, C, Cip, Gm, Ipm, K, Na, S, Sxt	Amc, Am, Fox, K, Te

[a] Isolates listed were resistant to 3 or more of the 14 antibiotics tested: An, amikacin; Am, ampicillin; Amc, amoxicillin–clavulanic acid; Fox, cefoxitin; Cro, ceftriaxone; C, chloramphenicol; Cip, ciprofloxacin; Gm, gentamicin; Ipm, imipenem; K, kanamycin; Na, nalidixic acid; S, streptomycin; Sxt, trimethoprim-sulfamethoxazole; Te, tetracycline.



Pecan Sample Number	Serotype	MPN or Original
PS-2011-0932	Tennessee	MPN
PS-2011-1012	Tennessee	MPN
PS-2013-0746	Tennessee	Original
PS-2011-0932	Livingstone	Original
PS-2011-1012	Livingstone	Original
PS-2013-0983	Rubislaw	Original
PS-2012-0056	6,7:y:-	Original
PS-2013-0321	Hartford	Original
PS-2010-0189	Newport	Original
PS-2013-1194	Newport	MPN
PS-2010-0229	Branderup	MPN
PS-2013-1010	Denver	Original
PS-2010-0173	Branderup	Original
PS-2010-0173	Branderup	MPN
PS-2010-0515	Branderup	Original
PS-2011-1412	Branderup	Original
PS-2011-0429	Mbandaka	Original
PS-2012-0744	Poona	Original
PS-2013-0490	Pensacola	Original
PS-2013-0770	4,5,12:k:1,7	Original
PS-2010-0227	Enteritidis PT8	Original
PS-2011-0803	Tucson	Original
PS-2010-0038	Thompson	Original
PS-2010-0179	6,7:-:1,5	Original
PS-2013-0026	Bredeney	Original
PS-2013-1194	Newport	Original
PS-2011-0123	Tennessee	MPN
PS-2011-0123	Enteritidis PT8	Original
PS-2010-0229	Enteritidis PT8	Original
PS-2010-0235	Enteritidis PT8	Original
PS-2010-0239	Enteritidis PT8	Original
PS-2013-0699	Seftenberg	Original
PS-2013-0832	Oranienburg	Original
PS-2011-0932	Cubana	Original
PS-2013-0213	6,7:-:1,6	Original
PS-2011-0123	Enteritidis RDNC	MPN
PS-2012-0823	Oranienburg	Original
PS-2013-1218	III 45:z4,z23:-	Original
PS-2013-0699	Javiana	MPN
PS-2013-0490	Javiana	MPN
PS-2013-0442	Javiana	MPN
PS-2013-0323	Javiana	Original
PS-2012-0424	Javiana	Original
PS-2013-1093	Javiana	Original
PS-2012-0713	Javiana	Original
PS-2010-0293	Drac	Original
PS-2010-0173	Michigan	MPN
PS-2013-0323	Javiana	MPN
PS-2011-1463	Saintpaul	Original
PS-2010-0316	iii-48:g,z51:-	Original
PS-2010-0319	iii-48:g,z51:-	Original
PS-2013-0486	Typhimurium var 5-	Original
PS-2010-0774	San Diego	Original
PS-2013-0442	Rough O:d:e,n,z15	Original
PS-2013-0742	III-61:l,v:1,5,7	Original

FIGURE 1. *PFGE fingerprints of typed* Salmonella *isolates obtained from in-shell pecans during the 4-year survey period.*

Salmonella (17). In one study *(11),* the frequency of *Salmonella*-positive samples (84%) in recalled almond samples in 2000 to 2001 was significantly higher than that reported in other nut studies. In all previous studies, nut samples were collected after shelling and hulling, whereas in the present study, pecan samples were obtained before shelling. Pecan shelling involves the use of hot water, cold water, chlorinated water, or a combination of treatments; therefore, the present study was conducted with in-shell pecans to determine the pathogen prevalence naturally present on the pecan surface. Four varieties of pecans were included, but no significant differences in *Salmonella*

prevalence was found among these varieties. *Salmonella* prevalence was also studied in different grades of peanuts in a similar study, and significantly higher prevalence was found in splits (1.46%; $P < 0.05$) than in medium (0.33%) and no. 1 (0.91%) grades *(19)*. In other studies of in-shell nuts, *Salmonella* prevalence was 0.9% in 2006 and 2.2% in 2007 on in-shell almonds *(4)* and 0.14% on in-shell walnuts collected over three harvest years, 2011 to 2013 *(12)*.

