

## Growth and Survival of *Enterobacteriaceae* and Inoculated *Salmonella* on Walnut Hulls and Maturing Walnut Fruit

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MS 14-075: Received 14 February 2014/Accepted 2 May 2014

### ABSTRACT

Postharvest contamination of in-shell walnuts may occur when the fruit is dropped to or harvested from the orchard floor or as the outer hull is removed with mechanical abrasion and water. To evaluate the effect of maturity on the potential for microbial contamination, ‘Howard’ walnut fruits were collected weekly from the tree canopy, from 6 to 7 weeks before to 1 week after typical commercial harvest. The numbers of microorganisms able to form colonies on plate count agar, MacConkey agar (presumptive *Enterobacteriaceae*), or violet red bile lactose agar (presumptive coliforms) were compared on whole walnut fruits collected by hand directly from the tree or after exposure to the orchard floor for 10 min or 24 h. *Salmonella* Enteritidis PT 30 was inoculated at <1 to 8 log CFU/g onto 5-g hull pieces (from walnut fruit of different maturities) and stored at ambient temperature (23 to 26°C) in unsealed bags (38 to 90% relative humidity [RH] within bag) or in low humidity (20 to 45% RH) or high humidity (68 to 89% RH) for up to 14 days. *Salmonella* at 2 or 5 log CFU/ml was inoculated onto hulls before or up to 14 days after blending with water. As the walnut fruit matured, the indigenous bacterial levels on the surface increased, irrespective of whether fruit was collected from the tree or the ground. The RH influenced the growth of inoculated bacteria on hull pieces: *Salmonella* declined to <0.3 log CFU/g within 24 h at low RH but multiplied from 2 to 6 log CFU/g over 14 days of storage at >40% RH. *Salmonella* populations declined to <1 CFU/ml within 24 h in freshly blended green hulls but survived or multiplied in blended brown hulls or in blended green hulls that had been stored for 24 h or more before being inoculated.

Persian, or English, walnuts (*Juglans regia* L.) are harvested in late August to mid-November in California. Walnut fruits have a fleshy, high-moisture, green-colored hull that surrounds the nut shell; the hull is botanically a pericarp but is also known as a shuck or husk. Walnuts are ideally harvested when both the kernel and hull have reached physiological maturity. At this point, the kernel has filled the shell and the packing tissue has turned brown. For most commercial varieties, hull dehiscence, i.e., the splitting and separation of the drying green hull from the shell, occurs naturally shortly thereafter (32). The initially uniformly bright green color of the hull often progresses to a mottle of green and brown to mostly brown. Depending on moisture levels, the hulls may dehydrate or decompose. When mature, walnuts are mechanically shaken to the ground, mechanically swept up, and transported to a processing facility referred to as a ‘‘huller,’’ where the hulls are removed from the shell with both water and abrasive mechanical action (hulling) (32). After hulling and before storage, in-shell walnuts are dried at ≤43°C for 12 to 48 h (to moisture levels of less than 8%) (37). *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* can survive for extended periods on both

in-shell walnuts (7, 15) and walnut kernels (5, 6) under typical storage conditions.

California walnuts were epidemiologically linked to an *E. coli* O157:H7 outbreak in Canada in 2011 (9, 10). Walnuts were recalled in 2009 after the detection of *L. monocytogenes* (18) and in 2010 and 2012 after the isolation of *Salmonella* (11, 41). *Salmonella* was not isolated in any of the 595 ( $n = 74$ ,  $n = 441$ , and  $n = 80$ , respectively) 25-g English walnut samples analyzed in three studies (24, 25, 30) but was detected in one sample of prepacked mixed nuts (25 g,  $n = 329$ ) that contained walnuts (25). In a recent 4-year survey of California in-shell walnuts, the incidence of *Salmonella* contamination was 0% in 2010 (100 g,  $n = 935$ ), 0.2% in 2011 (375 g,  $n = 905$ ), 0.1% in 2012 (375 g,  $n = 999$ ), and 0.1% in 2013 (375 g,  $n = 1,000$ ); *E. coli* O157:H7 was not isolated from any of these samples (15).

Walnuts are among many different tree nuts that are shaken to the ground during harvest. The orchard floor is considered a potential source of contamination (19, 27, 39). *Salmonella* has been isolated from almond orchards (19, 39), and the organism can multiply in wet almond hulls (40) and in soils supplemented with almond hull extracts (13). In contrast, *Salmonella* died in water extracts of pecan shucks and in orchard soil saturated with water or pecan shuck extract (3). The influence of soil or water contact with

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walnut hulls on microbial contamination, growth, or survival has not been reported.

Walnuts can come into contact with water when they prematurely drop to the ground before irrigation is withdrawn or when it rains after the nuts are shaken to the ground. At the huller, walnuts hulls are removed with the aid of water and abrasive action. The hull-free in-shell walnuts briefly come into contact with the resulting slurry of water, crushed walnut hulls, leaves, broken shells and kernels, and other debris. Meyer and Vaughn (28) suggested that *E. coli*-contaminated hulling wastewater of black walnuts (*Juglans nigra* L.) could pose a risk for contamination of in-shell walnuts. Walnut fruit and other vegetative materials contain a diverse array of phenolic compounds (35). These compounds have been correlated with microbial toxin inhibition, mycostatic or bacteriostatic properties, and mycocidal or bactericidal properties (21, 26, 31, 33). The effect of the presence and release of some of these walnut secondary metabolites on indigenous microbial populations during walnut hulling is unknown.

In the development of a comprehensive food safety plan, the microbial hazards associated with walnuts during preharvest and hulling should be defined. In this study, the survival and growth of indigenous microbiota and inoculated *Salmonella* Enteritidis PT 30 on walnut hulls were characterized at different stages of walnut maturity during storage at different relative humidity (RH) levels. In addition, the survival and growth of inoculated *Salmonella* Enteritidis PT 30 in crushed and blended hulls was determined under conditions simulating commercial hulling.

## MATERIALS AND METHODS

**Walnut samples.** For three seasons, from mid-August to mid-October, 'Howard' walnuts, a variety that matures in midseason (17), were collected as whole fruit (in-shell walnut surrounded by a hull) from two tree canopies within research orchards at the University of California, Davis. Weekly collection (for up to 9 weeks) began approximately 7 weeks before the beginning of the typical commercial harvest period for that cultivar in California. Walnuts were handpicked from the tree canopy and collected directly into bags or dropped onto the orchard floor and then collected after 10 min or 24 h. Single walnuts were placed separately into 532-ml (18-oz) Whirl-Pak bags (Nasco, Modesto, CA) at the collection site, and the samples were transported in sampling bags under ambient conditions to the nearby laboratory (~15 min) and then processed immediately or refrigerated at 4°C and processed within 2 h.

**Culture and growth conditions.** *Salmonella* Enteritidis PT 30 (ATCC BAA-1045), isolated from raw almonds associated with an outbreak (19), was chosen as a model organism to study bacterial survival. Studies in our laboratory have shown that harvested walnut fruit can have aerobic plate counts (APC) and levels of *Enterobacteriaceae* that exceed 6 log CFU per nut (4, 15), which necessitated the use of antibiotic-resistant strains for inoculation studies. A mutant of *Salmonella* Enteritidis PT 30 capable of growing in medium supplemented with 50 µg/ml rifampin (Rif) (Sigma-Aldrich, St. Louis, MO) was isolated and used in all inoculation studies. Unless otherwise specified, culture media were obtained from BD (Franklin Lakes, NJ). Isolates were stored at -80°C in tryptic soy broth (TSB) supplemented with 15% glycerol (Fisher Scientific, Fair Lawn, NJ).

