Prevalence of *Escherichia coli* O157:H7 and *Salmonella* on Inshell California Walnuts

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ABSTRACT

Inshell walnuts collected from California walnut handlers over four harvests were evaluated for the presence of *Escherichia coli* O157:H7 and *Salmonella*. *E. coli* O157:H7 was not detected in any of 2,903 375-g samples evaluated in 2011, 2012, and 2013 (<0.034% prevalence; 95% confidence interval [CI], 0 to 0.13%). *Salmonella* was not isolated from any of the 935 samples in 2010 (100 g evaluated; <0.11% prevalence; 95% CI, 0 to 0.41%) but was isolated from 2 of 905 (375 g; 0.22% prevalence; 95% CI, 0.061 to 0.80%), 1 of 998 (375 g; 0.10% prevalence; 95% CI, 0.018 to 0.56%), and 1 of 1,000 (375 g; 0.10% prevalence; 95% CI, 0.018 to 0.56%) samples in 2011, 2012, and 2013, respectively, for an average annual prevalence of 0.14% (375 g; 95% CI, 0.054 to 0.35%). The levels of *Salmonella* in positive samples determined by a modified most-probable-number (MPN) method were estimated to be 0.32 to 0.42 MPN/100 g (95% CI, 0.045 to 3.6 MPN/100 g).

MATERIALS AND METHODS

Walnut samples. Inshell walnuts were collected over a 4-year period (2010 to 2013) from 15 walnut handlers (processors) that were located throughout the five walnut-growing regions of California (Sacramento Valley, Northern San Joaquin Valley, Southern San Joaquin Valley, North Coast, and Central Coast). These 15 handlers represent approximately 50% of the total crop output in the state. The same handlers contributed samples in each of the 4 years of the study, with the exception of a single handler (replaced in 2012). A total of 935, 905, 998, and 1,000 samples, each ~1 kg, were collected in 2010, 2011, 2012, and 2013, respectively.

In general, each sample consisted of a single cultivar of walnuts representing 1 of 24 different cultivars produced in California (of the approximately 37 varieties grown commercially); handlers were instructed to sample each cultivar in approximate proportion to its annual production volume for that facility. In 18 cases, the samples consisted of a mixture of two cultivars. Samples were collected from incoming trucks arriving at the handler from various hullers. The number of samples collected from each handler was proportional to the amount of walnuts produced by the facility; approximately 20 samples were taken annually from the smallest handler and 250 samples from the largest. Samples were collected either by DFA of California (Fresno, CA) staff (60% of samples) or the handlers’ quality control staff (40% of samples). Samples were shipped to DFA of California for microbial analysis, coded to blind the origin, and stored at 4°C before testing (to minimize changes in microbial populations as previously demonstrated for *Salmonella* (7)); samples were tested within 3 months of collection. Subsamples (100 g in 2010 and 375 g in 2011, 2012, and 2013) of walnuts were analyzed for *Salmonella* (2010 to 2013) by AOAC official method 2001.09 (2) and separately for *E. coli* O157:H7 (375 g from 2011 to 2013) by AOAC Research Institute method no. 060903.
Enrichment for *Salmonella* from walnuts. In accordance with AOAC official method 2001.09, survey walnuts were mixed, and 100-g, 250-g, or 375-g subsamples were combined with 900, 2,250, or 3,375 ml of buffered peptone water (BD, Franklin Lakes, NJ), respectively, in sterile 946-ml or 4,000-ml plastic jars (Bel-Art Products, Pequannock, NJ) and shaken for 120 s. Following mixing, the samples were loosely capped and incubated at 35 ± 2°C for 18 to 24 h. The overnight preenrichment culture was then subjected to immunocentrifugation by the automated mini-VIDAS system (bioMérieux, Hazelwood, MO). Preenrichment broth (800 µl) was processed on an immunocentrifugation *Salmonella* (ICS) test strip (bioMérieux), and the resulting concentrate was used to inoculate a 2-ml vial of ICS broth (bioMérieux); vials were incubated at 41 ± 1°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* by using the mini-VIDAS system. The VIDAS screening system is based on an enzyme-linked fluorescence assay; a relative fluorescence value greater than 0.23 was considered a positive result.

