Survival of foodborne pathogens on inshell walnuts☆

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The survival of Salmonella enterica Enteritidis PT 30 or five-strain cocktails of S. enterica, Escherichia coli O157:H7, and Listeria monocytogenes was evaluated on inshell walnuts during storage. Inshell walnuts were separately inoculated with an aqueous preparation of the pathogens at levels of 10 to 4 log CFU/nut, dried for 24 h, and then stored at either 4 °C or ambient conditions (23–25 °C, 25–35% relative humidity) for 3 weeks to more than 1 year. During the initial 24-h drying period, bacterial levels declined by 0.7 to 2.4 log CFU/nut. After the inoculum dried, further declines of approximately 0.1 log CFU/nut per month of Salmonella Enteritidis PT 30 levels were observed on inshell walnuts stored at 4 °C; at ambient conditions the rates of decline ranged from 0.55 to 2.5 log CFU/nut per month. Rates of decline were generally greater during the first few weeks of storage, particularly at lower inoculum levels. The survival of the five-strain cocktails inoculated at very low levels (under 400 CFU/nut) was determined during storage at ambient conditions. The pathogens could be recovered by either enumeration or enrichment from most samples throughout the 3-month storage period; reductions in bacterial levels from the beginning to end of storage were 0.7, 0.2, and 2.3 log CFU/nut for Salmonella, E. coli O157:H7, and L. monocytogenes, respectively. For 6% of all nut samples (14 of 234 samples), pathogens were isolated from the second but not first 24-h enrichment, suggesting that bacterial cells were viable but not easily culturable. Salmonella-inoculated walnuts were exposed for 2 min to water or a 3% solution of sodium hypochlorite (to mimic commercial brightening) either 24 h or 7 days after inoculation; treated nuts were dried for 24 h and held at ambient conditions. Salmonella levels were reduced by less than 0.5 log or 2.4 to 2.6 log CFU/nut on water- or chlorine–treated walnuts, respectively, regardless of postinoculation treatment time. Additional reductions of 2.6 and 2.1 log CFU/nut were observed for water- and chlorine-treated walnuts, respectively, after storage for 2 weeks at ambient conditions. Bacterial foodborne pathogens are capable of long-term survival on the surface of inshell walnuts even when initial levels are low.

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of California inshell walnuts. E. coli O157:H7 was not detected; Salmonella was detected in none of the samples in 2010 (100 g, n = 935), in 0.2% of samples in 2011 (375 g, n = 905), and in 0.1% of samples in 2012 (375 g, n = 999) (Eidsath, 2012).

The United States is the leading exporter of the Persian or English walnut (Juglans regia L.); 99% of the U.S. production (470,000 metric tons projected in 2012) is grown in California (California Walnut Commission, 2012; USDA FAS, 2012; USDA NASS, 2012). Shortly after harvest, walnuts are hulled to remove the fleshy husk and then dried with forced air; dried inshell walnuts may be stored prior to packaging or shellling. In 2011, approximately 40% of edible California walnut kernels were sold in-the-shell (estimated average 44% kernel weight) and the majority of these inshell walnuts (94%) were exported (California Walnut Board, 2012); the remaining 6% were removed from storage as needed, then cracked and sold as kernels.

Traditionally, most of the inshell walnuts sold in North America undergo a shell-lightening (or “brightening”) treatment by direct surface application of sodium hypochlorite at a concentration of 3 to 4% (30,000–40,000 µg/ml or ppm in solution). The solution is sprayed onto the nuts, which are then mechanically mixed for approximately 2 min in a barrel trommel, and dried with or without forced air (Lindsay, 2010). Although the purpose of this treatment is to lighten shells, sodium hypochlorite is also a common disinfectant; it is unknown to what extent brightening impacts the microbial load on walnut shells.