**Inoculum preparation.** The inoculum was prepared as described by Uesugi et al. (38). The frozen stock culture was streaked onto tryptic soy agar (TSA; TSB and 1.5% granulated agar) and incubated at 37 ± 2°C for 24 ± 3 h. At two consecutive 24-h intervals, a 10-µl sterile loop of the culture was transferred into 10 ml of TSB and incubated at 37 ± 2°C for 24 ± 3 h. The second overnight broth culture (1 ml) was spread over 150- by 15-mm TSA plates that were then incubated at 37 ± 2°C for 24 ± 3 h. To collect the resulting bacterial lawns, 9 ml of 0.1% peptone was added to each plate and the lawn was loosened with a sterile spreader (Lazy-L Spreader, Andwin Scientific, Tryon, NC). The cell suspensions were diluted, if appropriate, with 0.1% peptone to obtain inocula with bacterial populations ranging from 5 to 11 log CFU/ml.

*Salmonella* populations in the final inoculum were determined after serial dilution in Butterfield's phosphate buffer and plating of the appropriate dilutions onto TSA and bismuth sulfite agar (BSA) supplemented with rifampin (50 µg/ml). Cultures on TSA and BSA plates were incubated at 37 ± 2°C for 24 ± 3 h and 48 ± 3 h, respectively.

**Inoculation and storage procedures.** To assess whether walnut hulls could support the growth of inoculated bacteria, we examined hull pieces and hull slurries. Hulls were removed from the walnut shells with a sterile scalpel. Five-gram pieces of hull from fruit at different maturities (from 6 weeks before to 1 week after typical commercial harvest) were placed on individual weigh boats, and each piece was spot inoculated with 20 µl of *Salmonella* in a biosafety hood (NuAire, Plymouth, MN). Up to three inoculation levels were used, depending on the study, high (~8 log CFU/g), moderate (~4 log CFU/g), and low (~1 to 2 log CFU/g). After inoculation, one set of samples was immediately processed to recover an initial inoculation concentration, while all other samples were allowed to dry in the hood for 1 h before storage. Some of the dried inoculated hull pieces were left on the weigh boats and stored in plastic lidded bins under high- or low-RH conditions (high humidity, 68 to 89% RH, 23 to 26°C; low humidity, 20 to 45% RH, 24 to 25°C). The high-humidity condition was achieved by placing paper towels saturated with tap water at the bottom of a plastic lidded bin and keeping the bin closed. The low-humidity condition was created by keeping a plastic lidded bin partially open to the inherently low-humidity ambient air in our laboratory (25 to 35% RH, 23 to 25°C). Other dried inoculated hull pieces were placed in unsealed 118-ml (4-oz) Whirl-Pak bags that were stored in plastic lidded bins partially open to the ambient air; the conditions within the bags were 38 to 90% RH and 23 to 26°C. Temperature and humidity data loggers (TempTale 4, Sensitech, Inc., Beverly, MA) were placed in the bins (high and low humidity) or in the bags containing hull pieces. Inoculated samples were stored for up to 2 weeks to simulate the time period in the orchard when hulls may separate from the in-shell walnuts, fall to the ground, and remain on the orchard floor until harvest.

To mimic the slurry that is produced during the commercial hulling process, a 1:2 (wt/vol) mixture of hull pieces removed from mature hand-harvested walnut fruit and 0.1% peptone was blended at high speed for 2 min in a commercial blender (Waring, Torrington, CT). Blending the hulls results in a uniform slurry that is a more homogenous mixture than the slurries found in hulling operations; however, blending is both standardized and repeatable and does replicate the most severely crushed hull pieces that result from hulling. Aliquots (15 ml) of this hull-peptone slurry were transferred to 50-ml centrifuge tubes (BD) and inoculated immediately or after holding at ambient conditions for 24 h or 3,

7, or 14 days with 20  $\mu$ l of *Salmonella* to a target initial concentration in the slurry of 2 log CFU/ml.

In a separate experiment, hulls (12.5 g) that were mostly green or mostly brown were combined in a blender jar with well water collected from a California farm (100 ml) and inoculated with 50  $\mu$ l of *Salmonella* at target final concentrations of 5 log CFU/ml. This proportion of water was needed to liquefy the drier brown hulls. The hulls and water were mixed by manually shaking the blender jar for 2 min and then blended at high speed for 2 min. Inoculated mixtures were held for up to 24 h under ambient conditions. Aliquots of the liquid were analyzed for microbial levels after mixing (before blending), after blending, and after holding for 30, 60, and 120 min and 24 h under ambient conditions.

**Moisture content and water activity of hull pieces.** The moisture content and water activity of hull pieces were evaluated during storage. Hull pieces (4 g) were cut into 8 to 12 smaller portions that were placed in individual foil pans (0.6 by 10.2 cm), and the percent moisture or water activity was determined with a moisture analyzer (HG63 Halogen Moisture Analyzer, Mettler-Toledo, Columbus, OH) or a water activity meter (AquaLab model CX2, Decagon Devices, Pullman, WA), respectively.

**Enumeration.** To recover indigenous *Enterobacteriaceae* and coliforms on whole walnut fruit, 10 ml of 0.1% peptone was added to a single walnut in a sterile 530-ml Whirl-Pak bag, and each bag was rubbed by hand and periodically shaken in a 30-cm arc for 2 min.

*Salmonella*-inoculated hull pieces (5 g) were added to 10 ml of 0.1% peptone in a sterile 530-ml Whirl-Pak bag and homogenized by stomaching (Stomacher 400 Circulator, Seward Laboratory Systems, Inc., Bohemia, NY) for 4 min at high speed (~230 rpm). Blended hull-peptone slurries (inoculated with *Salmonella* at a level of log 2 CFU/ml) were mixed with a vortex mixer for approximately 15 s. For recovery of APC and *Salmonella* from green- or brown-hull-water slurries (inoculated with *Salmonella* at a level of log 5 CFU/ml), sampling was carried out immediately before and after the blending step and after subsequent storage (up to 24 h) under ambient conditions. The slurry samples were manually shaken for 30 s before serial dilution and plating.

Serial dilutions were made in Butterfield's phosphate buffer, and the appropriate dilutions were plated onto plate count agar for counts of aerobic bacteria (APC), MacConkey agar (BD-Thermo Fisher Scientific-Remel, Lenexa, KS) for presumptive *Enterobacteriaceae* counts, violet red bile lactose agar (VRBLA; Oxoid, Thermo Fisher Scientific) for presumptive coliforms, and TSA and BSA for *Salmonella*. All colonies on MacConkey agar were counted as presumptive *Enterobacteriaceae*. Dark-red to purple colonies 0.5 mm in diameter or greater with or without a surrounding purple zone were counted as coliforms. All media used in the enumeration of *Salmonella* were supplemented with rifampin (50 mg/liter). Plate count agar and VRBLA were incubated at  $35 \pm 2^\circ\text{C}$  for  $24 \pm 3$  h, MacConkey agar and TSA were incubated at  $37 \pm 2^\circ\text{C}$  for  $24 \pm 3$  h, and BSA plates were incubated at  $37 \pm 2^\circ\text{C}$  for  $48 \pm 3$  h. Colonies on plates were counted after incubation, and bacterial population levels were determined.