When a sample was positive for *Salmonella* by the VIDAS system, the remaining (unboiled) portion of the ICS broth culture from the vial was streaked onto three selective agars: CHROMagar *Salmonella*, Hektoen enteric (HE), and xylose lysine deoxycholate (XLD). Unless otherwise noted, media were obtained from BD. Plates were examined for typical *Salmonella* colonies after incubation at 35 ± 2°C for 24 h. When present, suspect colonies from each of the three media were checked for purity by restreaking them onto plates of MacConkey agar (BD Diagnostic Systems, Sparks, MD), MacConkey agar plates were incubated at 35 ± 2°C for 24 h and examined for typical *Salmonella* colonies. The identity of presumptive *Salmonella* colonies was confirmed with API 20E test strips (bioMérieux) according to the manufacturer’s instructions.

When at least 50 g remained of the *Salmonella*-positive samples, additional subsamples were enriched for *Salmonella* using a modification of the U.S. Food and Drug Administration Bacteriological Analytical Manual (FDA BAM) method (1). Lactose broth was added at a ratio of 1:9 for each of 2 50-g (2012), 1 120-g (2012), or 10 50-g (2013) subsamples. Each bag was vigorously hand shaken through a 30-cm arc for 1 min. All samples were incubated at 37 ± 2°C for 24 h.

After incubation, 0.1 and 1 ml of each lactose broth sample was transferred into 9.9 ml of Rappaport-Vassiliadis R10 broth and 9 ml of tetraionate broth, respectively, and incubated at 42 ± 2°C in a water bath and at 37 ± 2°C in an incubator, respectively, for 24 h. Each enrichment was streaked onto XLD agar, HE agar, and bismuth sulfite agar and incubated at 37 ± 2°C for 24 h (XLD and HE agars) or 48 h (bismuth sulfite agar). A single colony that displayed typical characteristics of *Salmonella* on one of the three plates was selected from each enrichment (tetraionate and Rappaport-Vassiliadis R10 broths) and restreaked onto both XLD agar and HE agar. A single isolated colony that displayed typical characteristics of *Salmonella* on one of these plates was selected and then streaked onto tryptic soy agar (TSA) and incubated at 37 ± 2°C for 24 h. Isolated colonies were selected and stabbed and streaked into triple sugar iron agar and lysine iron agar slants and incubated at 37 ± 2°C for 24 h. Isolates that were characteristic of *Salmonella* on triple sugar iron agar or lysine iron agar were confirmed with a *Salmonella* latex test (Oxoid, Ogdensburg, NY). Most-probable-number (MPN) values and confidence intervals (CIs) were determined, using the Excel spreadsheet provided in the FDA BAM method (8), for the following dilution series: one 375-g replicate (the positive original enrichment), total 50-g replicates (2011 and 2013), or one 120-g replicate (2012).

Enrichment for *E. coli* O157:H7 from walnuts. In accordance with AOAC Research Institute method no. 060903, 375-g subsamples were combined with 1,125 ml of buffered peptone water with added vancomycin in sterile 4,000-ml plastic jars (Bel-Art Products) and shaken for 120 s. Following mixing, the samples were loosely capped and incubated at 42 ± 2°C for 10 to 24 h. Preenrichment broth (500 µl) was added to an *E. coli* phage technology strip (bioMérieux) and heated for 5 ± 1 min prior to performing the VIDAS assay. When a positive result was obtained, a second sample (500 µl) was taken from the unboiled enrichment broth and subjected to a VIDAS immunocentrifugation *E. coli* assay. The immunocentrifugated solution was then collected with a calibrated swab and streaked onto sorbitol MacConkey agar with cefixime and tellurite plates and incubated at 37 ± 2°C for 18 to 24 h.

After incubation, plates were examined for colonies typical of *E. coli* O157:H7 (colorless or neutral/gray with a smoky center). Two or more colonies were picked and tested with a latex agglutination test for the O157 and H7 antigens (Remel, Lenexa, KS). Up to 10 additional colonies were streaked onto TSA supplemented with yeast extract and incubated at 37 ± 2°C for 18 to 24 h. The identity of presumptive *E. coli* O157:H7 colonies was confirmed with API 20E test strips according to the manufacturer’s instructions.

Identification of *Salmonella* isolates. Confirmed *Salmonella* isolates from survey walnuts were stored at −80°C in tryptic soy broth containing 15% glycerol. Agar slants for all cultures were submitted to the California Animal Health and Food Safety Laboratory System (Davis, CA) for serotyping. Isolates serotyped as *Salmonella enterica* serotype Enteritidis were submitted to the National Veterinary Services Laboratory (Ames, IA) for phage typing.