The routes of contamination of tree nuts have not been definitively determined, but there are a number of potential opportunities for introduction of foodborne pathogens to walnuts through direct contact with contaminated soil during harvest, during postharvest hulling and drying, during cracking and shellling, or during further processing (Blessington, 2011; Meyer and Vaughn, 1969; Weinzil, 1929). Foodborne pathogens can survive for extended periods on walnut kernels (Blessington et al., 2012) and Salmonella has been shown to survive on the shells of pecans and hazelnuts (Beuchat and Heaton, 1975; Beuchat and Mann, 2010a, 2010b; Komitopoulou and Peñalosa, 2009). Survival of foodborne pathogens on inshell walnuts has not been documented. The objectives of this study were to evaluate the survival of Salmonella, E. coli O157:H7, and L. monocytogenes during storage of inshell walnuts, and to determine the impact of a brightening treatment on reducing Salmonella levels on inoculated inshell walnuts.

2. Materials and methods

2.1. Walnut samples

Inshell walnuts, J. regia L. cv. Hartley and cv. Chandler, were obtained from a San Joaquin county processor in California. The walnuts had been hulled and dried (to ~8% moisture) at a commercial huller-dehydrator and had been stored at the processor for 1 to 6 months after harvest. For the inoculation studies, the inshell walnuts were used within 1 month of receipt; for the brightening study, the walnuts were stored for up to 11 months at ambient conditions in the laboratory (23–25 °C, 25–35% relative humidity) in a closed container. Walnuts with missing shell or those with major visible cracks were discarded.

2.2. Bacterial cultures

The pathogens used in this study were as follows: S. enterica Enteritidis PT 30 (ATCC BAA-1045), isolated from raw almonds associated with an outbreak (Isaacs et al., 2005); S. enterica Enteritidis PT 9c, a clinical isolate from an outbreak associated with raw almonds (CDC, 2004); S. enterica Anatum (CAHFS D0307231), isolated from an almond survey (Danyiluk et al., 2007); S. enterica Oranienburg, isolated from pecans, (provided by Dr. Larry R. Beuchat, University of Georgia); S. enterica Tennessee (K4642), a clinical isolate from a peanut butter–associated outbreak (CDC, 2007); E. coli O157:H7 (H1730), a clinical isolate from a lettuce–associated outbreak; E. coli O157:H7 (CDC 658), a clinical isolate from a cantaloupe–associated outbreak; E. coli O157:H7 (F4546), a clinical isolate from an alfalfa sprout–associated outbreak; E. coli O157:H7 (Odwalla strain 223), isolated from an apple juice–associated outbreak; E. coli O157:H7 (EC4042), a clinical isolate from a spinach–associated outbreak (Kotewicz et al., 2008); L. monocytogenes (4b) (LJH552), isolated from tomatoes; L. monocytogenes (4b) (LCDC81-861), isolated from a raw cabbagge–associated outbreak; L. monocytogenes (4b) (Scott A), a clinical isolate from a milk–associated outbreak; L. monocytogenes (1/2a) (V7), isolated from milk in a milk–associated outbreak; and L. monocytogenes (4b) (101 M), isolated from beef in a beef–associated outbreak. E. coli K12 was used as a pathogen substitute, for safety reasons and to mimic similar viscosity and chemical characteristics of inoculation liquid, in experiments in which the moisture content and water activity of the walnut shells and kernels were analyzed before, during, and after inoculation.

Many of the inshell walnuts used in this study had high initial populations of bacteria (>5 log CFU/nut) and yeasts and molds (>3 log CFU/nut), which necessitated the use of antibiotic-resistant strains. Mutants of Salmonella, E. coli O157:H7, and L. monocytogenes able to grow in media supplemented with rifampicin (Rif) (Sigma-Aldrich, St. Louis, MO) at 50 µg/ml were isolated and used in experiments in which the inoculation level was near the indigenous microbiota level. Unless otherwise specified, culture media were obtained from BD (Franklin Lakes, NJ), and were supplemented with Rif. The isolates were stored at −80 °C in tryptic soy broth (TSB) supplemented with 15% glycerol (Fisher Scientific, Fair Lawn, NJ).