**Determination of AC.** The antioxidant capacity (AC) of the hull tissue was measured with a colorimetric assay, described by Brand-Williams et al. (8), in which the reduction of a 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution is positively correlated with a decrease in absorbance. Each hull slurry sample (5 ml) was combined with 15 ml of methanol in a 50-ml centrifuge tube and

centrifuged at  $3,210 \times g$  (3,000 rpm) for 15 min (Allegra 6, Beckman Coulter, Brea, CA). The supernatant was collected for analysis. The sample extracts and a methanol control were allowed to react with DPPH until stabilization at 24 h. Absorbance was measured at 515 nm (DU-800 spectrophotometer, Beckman Coulter), and the reduction in absorbance between the methanol control and the samples was calculated. The absorbance readings of aqueous solutions of ascorbic acid (0 to 160  $\mu$ g/ml) were used to construct a standard curve, which was used to convert the change in absorbance into AC. AC was reported as ascorbic acid equivalents and determined using the equation  $y = 219.02x$ , where  $x$  is the change in absorbance (calculated by subtracting the sample absorbance from the blank of methanol and DPPH at 515 nm) and  $y$  is the micrograms of ascorbic acid equivalents per gram.

**Experiment design and statistical analysis.** In the experiment with hull-water slurries generated from mostly green or mostly brown hulls, triplicate samples were used to estimate APC in uninoculated samples or *Salmonella* populations in inoculated samples. In all other experiments, six replicates were used to estimate the population at each sampling time. Three replicates were used to estimate moisture content and water activity at each sampling time. Four replicates were used to estimate AC. Analyses of variance, full-factorial regression models,  $t$  tests, and post-hoc Tukey's honestly significant difference multiple-comparison tests were performed with the JMP 8 software package (SAS Institute, Cary, NC). Differences between the mean values were considered significant at a  $P$  value of  $<0.05$ . Baranyi and linear regression models of microbial behavior were developed with the aid of DMFit (2) and JMP 8.

## RESULTS AND DISCUSSION

**Natural microbiota.** For most commercial walnut varieties, the hull splits open and separates from the shell shortly after the walnuts have reached physiological maturity. Over time, the walnut hull color changes from mostly bright green to mostly brown and the hull may become dehydrated or begin to decompose. In preliminary experiments in a commercial orchard that was near harvest maturity, the *Enterobacteriaceae* levels on walnut fruit collected from the tree canopy were lower than those on fruit collected from the orchard floor (4). The higher levels observed on the latter may have been associated with the location of the fruit (orchard floor versus tree canopy) or the advanced maturity (beginning of bacterial decay) of the fruit found on the ground.

To further explain these differences, the present study was conducted to analyze the effects of both fruit maturity and fruit location on the levels of APC, presumptive *Enterobacteriaceae*, and presumptive coliforms on walnut fruits. Walnut fruits were collected weekly over a 9-week period from mid-August to mid-October directly from the tree canopy or were dropped to the orchard floor and then collected after 10 min or 24 h. The typical commercial harvest time for Howard walnuts in this location corresponded to approximately week 8 in this study. Increases in both *Enterobacteriaceae* and coliform levels were observed as walnuts matured (Fig. 1). The *Enterobacteriaceae* levels observed in samples collected at weeks 7 to 9 (approximately 4 to 5 log CFU per nut) were each significantly greater than those observed at weeks 1 to 5 (approximately 1

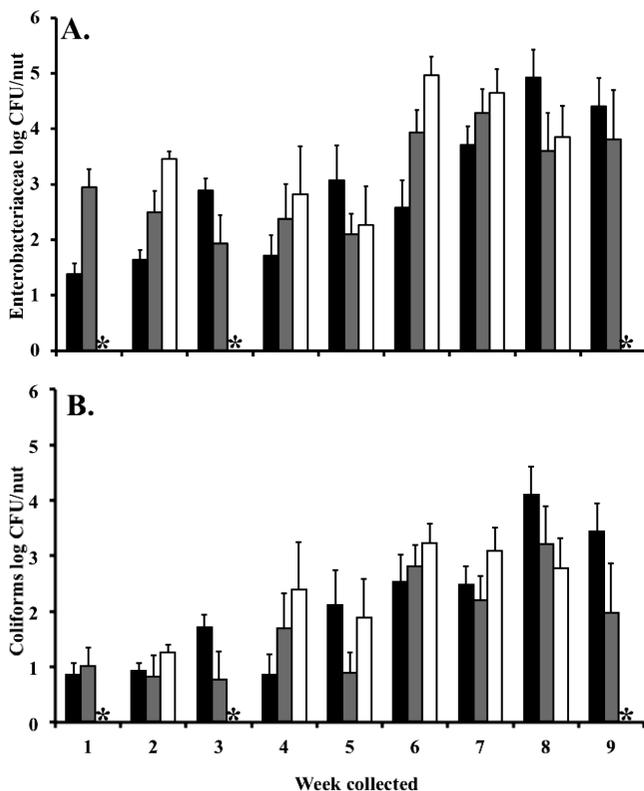


FIGURE 1. Enterobacteriaceae (A) and coliform (B) levels on maturing 'Howard' walnuts handpicked from the tree canopy and collected directly (black bars) or handpicked from the tree, dropped onto the orchard floor, and collected after 10 min (gray bars) or 24 h (white bars). Samples were obtained weekly from the same two trees beginning in mid-August (week 1) through the second week of October (week 9); the trees would have been commercially harvested at approximately week 8. An asterisk indicates 24-h samples were not collected. Values represent averages, and error bars indicate standard errors.

to 3 log CFU per nut), although not at week 6, and the levels of coliforms observed at weeks 6 to 9 (approximately 4 to 5 log CFU per nut) were each significantly greater than those observed at weeks 1 to 3 (approximately 1 to 3 log CFU per nut). Fruit maturity was more influential in determining Enterobacteriaceae and coliform counts; the maturity of walnut fruit ( $P < 0.001$ ) and the interaction between maturity and location ( $P < 0.001$ ,  $P = 0.03$ ) were significant factors, whereas the location of walnut collection ( $P = 0.14$ ,  $P = 0.28$ ) was not.

The isolation of Enterobacteriaceae and coliform organisms from walnuts is not unexpected. Enterobacteriaceae, including coliforms (42) and *E. coli* (14, 21, 24, 25, 28, 36), have been isolated from black and English walnut kernels. The types and numbers of Enterobacteriaceae and coliforms capable of forming colonies on laboratory media differ with medium composition and incubation temperature and time. Many genera, including *Enterobacter*, *Klebsiella*, and *Citrobacter*, are coliform organisms commonly associated with soils and plant material but not necessarily fecal matter. In the current study, the VRBLA was incubated at 35°C rather than at higher temperatures that are more selective for *E. coli* or thermotolerant coliforms (e.g., 42 to

44°C); none of the colonies arising on MacConkey agar or VRBLA were further identified.

The observed increase of indigenous Enterobacteriaceae and coliforms on mature walnut fruit may be associated with the increase in available nutrients and the high moisture content (approximately 70% [data not shown]) found in the split hulls closer to harvest. In a similar study, higher levels of total bacteria were observed on persimmon fruit at harvest than on fruit collected a month earlier (20).