Microbiological analysis of survey walnuts. For each survey year, every 10th sample and three of the four *Salmonella*-positive samples were screened for aerobic plate count (APC), *E. coli*, yeasts, and molds. For these tests, a 90-g sample of walnuts was combined with 90 ml of sterile Butterfield’s phosphate buffer (3M, St. Paul, MN) to obtain a 109 dilution and then shaken 50 times through a 30-cm arc. The mixture was allowed to stand for 3 to 5 min and then shaken five times through a 30-cm arc to resuspend the sample before serial dilution in Butterfield’s phosphate buffer and transfer to media. APCs were determined by AOAC official method 966.23 (3). *E. coli* was quantified via MPN analysis by a modified version of AOAC official method 966.24 (4); the modification was to use API 20E test strips to confirm positive *E. coli* colonies. Yeast and mold counts were determined by following the FDA BAM method (33), except that tempered agar pour plates rather than spread plates of Phytone yeast extract agar were used. This modified method is commonly used by the nut and dried fruit industry. When evaluated previously, counts from pour and spread plates were not significantly different (data not shown).

Statistical analysis. A comparison of difference in *Salmonella* prevalence on walnuts and other tree nuts and peanuts was performed using Fisher’s exact test. Statistical tests of these hypotheses used a level of significance of 0.05 and were performed with JMP 11 software (SAS Institute, Inc., Cary, NC). The 95% Wilson score CIs were determined with JMP 11 software.
RESULTS

Prevalence of Salmonella and E. coli O157:H7 on inshell walnuts. A total of 3,838 inshell walnut samples collected from the 2010 through 2013 California harvests were analyzed. Our goal was to sample many of the major cultivars at rates roughly proportional to the harvested volume for each cultivar (Table 1). Cultivar Chandler was sampled at a lower proportion than the harvested volume, and cultivars Howard, Hartley, and Tulare were sampled at higher proportions. None of the 935 100-g-walnut samples from the 2010 harvest were positive for Salmonella (<0.11% prevalence; 95% CI, 0 to 0.041%) (Table 2). In subsequent years, the sample size was increased to 375 g; in some cases, the amount of sample available for analysis was limited to 100 g (two samples in 2011 and three samples in 2012) or 250 g (seven samples in 2012). Four of the 375-g samples were positive for Salmonella: 2 of 905 (0.22% prevalence; 95% CI, 0.061 to 0.80%; cultivar Howard and cultivar Serr), 1 of 998 (0.10% prevalence; 95% CI, 0.018 to 0.56%; cultivar Howard), and 1 of 1,000 (0.10% prevalence; 95% CI, 0.018 to 0.56%; cultivar Vina) in 2011, 2012, and 2013, respectively, for an average annual prevalence of 0.14% (95% CI, 0.054 to 0.35%). In early 2011, separate outbreaks of E. coli O157:H7 gastroenteritis were epidemiologically linked to consumption of inshell hazelnuts (15) and inshell walnuts (10). Therefore, separate subsamples of inshell walnuts were analyzed for E. coli O157:H7. None of the 2,903 375-g samples evaluated in 2011, 2012, and 2013 were positive for E. coli O157:H7 (<0.034% prevalence; 95% CI, 0 to 0.13%).

Levels of Salmonella on inshell walnuts. Small amounts of three of the four Salmonella-positive walnut samples (2 50-g, 1 120-g, or 10 50-g subsamples) were available for further analysis; one of these secondary samples was positive after enrichment (Table 2). The estimated levels of Salmonella in these walnut samples were 0.42 (95% CI, 0.047 to 3.6), 0.38 (95% CI, 0.045 to 3.2), and 0.32 (95% CI, 0.077 to 1.3) MPN/100 g, respectively (Table 2).

Serotyping of Salmonella isolates. Salmonella isolates were serotyped as S. enterica serovars Saintpaul, Muenchen, Enteritidis phage type RDNC (routine dilution, no conformity), and Bovismorbificans (Table 2). All isolates from a single sample (three to nine isolates per sample) were the same serotype. Both positive subsamples from 2013 (one from the original 375-g subsample and the other from 1 of 10 50-g subsamples) were identified as Salmonella Bovismorbificans.