2.3. Inoculum preparation

The single-strain inocula were prepared as described by Uesugi et al. (2006). The frozen stock culture was streaked for isolation onto tryptic soy agar (TSA: tryptic soy broth plus 1.5% granulated agar) and incubated at 37 ± 2 °C for 24 ± 3 h. A 10-µl sterile loop of this culture was transferred into 10 ml of TSB and incubated at 37 ± 2 °C for 24 ± 3 h; this transfer procedure into TSB was repeated once. An aliquot (1 ml) of the second overnight culture was spread over large TSA plates (150 by 15 mm) and incubated at 37 ± 2 °C for 24 ± 3 h. The resulting bacterial lawn was collected by adding 9 ml of a 0.1% peptone to each plate and scraping the surface of the plate with a sterile spreader (Lazy-L Spreader, Andwin Scientific, Tryon, NC). The harvested cells (11 log CFU/ml) were diluted, as appropriate, with 0.1% peptone to inoculum levels ranging from 4 to 11 log CFU/ml. The five-strain mixtures of Salmonella, E. coli O157:H7, or L. monocytogenes were prepared by growing each strain separately (under the conditions described above) and then combining equal volumes of each strain to produce the target inoculum. The populations in the individual and final mixed inocula were determined by serial dilution in Butterfield’s phosphate buffer (BBP) and plating onto media as described below.

2.4. Inoculation procedures

Inshell walnuts were inoculated as described by Uesugi et al. (2006) for almond kernels. Inshell walnuts (400 g) were weighed into a sterile bag, inoculum (25 ml) was added, and the sealed bag was shaken and rubbed by hand for 2 min. Inoculated walnuts were spread onto four layers of filter paper (57 by 46 cm sheets; Qualitative P-5 Grade, Fisher Scientific) that was placed into a sealed plastic container (leaving a 3- to 5-cm gap to allow for air exchange). Walnuts were dried under ambient conditions for 24 ± 2 h. After drying, inshell walnuts were placed in sterile plastic bags and manually mixed by shaking for 2 min.

2.5. Storage conditions

To evaluate pathogen survival on inshell walnuts, inoculated and control nuts were stored in unsealed bags within closed plastic
containers held at refrigerator (4 °C) or ambient conditions for periods of 12 weeks to 3 years, depending upon the experiment. Condensate was not observed in the bags or on the walnuts during storage. Data loggers (TempTale 4, Sensitech Inc., Beverly, MA) were placed in each storage area to record temperature and relative humidity (RH).

2.6. Reduction of Salmonella on inshell walnuts washed in water or sodium hypochlorite

The reduction of Salmonella Enteritidis PT 30 during ambient storage was determined after exposing the inoculated inshell walnuts to water (control) or sodium hypochlorite. The inshell walnuts were inoculated with Salmonella at 9 log CFU/nut (wet) and dried for 24 h at ambient conditions as described above. The inshell walnuts were treated either immediately after the 24-h inoculum-drying period or after 7 days of ambient storage. Groups of six nuts were placed into 500-ml lidded jars (Nalgene, Rochester, NY) with either 20 ml of sterile distilled water (pH: 6.3) or a 3% solution of sodium hypochlorite (30,000 μg/ml or ppm; pH: 9.6). This ratio of walnuts to liquid was sufficient to visibly coat the nuts without producing significant excess liquid. The jars were vigorously shaken in a 10-cm arc for 2 min to mimic agitation of the nuts under commercial conditions. Water-washed, sodium hypochlorite-treated, and non-treated nuts were spread onto four layers of filter paper and dried under ambient conditions for 24 ± 2 h. After drying, nuts were stored for up to 2 weeks at ambient conditions and analyzed as previously described.

2.7. Enumeration

For pathogen enumeration, an individual inshell walnut (approximately 12 g) was added to 10 ml of 0.1% peptone or, for those samples in the brightening study, D/E neutralizing broth (both without Rif) in a sterile 532-ml (18-oz) Whirl-Pak bag (Nasco, Modesto, CA). Each bag was rubbed by hand and periodically shaken in a 10-cm arc for 2 min. The bacterial population density in the recovery liquid was determined by serial dilution in BPB and plated onto TSA for all inoculated or-water (pH: 6.3) or a 3% solution of sodium hypochlorite (30,000 μg/ml or ppm; pH: 9.6). This ratio of walnuts to liquid was sufficient to visibly coat the nuts without producing significant excess liquid. The jars were vigorously shaken in a 10-cm arc for 2 min to mimic agitation of the nuts under commercial conditions. Water-washed, sodium hypochlorite-treated, and non-treated nuts were spread onto four layers of filter paper and dried under ambient conditions for 24 ± 2 h. After drying, nuts were stored for up to 2 weeks at ambient conditions and analyzed as previously described.