Although the bacterial levels on walnuts collected from the orchard floor were not significantly different from the levels on corresponding walnuts collected directly from the tree, preharvest orchard preparation should not be overlooked. The isolation of *E. coli* from in-shell pecans increased from 4% to 23% when orchards were grazed before harvest (27). Significant amounts of orchard debris can be swept up as walnuts are harvested from the ground, and this debris may contact the in-shell walnuts or exposed kernels during hulling. Meyer and Vaughn (28) suspected that debris containing high coliform levels collected during harvest resulted in approximately 50% of a commercial crop of black walnut kernels ( $n = 408$ ) being contaminated with coliforms during hulling.

#### Behavior of inoculated *Salmonella* on walnut hulls.

Walnut hull pieces, harvested 3 weeks before a typical commercial harvest, were inoculated with *Salmonella* Enteritidis and allowed to dry for 1 h before being stored in unsealed bags under ambient conditions (23 to 26°C, 38 to 90% RH within the bags) (Fig. 2A). When inoculated at 8 log CFU/g, *Salmonella* declined to 7 log CFU/g in the first 24 h, and the populations did not change over the next 13 days. When inoculated at 2 and 4 log CFU/g, the *Salmonella* populations did not change in the first 24 h but then increased by 2 log CFU/g between 24 and 72 h and again by <1 log CFU/g after 72 h, to levels of 5 to 6.7 log CFU/g, respectively, at 7 days. The *Salmonella* populations initially inoculated at 2 and 4 log CFU/g increased by 1.3 and <1 log CFU/g, respectively, from days 7 to 14. The growth patterns of inoculated *E. coli* O157:H7 on walnut hulls were comparable to those of *Salmonella* under similar conditions (4).

Although fruit maturity affected the levels of endogenous Enterobacteriaceae and coliforms on whole walnut fruit (Fig. 1), hull maturity had little to no effect on the behavior of *Salmonella* inoculated on the hull pieces (Fig. 2B). With the exception of hulls collected 1 week before the typical commercial harvest, *Salmonella* levels increased in the first 24 to 48 h after inoculation. Between 2 to 3 days after inoculation, the counts of *Salmonella* increased by 5 to 6 log CFU/g on walnut hulls regardless of hull maturity. The small differences in the growth of *Salmonella* on hulls harvested at different maturity levels may be associated with an unaddressed factor, such as inherent moisture levels or available nutrients.

*Salmonella* has been shown to multiply in hull materials from other nuts, given adequate available moisture. *Salmonella* in almond hull slurries multiplied from initial levels of 3 to 4 log CFU/ml to 7 log CFU/ml

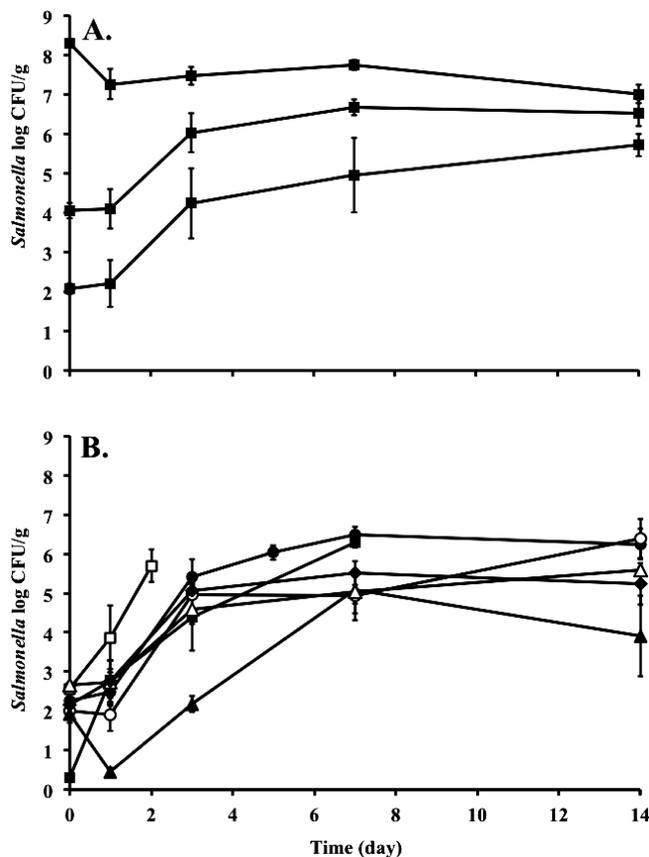


FIGURE 2. The behavior of inoculated *Salmonella* populations on 'Howard' walnut hull pieces in unsealed bags during storage under ambient conditions as affected by (A) inoculation level (high [8 log CFU/g], moderate [4 log CFU/g], and low [1 to 2 log CFU/g]) on hulls obtained from walnuts harvested and inoculated 3 weeks before typical commercial harvest and (B) hull maturity (inocula of <1 to 2.7 log CFU/g for all hull maturities). Walnut fruits were handpicked at 6 weeks before (■), 5 weeks before (□), 3 weeks before (●), 2 weeks before (○), 1 week before (▲), and same week as typical commercial harvest (△) and 1 week after (◆). Results shown are counts determined on BSA. Values represent averages, and error bars show standard errors.

within 24 h at 24°C (40), and in wet pecan shucks (water activity [ $a_w$ ] = 0.99), *Salmonella* grew from inoculation levels of 4 log CFU/g to 6 log CFU/g within 24 h at 21°C (3). The lag time of *Salmonella* on walnut hulls in the current study (approximately 1 day in all but one case) (Fig. 2) was longer than those observed previously on almond hulls or pecan shucks; however, both almond hulls and pecan shucks were soaked in water before inoculation, whereas the growth of *Salmonella* on walnut hulls shown here was dependent on the inherent moisture of the senescing hull and the high RH within the storage bag. Prematurely fallen hulls may be present in the orchard for days to weeks prior to sweeping, but walnuts are typically collected from the orchard within 24 h of shaking (32), and this practice, as well as dry orchard conditions, should limit increases of *Salmonella* should the organism be present.

#### Effect of hull storage conditions on hull moisture.

Hulls on walnuts that have fallen from the tree canopy to lie on the orchard floor before harvest can vary in integrity:

some hull pieces may be dry and shriveled, while others can be moist and in various states of decay. Occasionally, conditions that lead to high humidity (e.g., rain, irrigation, and fog) can occur before or during the California walnut harvest, and these events can accelerate the hull dehiscence and also increase the moisture levels and rates of decay of fallen hull pieces (32). Mature walnut trees with a large canopy can also lead to within-orchard microenvironments that support zones of high humidity in the otherwise dry locale. Approximately 1 month before harvest, the temperature and humidity levels under a walnut canopy can fluctuate between 10 and 30°C and between 30 and 90% RH; frequently, for periods of 6 h or more, the humidity levels can be greater than 70% (22).

The RH levels of the various storage environments and the moisture content and water activity of hull pieces were monitored over a 2-week period for hulls placed in (i) unsealed bags, (ii) on weigh boats in a partially open plastic bin under ambient (low humidity) conditions, and (iii) on weigh boats in a closed plastic bin under high-humidity conditions. After 24 h, the humidity levels equilibrated, depending on the study, to maxima of 90% RH within the unsealed bags, 45% RH in the partially open bin, and 89% RH in the closed bin. The temperatures recorded were similar under all three conditions (23 to 26°C). Within 24 h of storage, the walnut hull pieces on weigh boats in the low-humidity bin were shriveled and had obviously lost moisture. The hull pieces in unsealed bags or on weigh boats in the high-humidity bin did not change in appearance for 3 to 7 days but became moist and flaccid and began to deteriorate after this time.