Background microbiota on inshell walnuts. A total of 386 samples were analyzed for APC, E. coli, yeasts, and molds (Fig. 1), including three of the four Salmonella-positive samples. APCs ranged from <1.00 to >5.40 log CFU/g, with a mean of 3.30 ± 0.92 log CFU/g and a median of 3.43 log CFU/g; the APCs for positive samples (2.40, 2.83, and 2.92 log CFU/g) were not significantly different (P > 0.05) from the mean APC of negative samples. E. coli was detected in 10 samples (2.6%) with concentrations ranging from −0.40 to 2.04 log MPN/g and mean and median concentrations of 0.84 and 0.75 log MPN/g, respectively; E. coli was not detected (≤−0.44 log MPN/g) in the Salmonella-positive samples. Yeasts were detected in 26 samples (6.7%), with populations ranging from 1.00 to 5.18 log CFU/g (average and median concentrations of 2.02 and 1.74 log CFU/g, respectively); yeasts were not detected in Salmonella-positive samples (≤1.00 log CFU/g). Populations of molds ranged from 1.00 to 5.00 log CFU/g (average and median concentrations of 2.45 and 2.36 log CFU/g, respectively); the levels of molds in Salmonella-positive samples were 2.40, 2.56, and 2.34 log CFU/g.

DISCUSSION

Much of the available data for the prevalence of foodborne pathogens on nuts is derived from retail surveys using small sample sizes (25 g) (21). In the current study, inshell walnuts were collected shortly after harvest from a representative group of handlers across California that process approximately half of the total production volume harvested in the state. Samples were stored refrigerated and

### TABLE 1. Proportion of walnuts sampled to walnuts produced in California in 2010 to 2013 for each walnut cultivar

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
<th>4-yr avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chandler</td>
<td>31/42</td>
<td>14/44</td>
<td>23/42</td>
<td>47/46</td>
<td>29/43</td>
</tr>
<tr>
<td>Howard</td>
<td>14/11</td>
<td>19/13</td>
<td>24/12</td>
<td>11/14</td>
<td>17/13</td>
</tr>
<tr>
<td>Hartley</td>
<td>16/14</td>
<td>14/12</td>
<td>11/12</td>
<td>12/11</td>
<td>13/12</td>
</tr>
<tr>
<td>Tulare</td>
<td>13/3</td>
<td>18/12</td>
<td>18/14</td>
<td>12/13</td>
<td>15/12</td>
</tr>
<tr>
<td>Vina</td>
<td>7.0/7.1</td>
<td>9.9/5.9</td>
<td>7.8/6.1</td>
<td>4.9/5.0</td>
<td>7.4/6.0</td>
</tr>
<tr>
<td>Serr</td>
<td>6.5/7.2</td>
<td>12/5.7</td>
<td>8.0/5.7</td>
<td>4.1/4.5</td>
<td>7.5/5.8</td>
</tr>
<tr>
<td>Ashley</td>
<td>2.7/1.9</td>
<td>2.7/1.3</td>
<td>1.4/1.3</td>
<td>0.8/1.2</td>
<td>1.9/1.4</td>
</tr>
<tr>
<td>Payne</td>
<td>2.5/1.6</td>
<td>1.2/1.2</td>
<td>1.9/1.3</td>
<td>0.7/1.1</td>
<td>1.6/1.3</td>
</tr>
<tr>
<td>Franquette</td>
<td>0.0/0.95</td>
<td>0.5/0.85</td>
<td>0.2/0.10</td>
<td>2.2/0.79</td>
<td>0.74/0.90</td>
</tr>
<tr>
<td>Tehama</td>
<td>1.8/1.0</td>
<td>0.5/0.73</td>
<td>0.2/0.81</td>
<td>0.5/0.64</td>
<td>0.77/0.80</td>
</tr>
<tr>
<td>Eureka</td>
<td>0.11/0.57</td>
<td>0/0.34</td>
<td>0.3/0.10</td>
<td>0.7/0.32</td>
<td>0.28/0.56</td>
</tr>
<tr>
<td>Other</td>
<td>5.9/3.8</td>
<td>7.8/3.6</td>
<td>4.3/3.7</td>
<td>4.0/2.8</td>
<td>5.5/3.5</td>
</tr>
</tbody>
</table>
TABLE 2. Summary of walnut surveys from 2010 to 2013

<table>
<thead>
<tr>
<th>Survey yr</th>
<th>No. of samples</th>
<th>Sample size (g)</th>
<th>No. of samples positive for Salmonella (95% CI)</th>
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<tr>
<td>2010</td>
<td>935</td>
<td>100</td>
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<tr>
<td>2011</td>
<td>905</td>
<td>375</td>
<td>2 (Howard, Serr) 0.22 (0.06–0.80)</td>
</tr>
<tr>
<td>2012</td>
<td>998</td>
<td>375</td>
<td>1 (Howard) 0.10 (0.008–0.65)</td>
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<tr>
<td>2013</td>
<td>1,000</td>
<td>375</td>
<td>1 (Vina) 0.10 (0.008–0.65)</td>
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Additional enrichment of positive samples from Salmonella-positive samples

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</tr>
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</table>

Note: NA, not applicable; RDNC, routine dilution, no conformity.