2.8. Moisture content and water activity of walnut shells and kernels

Moisture content and water activity were compared for shells and kernels obtained from uninoculated inshell walnuts and E. coli K12-inoculated inshell walnuts (10 log CFU/nut) immediately after inoculation or after drying on filter paper for 24 h under ambient conditions. Inshell walnuts were cracked with a culinary nut cracker, kernels and shells were separated, and pieces were reduced (to ~1 cm) with a mortar and pestle. Moisture content and water activity of the sieved samples was measured with a dual moisture content and water activity meter (AquaLab model 4TE DUO, Decagon Devices, Pullman, WA).

2.9. Experiment design and statistical analysis

Six replicates per experiment were used to enumerate the population density at each sampling time, and three replicates per experiment were used to estimate moisture and water activity of nut samples. When enumerated bacterial values obtained were below the LOD (10 CFU/nut) but positive through enrichment of the remaining sample, the bacterial concentration was analyzed with an assigned value of just below the LOD or 9 CFU/nut (0.9 log CFU/nut). When results were negative after enrichment, the bacterial concentration was analyzed with an assigned value of 0.1 CFU/nut (<1 CFU/nut) or ~0.9 log CFU/nut. Population declines were normalized by the initial wet-nut level or dry-nut level. Analyses of variance and post-hoc Tukey’s HSD multiple comparison tests were performed with the JMP 8 software package (SAS Institute, Cary, NC). Differences between the mean values were considered significant at P < 0.05. Baranyi, Campert, and linear regression models of microbial behavior were developed with the aid of DMFit (Baranyi and Roberts, 1994; Zwietering et al., 1991) and JMP 8. Rates of bacterial decline during storage were converted from log CFU per nut per day to log CFU per nut per month by multiplying by 30.4.

3. Results and discussion

3.1. Influence of inoculation on moisture content and water activity of walnut shells and kernels

Shell moisture content and water activity were affected by the aqueous inoculation procedure, initially increasing by more than 1% (from 3.9 to 5.1%) and 0.30 (from 0.28 to 0.60), respectively. After drying at ambient conditions for 24 h, inoculated shells differed from the uninoculated shells in moisture content and water activity by ~0.05% (4.3%) and <0.01 (0.41 and 0.42), respectively. Kernel moisture and water activity for inoculated walnuts differed by ~0.2% (from 3.9 to 4.1%) and <0.1 (from 0.28 to 0.34), respectively, from the uninoculated controls immediately after inoculation and no differences in moisture (4.3%) and water activity (0.42) were observed after drying.

3.2. Influence of temperature and inoculum level on survival of Salmonella Enteritidis PT 30 on inshell walnuts during storage

Inshell walnuts are typically stored in large silos or in warehouses in bins. Temperatures during storage are often at ambient during the cooler months after harvest; as ambient temperatures rise, walnuts may be transferred to cold storage (4 to 10 °C) to reduce the potential for development of rancidity. Inshell walnuts may also be stored, and are often distributed and retailed, at ambient temperature.

Inshell walnuts were inoculated with Salmonella at approximately 10 log CFU/nut and dried for 24 h at ambient conditions; after drying, population densities declined by 0.70 log CFU/nut to 9.5 log CFU/nut (Table 1). The inoculated dried walnuts were stored at 4 °C and ambient conditions. At 21 days of storage and all subsequent sampling times, Salmonella populations were significantly greater on walnuts stored at 4 °C (relative humidity ranged from 65 to 95%) than those stored under ambient conditions (Fig. 1A). After 20 weeks (139 days), populations on walnuts stored at 4 °C or ambient had declined by 0.5 or 2.7 log CFU/nut, respectively (Table 1, Fig. 1A). Although it is unusual for walnuts to be stored for more than 1 year, Salmonella levels were also determined on these nuts stored for 1.2 and 3.1 years (431 and 1143 days, respectively). After 1.2 and 3.1 years of storage, Salmonella
Salmonella Enteritidis PT 30

Ambient 10.0 ± 0.12 9.2 ± 0.04 83 – 0.001 Baranyi
Ambient 9.0 ± 0.10 8.9 ± 0.17 139 – 0.001 Baranyi-linear

Salmonella cocktail

Ambient 4.7 ± 0.33 2.3 ± 0.64 0.0 ± 0.99 97 0.001 Linear

E. coli O157:H7 cocktail

Ambient 3.58 ± 0.57 1.5 ± 0.49 0.0 ± 0.99 97 0.001 Linear

L. monocytogenes cocktail

Ambient 4.4 ± 0.37 2.5 ± 0.77 0.0 ± 0.99 97 0.001 Linear

* Samples stored longer than 27 days had counts lower than the LOD (1 log CFU/nut) and were not enriched; population values beyond 27 days were not used in the regression analysis.