The mean *Salmonella* population on in-shell pecans in the present study was 2.4 MPN/100 g (<0.47 to 39 MPN/100 g) based on combined results obtained with methods A and B. This mean is similar to that obtained in the previous almond study *(4)*: 2.3 MPN/100 g with method A and 2.1 MPN/100 g with method B. *Salmonella* levels in two peanut studies were <0.03 to 2.4 MPN/g (<3 to 240 MPN/100 g) *(10)* and 0.74 to 5.25 MPN/350 g (0.21 to 1.5 MPN/100 g) *(19)*. Both peanut studies included the three-tube MPN assay for analysis. The *Salmonella* levels obtained with a modified MPN method in the in-shell walnut study over three harvest years were 0.32 to 0.42 MPN/100 g *(12)*. Edible Brazil nuts were contaminated at <0.010 to 0.23 MPN/g (<1 to 23 MPN/100 g) as determined with a 10-tube MPN method *(17)*. The same group of researchers *(17)* also found *Salmonella* in 1 of 329 samples of mixed nuts (almond, Brazil nut, cashew, peanut, and walnut) tested, with a mean level of <0.010 MPN/g (1 MPN/100 g) obtained with a 10-tube MPN method *(17)*.

Similar to the almond studies *(4, 11),* where 19 of 31 samples (method A) *(4),* 23 of 29 samples (method B) *(4),* and 59 of 65 samples were not positive upon retesting, in the present study, a majority (31 of 44 samples; methods A and B) of the initially *Salmonella*-positive samples were not classified as positive after subsequent MPN analysis. Miksch et al. *(19)* reported similar results for peanuts, where 56 of 65 initially positive samples were not positive after retesting. The high frequency of negative samples obtained after retesting may be due to the sporadic nature of *Salmonella* contamination on pecans and the fact that all positive pecans were tested during the initial screening.

From the 42 *Salmonella*-positive in-shell pecan samples, 31 serotypes were isolated. Similarly, 35 serotypes from 81 *Salmonella*-positive samples and 23 serotypes from 53 *Salmonella*-positive samples were obtained from almond studies conducted for the periods 2001 to 2005 *(11)* and 2006 and 2007 *(4),* respectively. However, only two serotypes, *Salmonella* Tennessee and *Salmonella* Senftenberg, were isolated from Brazil nuts procured from a retail market in the United Kingdom *(17)*. Only *Salmonella* Anatum was isolated from the mixed nut sample tested by the same research group *(17)*. The high diversity of *Salmonella* serotypes recovered in the present study is presumed to be a result of the pecan production environment. Some serotypes obtained in the present and previous *(11)* studies were resistant to more than three antibiotics tested, which may be a matter of concern from a public health standpoint. Most of the isolates were resistant to either streptomycin or tetracycline. The increased resistance of *Salmonella* to these two antibiotics has become a concern worldwide *(21)*. In the present study, the multidrug-resistant isolates were resistant to more than one class of antibiotics.

In this study, different PFGE patterns were found within the same *Salmonella* serotype. In a few cases, the same strain (banding pattern confirmed 100% relatedness) was isolated over 2 years. The presence of identical strains over multiple years indicates the possibility of persistence of that strain in the environment or processing facility. Forty-six different PFGE banding patterns (original and MPN) were generated, suggesting the presence of at minimum 46 strains of *Salmonella* on the tested pecan samples. Similarly, a high diversity of *Salmonella* strains also was observed on peanut kernels in a previous study conducted over 3 years *(17)*.

Conditioning treatments used during the shelling of pecans may significantly reduce the *Salmonella* levels found on pecans *(7)*. Subsequent processing treatments can further reduce *Salmonella* populations to safer levels, provided recontamination does not occur. However, the presence of a few *Salmonella* cells on foods with a high fat content and low water activity can cause an outbreak *(14)*. *Salmonella* levels of 10 CFU/100 g or less have been recovered from contaminated chocolate bars associated with an outbreak, which emphasizes the need for proper preventive control measures for low-water-activity foods. The data obtained in the present study on the levels and prevalence of *Salmonella* serotypes will contribute to the development of product-specific quantitative microbial risk assessment models to determine the likelihood of illness associated with pecans and the necessary pathogen reductions required to obtain a safer product.

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