The humidity level during storage affected the moisture content and water activity of hull pieces (Table 1). The moisture content of the hull pieces on weigh boats stored in the low-humidity bin decreased from 88% ( $a_w$  = 0.98) to 78% ( $a_w$  = 0.94) in the first 24 h and then declined rapidly to 22, 15, and 10% moisture ( $a_w$  = 0.69, 0.43, and 0.40) by days 3, 7, and 14, respectively. The moisture content of hull pieces on weigh boats stored in the closed high-humidity bin increased slightly, from 88 to 92% over the 14 days of storage; the water activity values did not change ( $a_w$  = 0.97 to 0.99). The moisture content (88 to 89%) and water activity (0.98 to 0.99) of hull pieces in the unsealed bags stored in a partially open bin under ambient conditions were not affected during 14 days of storage, even though the humidity within the bags remained high. A wide range of RH levels was observed among the samples of hull pieces that were stored in separate unsealed bags (38 to 90% RH). This variability may have been due to differences in senescence and tissue breakdown among the different hull pieces.

**Effect of hull storage conditions on the survival of *Salmonella*.** The behavior of *Salmonella* on green hulls was affected by the RH in the storage environment (Fig. 3). Within 24 h, *Salmonella* populations decreased from initial levels of 2 to 3 log CFU/g to undetectable levels (<0.3 log CFU/g) on hull samples in the low-humidity bin, to a level of 0.6 log CFU/g in the high-humidity bin, and to 1.6 log

TABLE 1. Moisture content and water activity of hull pieces held under different relative humidity conditions<sup>a</sup>

Time (days)	% moisture content			Water activity		
	Low humidity	High humidity	Unsealed bags	Low humidity	High humidity	Unsealed bags
0	88 ± 2.2	88 ± 2.2	88 ± 2.2	0.98 ± 0.01	0.98 ± 0.01	0.98 ± 0.01
1	78 ± 5.1	87 ± 1.3	89 ± 0.9	0.94 ± 0.03	0.99 ± 0.00	0.98 ± 0.00
3	22 ± 2.6	88 ± 2.1	88 ± 0.6	0.69 ± 0.08	0.99 ± 0.00	0.99 ± 0.00
7	15 ± 0.4	90 ± 0.9	89 ± 1.4	0.43 ± 0.00	0.99 ± 0.00	0.99 ± 0.00
14	10 ± 2.1	92 ± 0.9	89 ± 1.1	0.40 ± 0.01	0.99 ± 0.00	0.98 ± 0.01

<sup>a</sup> The hulls were held on open weigh boats in low-humidity (20 to 45% RH, 23 to 25°C) or high-humidity (68 to 89% RH, 23 to 26°C) bins or in unsealed bags (38 to 90% RH, 24 to 26°C) under ambient conditions.

CFU/g in unsealed bags. For hulls stored at higher humidity (unsealed bags and high-humidity bin), the populations of *Salmonella* then recovered and increased to 3 and 5 log CFU/g at days 3 and 7, respectively. After day 7, mold growth was visible on hull samples in the high-humidity bin, possibly causing the observed reduction of the levels of *Salmonella* on hull pieces stored under these conditions. These data suggest that *Salmonella* populations might increase if in contact with walnut hulls in a humid (>40% RH) or wet orchard environment but are likely to rapidly decrease when conditions are dry.

Danyluk et al. (13) suggest that leeching of almond hull nutrients into orchard soil via rain events may be a mechanism for the persistence of *Salmonella* in the almond production environment. Although *Salmonella* grew on high-moisture pecan shucks, significant reductions of the organism were observed in pecan shuck extract and soils mixed with the extract (3). Walnut growers may wish to evaluate current orchard management practices to reduce the opportunity for water-exposed prematurely dropped fruit to be collected with the harvested crop.

**Behavior of inoculated *Salmonella* on intact and blended walnut hulls.** As walnuts enter the hulling facility, they pass through a tank of water (float tank) that permits heavy materials like stones to separate from the floating

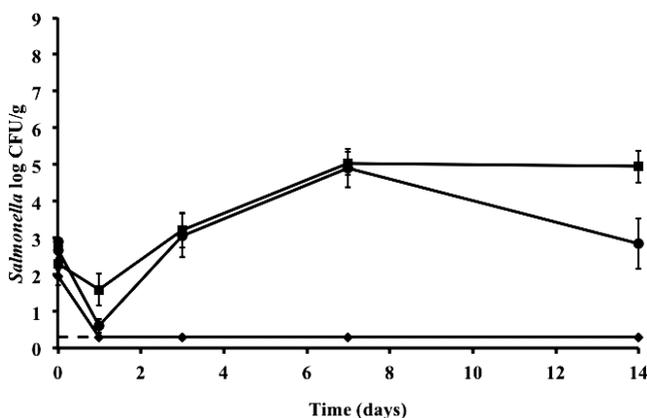


FIGURE 3. Behavior of inoculated *Salmonella* populations on green 'Howard' walnut hull pieces placed in unsealed bags (24 to 26°C, 38 to 90% RH within bags) (■), in a high-humidity bin (23 to 26°C, 68 to 89% RH) (●), or in a low-humidity bin (24 to 25°C, 20 to 43% RH) (◆). Results shown are counts determined on BSA. Values represent averages, and error bars show standard errors.

product. The hull surrounding the walnut shell, if still present, is removed with abrasion during a relatively rapid hulling process. This action produces a slurry made up of hull pieces and hull liquid along with other orchard debris that can end up in the float tank and can coat the hulled in-shell walnuts before they are rinsed with water or sanitizer (15). In the present study, hull pieces were blended with peptone to mimic the production of a hull slurry.

The behavior of *Salmonella* populations on green intact or green blended walnut hull pieces stored under ambient conditions over a period of 2 weeks differed strikingly (Fig. 4). The populations of *Salmonella* increased from 2 to 5 log CFU/g within 72 h on intact hull pieces stored in unsealed bags (>40% RH within bags), while the levels of *Salmonella* on blended hulls decreased to less than 1 CFU/ml in 24 h.

Slurries prepared by blending green-colored hulls and peptone (1:2, wt/vol) were held at ambient conditions for 1 to 14 days before inoculation with *Salmonella*. The age of the prepared slurry had a pronounced effect on the postinoculation behavior of *Salmonella* (Fig. 5A). The *Salmonella* levels in the hull-peptone slurries inoculated immediately after blending were reduced by over 2 log CFU/ml to undetectable levels (<1 CFU/ml) within 24 h

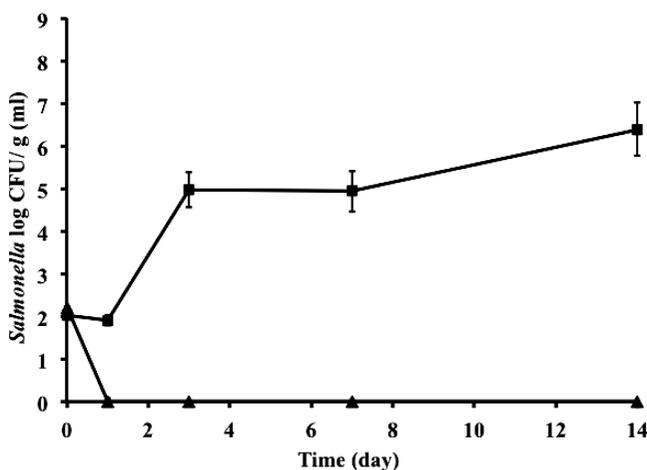


FIGURE 4. Behavior of *Salmonella* populations after inoculation of green 'Howard' walnut hull pieces placed in unsealed bags (38 to 90% RH within bags) (■) or in 1:2 (wt/vol) slurries of green hulls and 0.1% peptone (▲), followed by storage under ambient conditions (23 to 25°C). The lower limit of detection is 1 CFU/ml.