A

B

Fig. 1. Survival of Salmonella Enteritidis PT 30 on inshell walnuts during storage. Storage began on day 0 (24 h after inoculation). Results are mean plate counts (and standard error) on BSA + Rif: A) influence of storage temperature, 4 °C (closed square) and ambient conditions (open square); and B) influence of inoculum level (initial inoculum levels: 6, 8, and 10 log CFU/nut) on walnuts stored at ambient conditions. * indicates at least one replicate was below the LOD (1 log CFU/nut).
approach if the rates of decline of the pathogen are similar across a wide range of inoculum levels, however, the survival dynamics of various inoculation concentrations may be incongruent. Inshell walnuts were inoculated at 10, 8, and 6 log CFU/nut and stored for 90 days at ambient conditions. Inoculation level influenced the survival of *Salmonella* on inshell walnuts during both drying of the inoculum and subsequent storage. During the initial 24-h drying period, a greater reduction in *Salmonella* populations was observed for walnuts inoculated at 6 and 8 log CFU/nut (2.0- and 1.5-log CFU/nut reductions, respectively) than for walnuts inoculated at 10 log CFU/nut (0.7-log CFU/nut reduction) (Table 1). Inoculum level similarly impacted survival of *Salmonella* on walnut kernels (Blessington et al., 2012) and almond kernels and inshell pistachios (Kimber et al., 2012) during postinoculation drying but not during long-term storage of walnut and almond kernels (Blessington et al., 2012; Uesugi et al., 2006) or of inshell pecans (Beuchat and Mann, 2010a).

During the first 4 weeks of ambient storage after drying, bacterial populations declined more rapidly for inshell walnuts inoculated at 6 or 8 log CFU/nut than for walnuts inoculated at 10 log CFU/nut (Fig. 1B). Similarly for medium pecan pieces, the decline of *Salmonella* was greater within the first few weeks of storage when inoculated at moderate (5 log CFU/g) compared to high (7 log CFU/g) levels (Beuchat and Mann, 2010a). When walnuts were inoculated at 6 log CFU/nut, populations of *Salmonella* fell below the LOD (1 log CFU/nut) in three out of six samples after 4 weeks and in all six samples by 8 weeks of storage. At an initial inoculum of 8 log CFU/nut Salmonella levels were above the LOD through 8 weeks and fell below the LOD in two of six samples at 12 weeks of storage.

### 3.3. Survival of low-level cocktails of *Salmonella*, *E. coli O157:H7*, and *L. monocytogenes* inoculated on inshell walnuts

Inshell walnuts were inoculated at 4 log CFU/nut with five-strain cocktails of *Salmonella*, *E. coli O157:H7*, or *L. monocytogenes* (4 to 5 log CFU/ml); survival was evaluated over 14 weeks (97 days) of ambient storage (Table 2). During the 24-h drying period, *Salmonella*, *E. coli O157:H7*, and *L. monocytogenes* declined by 2.1, 2.2, and 1.9 log CFU/nut, respectively, as determined on TSA; declines among the genera were not significantly different. Thus at the beginning of ambient storage, bacterial populations were near the limit of detection by plating (10 CFU/nut) and average counts were estimated using a combination of plate counts and enrichment data. A range of bacterial levels was observed among samples within a single time point, sometimes resulting in a standard deviation that was larger than the average counts, in part, a product of the combination of enumerated and assigned values for samples (Table 2). This is an inherent limitation of microbiological data. Gathering statistically sound plate count data is only possible when using higher inoculum concentrations, but such treatments are less likely to mimic natural contamination scenarios. Lowering the limit of detection for enumeration by filtration or including MPN determinations would add time and cost to the analysis but should be considered for future studies. Differences among samples in nut shell topography and shell integrity (e.g., small cracks) may also have contributed to this variation by influencing our ability to remove inoculated organisms with our sampling procedure.