Additional enrichment of positive samples from Salmonella-positive samples

The presence of *E. coli* was not predictable of the presence of *Salmonella* in almonds (17) or in seeds (36); *E. coli* was not detected in the four positive walnut samples (<0.30 MPN/g).

Determining the source of *Salmonella* in outbreaks or recalled product is often challenging, and in many cases, a definitive source remains unknown. Contamination in the orchard during harvest was considered the most likely source of *Salmonella* for outbreak-associated almonds (22, 34); postroasting contamination was suspected for several peanut butter outbreaks (12, 14). In the current study and other large-scale nut surveys (5, 9, 17, 27), the samples were collected at processor receipt and prior to any sorting, grading, or processing. Thus, contaminants in these surveys could only have been introduced before any processing steps, namely in the orchard or during harvest or immediate postharvest handling.

There are a number of points during harvest and postharvest handling when it is feasible that *Salmonella* could be introduced to walnuts. Walnut fruits consist of...
a kernel surrounded by a hard shell and fleshy hull. At harvest, walnuts are mechanically shaken to the ground and then mechanically swept up. The walnut fruits are separated from rocks, sticks, leaves, and other debris in a process that includes rapid passage through a water-filled tank to separate sinking rocks from the floating walnuts. APC and coliform counts in the float tank water can be 6 log CFU/ml or greater (6). Walnuts are mechanically hulled under wet conditions to remove the flesh from the fruit, leaving the inshell nut. Hulled walnuts are dried for 4 to 48 h at ≤43°C (23); APC reductions of 0.1 to 2.0 log CFU per nut were observed during drying (6, 19). Inoculated Salmonella and E. coli O157:H7 declined by 2.6 and 3.0 log CFU per nut, respectively, on inshell walnuts dried under simulated commercial conditions and by 0.29 and 2.0 log CFU per nut, respectively, during the first week of simulated commercial storage (18). Walnuts are typically stored in the shell at ambient temperature and are processed (including cracking and removing shells) as needed to fill orders. Salmonella and E. coli O157:H7 declined at rates of 0.19 and 0.17 log CFU/month in simulated commercial storage (10°C and 65% relative humidity) of inshell walnuts (18). Both E. coli O157:H7 and Salmonella could be recovered from inoculated inshell walnuts by plating or enrichment for extended periods (97 days at 23 to 25°C [initial levels of 1.5 log CFU per nut] or 360 days at 10°C [initial levels of ≥8.0 log CFU per nut]) (7, 18). It is not known how well these studies translate to naturally contaminated inshell product. However, these experimental data suggest that 2- to 5-log reductions may occur from the time of hulling to the point at which our samples would have been collected.

Each of the four positive walnut samples in this study yielded a unique Salmonella isolate; the limited number of positive samples in the current survey makes it difficult to assess the potential environmental source(s) of Salmonella in walnuts. A wide range of Salmonella serovars were isolated from raw almonds (49 different serovars in 151 positive samples) (5, 17) and from raw, shelled peanuts (13 different serovars in 22 positive samples) (9). The diversity of Salmonella serovars observed in these larger studies suggests that Salmonella may be introduced to these nuts via several environmental sources rather than from a point source or reservoir.

Salmonella is widely considered to be the pathogen of concern in low-moisture foods, including nuts (20, 32). When performing a hazard analysis as part of a food safety plan, walnut handlers should consider a broad array of sources of information, including survey and survival data and information on recalls and outbreaks for walnuts, as

FIGURE 1. Distribution of populations of APC, E. coli, molds, and yeasts on inshell walnuts in survey sampling conducted from 2010 to 2013 (n = 386).
well as other tree nuts. A key element in quantitative microbiological risk assessments is an exposure assessment, which considers consumption estimates and known prevalence and concentrations of specific pathogens on the crop(s) being assessed. The data presented here can be used in subsequent risk assessments to guide the walnut industry in the development of targeted processes or practices that further reduce the risk of foodborne illness linked to consumption of walnuts.

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