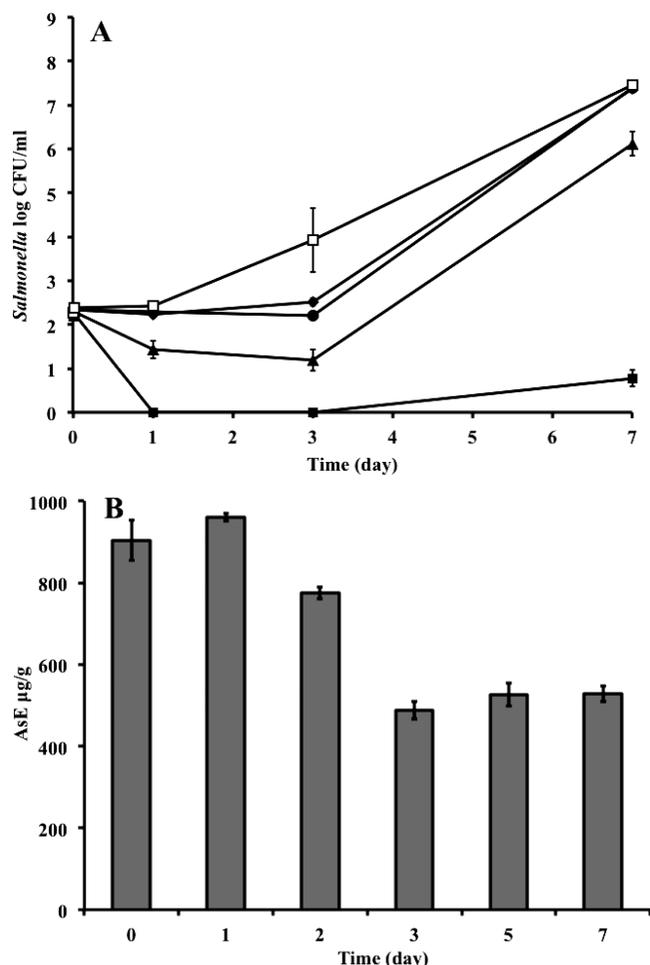


FIGURE 5. (A) Behavior of *Salmonella* populations in 1:2 (wt/vol) slurries of green 'Howard' walnut hulls and 0.1% peptone blended for 2 min and inoculated immediately after blending (■), 1 day after blending (▲), 3 days after blending (●), 7 days after blending (◆), and 14 days after blending (□), followed by storage at ambient temperature (23 to 25°C); the lower limit of detection is 1 CFU/ml. (B) Antioxidant capacities of 2-min-blended green 'Howard' hulls as analyzed immediately after blending (0 days), or 1, 2, 3, 5, and 7 days after blending. Values were determined as ascorbic acid equivalents (AsE).

after inoculation; *Salmonella* levels in four of six samples were below 1 CFU/ml after 3 days of storage. After 7 days of storage, detectable levels of *Salmonella* were recovered (1 to 13 CFU/ml in six samples). In contrast, the levels of *Salmonella* in the hull-peptone slurries inoculated 1, 3, and 7 days after blending initially remained the same or decreased slightly and then increased during storage. *Salmonella* inoculated into the hull-peptone slurry 14 days after blending increased by less than 0.5 log CFU/ml within 24 h after inoculation and by 5 to approximately 7 log CFU/ml within 7 days.

Observed reductions of *E. coli* levels on inoculated walnut kernels were attributed to phenolic-rich tannins present in the walnut kernel skin (21). Similarly, reductions of inoculated *Salmonella* and *E. coli* levels in blended plum and pomegranate extracts were correlated to the levels of antioxidant-rich phenolic compounds in the extracts (12, 29). The majority of plant phenolic compounds are located

within the cell vacuole (1). This may explain why the growth of inoculated *Salmonella* was not inhibited on green-hull pieces stored at high humidity but was inhibited in freshly blended green hulls; *Salmonella* would not be exposed to broken vacuole components in intact hull pieces but would be exposed to such components when hull tissues were blended. After the hulls were blended, the color of the mixture darkened within a few hours, and a general progression from green to brown occurred over several days. Similarly, green hulls eventually turn brown when they split and are exposed to air; this color change is assumed to be a result of peroxidase and polyphenol oxidase enzymes (34).

The AC of uninoculated blended hulls was analyzed over a period of 7 days after blending (Fig. 5B). The AC values determined from hull samples immediately and up to 2 days after blending were significantly greater than the values determined at later sampling times. The decline in AC at day 3 correlated with a similar increase in the ability of *Salmonella* to survive and multiply in the hull-peptone slurry mixtures (Fig. 5A). It is possible that the enzymes peroxidase and polyphenol oxidase, which are associated with the manipulation of hull color (34), may have contributed to the reduction in AC of the blended hulls during ambient-temperature storage.

To further explore the potential correlation between the hull color at harvest and the survival of *Salmonella* in blended hulls, aqueous slurries were prepared with mostly green or mostly brown hulls and well water (1:8, wt/vol). The APC in uninoculated and levels of *Salmonella* in inoculated blended green or brown hulls were quantified under ambient conditions (23 to 25°C) over a 24-h period (Fig. 6). No differences in either APC or *Salmonella* levels were observed before or after blending the individual samples. APC were significantly lower on green hulls than brown hulls. Twenty-four hours after blending, the APC in the brown-hull-water slurries increased by over 3 log CFU/ml to 8 log CFU/ml, but they declined by 2 log CFU/ml in two of three green-hull-water slurries and increased by 2 log CFU/ml in one. The inoculated levels of *Salmonella* declined only in the green-hull slurries, by over 1 log CFU/ml within 30 min after blending and holding at ambient conditions; the inoculated levels of *Salmonella* increased by approximately 2 log CFU/ml to 7 log CFU/ml at 24 h after initial blending in the brown-hull slurries. During the same time, the inoculated levels of *Salmonella* in the green-hull slurries declined to under 1 CFU/ml; however, *Salmonella* was isolated in the green-hull slurries after 24- or 48-h enrichments.

The investigation of the behavior of indigenous aerobic microbiota in green- and brown-hull slurries was repeated with hulls obtained at a separate harvest date, and similar observations were made. Aerobic microbiota remained constant for at least 2 h and multiplied in brown-hull slurries after 24 h, while aerobic microbiota in green-hull slurries decreased by approximately 1 log CFU/ml within 30 min and by an additional 0.3 log within 24 h after blending (data not shown).