Bacterial decline at each sampling point during storage was calculated by subtracting the levels determined at the sampling point from the levels measured at the beginning of storage. The declines among the three genera were similar at all sampling points except for three; greater declines were observed in *L. monocytogenes* populations than in *Salmonella* and *E. coli O157:H7* populations at 27, 83, and 97 days of storage. Over 97 days of ambient storage, declines of *Salmonella* and *E. coli O157:H7* were estimated to be less than 1 log CFU/nut and the decline of *L. monocytogenes* was 2 log CFU/nut. These declines were less than the 2.8- to 3.8-log decline observed for *Salmonella Enteritidis* PT 30 on walnuts inoculated at 10 or 7.5 log CFU/nut, respectively, and stored for a similar length of time (83 to 139 days) (Table 1). These data are comparable to previous studies with other tree nuts; as populations decrease to near the standard LOD the rate of decline slows (Beuchat and Heaton, 1975; Beuchat and Mann, 2010a; Blessington et al., 2012; Kimber et al., 2012).

During the 97-day storage period, 78 inshell nuts were sampled per genera; of these, 73 *Salmonella*-, 66 *E. coli O157:H7*-, and 66 *L. monocytogenes*-inoculated nuts were positive by plate count or enrichment. Plate counts of at least 1 log CFU/nut were obtained for 49 *Salmonella*-, 23 *E. coli O157:H7*-, and 31 *L. monocytogenes*-inoculated nuts. At all time points during storage after the initial plating, all samples were subjected to a primary enrichment. Enriched broths were streaked

### Table 2

<table>
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<tr>
<th>Storage day</th>
<th><em>Salmonella</em></th>
<th><em>E. coli O157:H7</em></th>
<th><em>L. monocytogenes</em></th>
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<tr>
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<td>TSA + Rif (log CFU/nut)</td>
<td>TSA + Rif (log CFU/nut)</td>
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<td></td>
<td>E 1° 2° 3°</td>
<td>E 1° 2° 3°</td>
<td>E 1° 2° 3°</td>
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<td>3.9 ± 0.28</td>
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<td>1.5 ±0.45a</td>
<td>6 NA NA</td>
</tr>
<tr>
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<td>2.0 ± 1.2a</td>
<td>6 NA NA</td>
</tr>
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<td>1.4 ± 0.66a</td>
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</tbody>
</table>

* a Values are means ± standard deviation. Within rows, means with different letters are significantly different on the basis of decline values calculated from the initiation of storage (P < 0.05).

b Days before (−1) or during (0 to 97) storage; walnuts were placed in storage 24 h after inoculation (time 0).

c E, number of replicates enumerated at or above the LOD (1 log CFU/nut).

d 1°, number of replicates that were positive after the primary enrichment; 2°, number of replicates that were positive after the secondary enrichment; 3°, number of replicates that were positive after the tertiary enrichment; NA, not applicable.

e Values obtained from SMAC cultures were disregarded because indigenous microflora were not distinguishable from inoculated *E. coli O157:H7*. All replicate enrichments were streaked onto SMAC plates to confirm clear to white colonies typical of *E. coli O157:H7* on this medium.

f Colonies isolated from TSA + Rif per replicate per sampling day were restreaked onto MOX + Rif to confirm values obtained from TSA + Rif. All restreaked colonies were black colonies typical of *L. monocytogenes* on this medium.
onto selective/differential media for confirmation if enumerated values were below the LOD or if the previous enrichment was negative. An additional number of walnuts were positive after secondary or tertiary enrichment (Table 2); 14 nut samples (6% of the 234 nut samples evaluated) required additional enrichment beyond the initial 24 h for positive isolation.

Recovery of pathogens from dry foods presents a challenge as the cells may be severely injured. It is possible that standard and, especially, more rapid enrichment techniques may deliver false negative results (D’Lima and Suslow, 2009). As rapid methodologies are adapted to dry products, they should be validated using samples inoculated at low levels and held under dry conditions that may promote populations of difficult-to-culture cells that reflect naturally-contaminated samples. The influence of desiccation stress and injury on bacterial cell virulence is unknown, thus at this time the assumption is that the health risk from injured cells is similar to that from healthy cells (Lesne et al., 2000).

3.4. Rates of decline for inoculated pathogens on inshell walnuts during storage

As noted above, the decline of inoculated bacteria approaches a nonlinear pattern at lower inoculum levels and the most significant reductions occur within the first month of storage. Similar survivor curves have been observed for Salmonella inoculated on walnut kernels (Blessington et al., 2012), on inshell pecans (Beuchat and Heaton, 1975), and on inshell pistachios (Kimber et al., 2012). Nonetheless, rates of decline were calculated to allow for more direct comparison among a range of experiments. In each single-strain inoculation study, an analysis of variance was conducted and time was analyzed as a factor in determining bacterial populations during storage. In each study, the variance between time points exceeded that within time points, allowing for further analysis to assess trends to predict bacterial levels. The data for Salmonella Enteritidis PT 30 were fit to linear, Baranyi, and Compertz regression models. Best-fit models were selected based on their respective R² values. For comparison purposes the rates of decline for the non-linear curves of these models (DMFit and Compertz) were developed from a potential maximum rate of the model rather than an average (Baranyi and Roberts, 1994), which most closely represented die-off during initial storage. In two cases (Salmonella Enteritidis PT 30 inoculation levels of 7.5 and 5.7 log CFU/nut), the DMFit model resulted in the greatest R² value; however, the shapes of these models were unreasonable due to a greatly exaggerated predicted rate of decline (6 to 7 log CFU/nut/month). Thus, the linear model was chosen for these two data sets (Table 1).

Rates of decline for Salmonella Enteritidis PT 30 (inoculated at log 10 CFU/nut) from 139 to over 3 years of storage at 4 °C and ambient conditions were approximately 0.1 and 0.6 log CFU/nut per month, respectively (Table 1, Fig. 1A). In a separate 83-day ambient storage study the calculated rates of decline for inoculation levels of 10, 8, and 6 log CFU/nut were 1.3, 1.2, and 2.5 log CFU/nut per month, respectively (Table 1, Fig. 1B). When inoculated at 6 log CFU/nut, Salmonella levels on some of the samples fell to or below the LOD upon storage for 27 days. At 8 and 12 weeks all six samples initially inoculated at 6 log CFU/nut had Salmonella levels that were below the LOD. Enrichment procedures were not performed on these samples and data beyond 27 days were not included in the model determinations. The calculated decline rate for this inoculation level was 2.5 log CFU/nut per month. A similar rate of decline (2.3 log CFU/nut per month) was calculated for the 14-day storage of untreated inoculated inshell walnuts within the water washing/brightening study even though the inoculum level in that experiment was 9 log CFU/nut. In general, shorter storage times and lower inoculum levels resulted in greater calculated rates of decline.

The survival of Salmonella on inshell nuts has been described in a limited number of nut crops including pecans (Beuchat and Heaton, 1975; Beuchat and Mann, 2010a, 2010b), hazelnuts (Komitopoulou and Peñaloza, 2009), and pistachios (Kimber et al., 2012); the survival of E. coli O157:H7 and L. monocytogenes on nuts (inshell or shelled) has only recently been reported in almond and walnut kernels, and for inshell pistachios (Blessington et al., 2012; Kimber et al., 2012). The association between low-moisture foods and Salmonella contamination has been well described (Scott et al., 2009). Due to the number of outbreaks and recalls resulting from Salmonella contamination, it has been assumed that this bacterium has a greater ability to survive in dry environments. However, recent low-moisture food or ingredient outbreaks associated with pathogenic E. coli (CDC, 2011; CFIA, 2011a, 2011b; Neill et al., 2012) and the long-term viability of this pathogen on the surface of inshell walnuts and walnut kernels suggest that this organism should be considered in hazard assessments for the production and processing of walnuts and other tree nuts. L. monocytogenes populations declined more rapidly than either Salmonella or E. coli O157:H7 on both inshell walnuts and walnut kernels. L. monocytogenes would be of concern in products that support the growth of this pathogen and that use raw nuts as an ingredient.