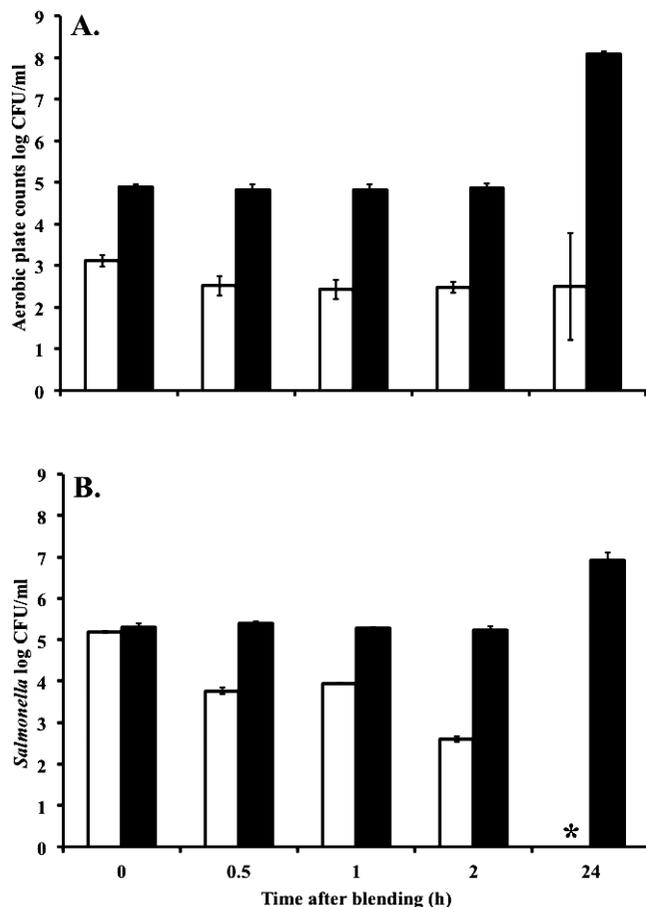


FIGURE 6. Behavior of indigenous aerobic microbiota (A) and inoculated *Salmonella* populations (B) in 1:8 (wt/vol) hull-water slurries of either green (white bars) or brown (black bars) 'Howard' walnut hulls. Hulls were collected at a single point during the harvest period; slurries were blended for 2 min and stored at ambient temperature (23 to 25°C). The lower limit of detection is 1 CFU/ml. The asterisk indicates levels were below the limit of detection but were positive through enrichment.

No declines of indigenous microbiota on in-shell walnuts were observed after the mechanical crushing and abrasive actions of hull removal in a limited sampling from several hulling facilities (4). Based on the results of the current study, those factors associated with the reduced levels of inoculated or indigenous microbiota in laboratory-blended green hulls were either not available, inactivated, or not present during commercial hulling. Further effort would be needed to identify and characterize the phytochemical compound(s) responsible for the antibacterial activities of freshly blended green walnut hulls in order to take advantage of this natural antimicrobial effect under commercial conditions.

A walnut harvest associated with a commercial huller can last for a few days to approximately 3 months, depending on the quantity and variety of the walnut supply and the maturation of the different varieties within a harvest period. The potential exists for hull liquid and/or slurries to remain on the hulling equipment and around the hulling facility for a number of days; these slurries could become a reservoir for contamination of in-shell walnuts or exposed

kernels. Waste streams from walnut hullers are often spread on fallow fields, and the water may be diverted back into orchards. Currently, there are no recommendations on how and how often hulling equipment should be cleaned and sanitized.

*Salmonella* is capable of survival and growth on moist walnut hulls and in hull slurries. When inoculated onto in-shell walnuts or kernels, *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* survive for several months under cool and ambient conditions (5–7, 15). Nut-specific guidance for Good Manufacturing Practices (16) is available, and the walnut industry has published best practices for both growers and processors (23). Walnut growers and producers should evaluate their production, harvest, and postharvest handling steps and strive to reduce opportunities for the introduction or amplification of *Salmonella* and other foodborne pathogens, such as by removing water-soaked premature drops, limiting the time between shaking the trees and picking up the fruit, and regularly washing and sanitizing hulling equipment and facilities. Many of the current best practices recommendations for walnuts are based on limited research available for other crops. Further characterizing the microbial ecology of walnut-specific operations and the effects of cleaning and sanitizing methods at hulling facilities will provide additional scientific backing for walnut-specific guidance.

## ACKNOWLEDGMENTS

T. Blessington was supported in part by a UC Davis Department of Plant Sciences Graduate Student Researcher award and the Oak Ridge Institute for Science and Education. Funding for this project was provided, in part, by the California Walnut Board. Cooperation and guidance were provided by several growers and processors of California walnuts. This project would not have been possible without the technical support of Dr. Anne-laure Moyne, Dr. Michelle Danyluk, Dr. Bruce Lampinen, Samuel Metcalf, Shirin Abd, Christopher G. Theofel, and Irene Zhao and the editorial assistance of Sylvia Yada.

## REFERENCES

- Amiot, M. J., A. Fleuriot, V. Cheyrier, and J. Nicolas. 1997. Phenolic compounds and oxidative mechanisms in fruit and vegetables, p. 51–86. In F. A. Tomás-Barberán and R. J. Robins (ed.), *Phytochemistry of fruits and vegetables*. Oxford University Press, New York.
- Baranyi, J., and T. A. Roberts. 1994. A dynamic approach to predicting bacterial growth in food. *Int. J. Food Microbiol.* 23:277–294.
- Beuchat, L. R., and D. A. Mann. 2010. Survival and growth of *Salmonella* in high-moisture pecan nutmeats, in-shell pecans, inedible nut components, and orchard soil. *J. Food Prot.* 73:1975–1985.
- Blessington, T. 2011. A microbiological hazard analysis of California walnut production and handling. Ph.D. dissertation. University of California, Davis.
- Blessington, T., E. J. Mitcham, and L. J. Harris. 2012. Survival of *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on inoculated walnut kernels during storage. *J. Food Prot.* 75:245–254.
- Blessington, T., C. G. Theofel, and L. J. Harris. 2012. A dry-inoculation method for nut kernels. *Food Microbiol.* 33:292–297.
- Blessington, T., C. G. Theofel, E. J. Mitcham, and L. J. Harris. 2013. Survival of foodborne pathogens on inshell walnuts. *Int. J. Food Microbiol.* 166:341–348.
- Brand-Williams, W., M. E. Cuvelier, and C. Berset. 1995. Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol.* 28:25–30.