Data generated from cocktail inoculations were not modeled because they were a combination of enumerated and assigned values based on positive and negative enrichments. The LOD was reached at 0, 6, and 13 days of storage for E. coli O157:H7, L. monocytogenes, and Salmonella, respectively and one or more samples were negative upon enrichment by day 34, 41, and 41, respectively. Given the 1 to 2 log CFU/month reductions calculated for low-level inoculum and short storage time samples (Table 1), the detection of Salmonella by plate count was not expected and the results further suggest rates of decline at these lower levels are not congruent with those observed at higher inoculation levels. It is not known whether low levels of indigenous bacterial contaminants would survive in a manner similar to this low-level inoculation, but normal commercial storage should not be assumed to significantly reduce bacterial contaminants on inshell walnuts. In addition, caution should be taken when calculated decline values from a limited number of studies at high inoculation levels are used in the development of risk assessments.

3.5. Impact of shell washing or brightening on Salmonella Enteritidis PT 30 survival

High concentrations of sodium hypochlorite (3%) are currently used to cosmetically lighten a small proportion of inshell walnuts (primarily markets in U.S. and Canada) to meet appearance standards. Alternative brightening methods such as 5% sodium hydroxide under alkaline conditions (pH 8–9) have also been explored (Fuller and Stafford, 1992, 1993) but were not evaluated in this study. Inshell walnuts were inoculated with Salmonella and exposed to water or sodium hypochlorite at 1 or 8 days after inoculation. In both cases, when compared to the corresponding untreated samples, Salmonella levels declined by 0.3 to 0.4 log CFU/nut after 2 min of exposure to water and by 2.4 to 2.6 log CFU/nut after 2 min of exposure to sodium hypochlorite (Fig. 2A). Additional population declines of approximately 1 log CFU/nut were observed after the treated nuts were dried at ambient conditions for 24 h. Salmonella levels continued to decline by a further 1.2, 2.7, and 2.1 log CFU/nut during 2 weeks of storage at ambient conditions on the untreated, water-washed, and hypochlorite-treated samples; total reductions were 1.2, 3.1, and 4.7 log CFU/nut, respectively.

Both the water and sodium hypochlorite treatments reduced the levels of inoculated Salmonella on the surface of inshell walnuts, especially after drying and storage. Water washing of dry inshell walnuts is not a current commercial practice. Introduction of water into a dry food facility without adequate controls to prevent both the cross contamination within the facility and the establishment of harborage sites for Salmonella would be problematic (Scott et al., 2009). Although adding appropriate levels of a suitable antimicrobial to maintain water quality might overcome some of these issues, an aqueous pre-shelling treatment for kernel production would face additional challenges. Walnuts are sorted into inshell and shelling streams prior to brightening to
remove those with significantly cracked or broken shells from the inshell stream. This sorting leaves a significant portion of exposed nutmeats in the shelling stream and contact of the kernel with an antimicrobial might negatively impact kernel flavor.

_Salmonella, E. coli O157:H7, and L. monocytogenes_ are capable of long-term survival on the surface of inshell walnuts even when initial levels are low. Walnut processors, processors, and those using walnuts as ingredients should consider these organisms when developing food safety plans and strive to minimize the opportunities for contamination and cross-contamination. Brightening treatments with sodium hypochlorite can reduce _Salmonella_ levels and may, in some cases, be an appropriate preventative control measure.

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Fig. 2. Reductions of _Salmonella Enteritidis_ PT 30 on inshell walnuts during water and brightening treatments and subsequent storage. Results are mean plate counts (and standard error) on BSA + Rif. (A) reductions compared to corresponding untreated samples on inshell walnuts 1 or 7 days after inoculation after shaking for 2 min in water or 3% sodium hypochlorite immediately after treatment (open bars) and 24 h after treatment (black bars); and (B) bacterial reductions on untreated (open squares), water treated (gray squares), and 3% sodium hypochlorite treated (black squares) inshell walnuts (1 day after inoculation) during storage at ambient conditions.

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<th>Log CFU/nt reduction</th>
<th>Time after inoculation (day)</th>
<th>Water</th>
<th>Sodium Hypochlorite</th>
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<tr>
<td>1 day</td>
<td>3</td>
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<td>8 days</td>
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