9. Canadian Food Inspection Agency. 2011. Health hazard alert—certain bulk and prepackaged raw shelled walnuts may contain *E. coli* O157:H7 bacteria. Available at: <http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-warnings/complete-listing/2011-04-04/eng/1359548340145/1359548340176>. Accessed 10 November 2013.
10. Canadian Food Inspection Agency. 2011. Health hazard alert—certain prepackaged raw shelled walnuts may contain *E. coli* O157:H7 bacteria. Available at: <http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-warnings/complete-listing/2011-09-01/eng/1357653790094/1357653790109>. Accessed 10 November 2013.
11. Canadian Food Inspection Agency. 2012. Health hazard alert—certain Compass brand in-shell California walnuts may contain *Salmonella* bacteria. Available at: <http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-warnings/complete-listing/2012-11-30b/eng/1354560606925/1354560614764>. Accessed 18 November 2013.
12. Cevallos-Casals, B. A., D. Byrne, W. R. Okie, and L. Cisneros-Zevallos. 2006. Selecting new peach and plum genotypes rich in phenolic compounds and enhanced functional properties. *Food Chem.* 96:273–280.
13. Danyluk, M. D., M. Nozawa-Inoue, K. R. Hristova, K. M. Scow, B. Lampinen, and L. J. Harris. 2008. Survival and growth of *Salmonella* Enteritidis PT 30 in almond orchard soils. *J. Appl. Microbiol.* 104:1391–1399.
14. Entis, P., B. Bennett, M. H. Brodsky, D. M. Burgener, V. L. Carlson, M. Carson, K. Catherwood, B. S. Ciebin, N. A. Cox, R. S. Dahiya, H. Daley, J. Devenish, J. Erickson, P. Fettig, R. Foster, J. Gorski, P. A. Hall, S. D. Hunt, J. Jagow, P. Jenkins, P. R. Jordan, F. Krieger, P. Kwan, J. M. Lanier, H. S. Lillard, A. Martin, M. E. Milling, H. Nakanis, E. Nath, J. Oggel, A. Okrend, V. Pongchairerks, D. Posey, J. Rodriguez, B. E. Rose, J. Sayfie, C. Taylor, T. Teramoto, and E. Vandellen. 1984. Enumeration of total coliforms, fecal coliforms, and *Escherichia coli* in foods by hydrophobic grid membrane filter: collaborative study. *J. Assoc. Off. Anal. Chem.* 67:812–823.
15. Frelka, J. 2013. The influence of postharvest handling practices on the microbiota of English walnuts (*Juglans regia* L.). M.Sc. thesis. University of California, Davis.
16. Grocery Manufacturers Association. 2010. Industry handbook for safe processing of nuts. GMA Nut Safety Task Force, Grocery Manufacturers Association, Washington, DC.
17. Hendricks, L. C., W. W. Coates, R. B. Elkins, G. H. McGranahan, H. A. Phillips, D. E. Ramos, W. O. Reil, and R. G. Snyder. 1998. Selection of varieties, p. 84–89. In D. E. Ramos (ed.), Walnut production manual, publication 3373. University of California, Division of Agriculture and Natural Resources, Oakland.
18. Hughlett, M. 15 October 2009. Elgin nut company Sanfilippo issues recall—products may be contaminated with *Listeria*, which can be fatal. Available at: [http://articles.chicagotribune.com/2009-10-15/news/0910140663\\_1\\_john-b-sanfilippo-nut-listeria](http://articles.chicagotribune.com/2009-10-15/news/0910140663_1_john-b-sanfilippo-nut-listeria). Accessed 11 November 2013.
19. Isaacs, S., J. Aramini, B. Ciebin, J. A. Farrar, R. Ahmed, D. Middleton, A. U. Chandran, L. J. Harris, M. Howes, E. Chan, A. S. Pichette, K. Campbell, A. Gupta, L. Y. Lior, M. Pearce, C. Clark, F. Rodgers, F. Jamieson, I. Brophy, and A. Ellis. 2005. An international outbreak of salmonellosis associated with raw almonds contaminated with a rare phage type of *Salmonella* Enteritidis. *J. Food Prot.* 68:191–198.
20. Izumi, H., Y. Tsukada, J. Poubol, and K. Hisa. 2008. On-farm sources of microbial contamination of persimmon fruit in Japan. *J. Food Prot.* 71:52–59.
21. Kokal, D. 1965. Viability of *Escherichia coli* on English walnut meats (*Juglans regia*). *J. Food Sci.* 30:325–332.
22. Lampinen, B. 2010. Personal communication.
23. Lampinen, B., and D. Lindsay. 2010. Quality counts—best practices for walnut growers. University of California and California Walnut Commission, Folsom, CA. Available at: <http://www.walnuts.org/walnuts/assets/File/QualityCountsBestPracticesBrochure.pdf>. Accessed 11 November 2013.
24. Little, C. L., W. Jemmott, S. Surman-Lee, L. Hucklesby, and E. de Pinna. 2009. Assessment of the microbiological safety of edible roasted nut kernels on retail sale in England, with a focus on *Salmonella*. *J. Food Prot.* 72:853–855.
25. Little, C. L., N. Rawal, E. de Pinna, and J. McLauchlin. 2010. Survey of *Salmonella* contamination of edible nut kernels on retail sale in the UK. *Food Microbiol.* 27:171–174.
26. Mahoney, N., and R. J. Molyneux. 2004. Phytochemical inhibition of aflatoxigenicity in *Aspergillus flavus* by constituents of walnut (*Juglans regia*). *J. Agric. Food Chem.* 52:1882–1889.
27. Marcus, K. A., and H. J. Amling. 1973. *Escherichia coli* field contamination of pecan nuts. *Appl. Microbiol.* 26:279–281.
28. Meyer, L. T., and R. H. Vaughn. 1969. Incidence of *Escherichia coli* in black walnut meats. *Appl. Microbiol.* 18:925–931.
29. Negi, P. S., and G. K. Jayaprakash. 2003. Antioxidant and antibacterial activities of *Punica granatum* peel extracts. *J. Food Sci.* 68:1473–1477.
30. NSW Food Authority. 2012. Report on the prevalence of *Salmonella* and *E. coli* in ready to eat nuts and nut products sold in Australia. Available at: <http://www.foodauthority.nsw.gov.au/science/market-analysis/ready-to-eat-nuts/>. Accessed 11 November 2013.
31. Oliveira, I., A. Sousa, I. C. F. R. Ferreira, A. Bento, L. Estevinho, and J. A. Pereira. 2008. Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia* L.) green husks. *Food Chem. Toxicol.* 46:2326–2331.
32. Olson, W. H., J. M. Labavitch, G. C. Martin, and R. H. Beede. 1998. Maturation, harvesting, and nut quality, p. 273–276. In D. E. Ramos (ed.), Walnut production manual, publication 3373. University of California, Division of Agriculture and Natural Resources, Oakland.
33. Pereira, J. A., I. Oliveira, A. Sousa, P. Valentão, P. B. Andrade, I. C. F. R. Ferreira, F. Ferreres, A. Bento, R. Seabra, and L. Estevinho. 2007. Walnut (*Juglans regia* L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. *Food Chem. Toxicol.* 45:2287–2295.
34. Piffaut, B., and M. Metche. 1991. Properties of peroxidase and polyphenol oxidase in natural complexes from walnuts (*Juglans regia*) and in active DL-DOPA copolymers. *J. Sci. Food Agr.* 57:493–506.
35. Pinney, K., J. M. Labavitch, and V. S. Polito. 1998. Fruit growth and development, p. 139–143. In D. E. Ramos (ed.), Walnut production manual, publication 3373. University of California, Division of Agriculture and Natural Resources, Oakland.
36. Riyaz-Ul-Hassan, S., V. Verma, A. Malik, and G. N. Qazi. 2003. Microbiological quality of walnut kernels and apple juice concentrate. *World J. Microbiol. Biotechnol.* 19:845–850.
37. Thompson, J. F., T. R. Rumsey, and J. A. Grant. 1998. Dehydration, p. 277–284. In D. E. Ramos (ed.), Walnut production manual, publication 3373. University of California, Division of Agriculture and Natural Resources, Oakland.
38. Uesugi, A. R., M. D. Danyluk, and L. J. Harris. 2006. Survival of *Salmonella* Enteritidis phage type 30 on inoculated almonds stored at –20, 4, 23, and 35°C. *J. Food Prot.* 69:1851–1857.
39. Uesugi, A. R., M. D. Danyluk, R. E. Mandrell, and L. J. Harris. 2007. Isolation of *Salmonella* Enteritidis phage type 30 from a single almond orchard over a 5-year period. *J. Food Prot.* 70:1784–1789.
40. Uesugi, A. R., and L. J. Harris. 2006. Growth of *Salmonella* Enteritidis phage type 30 in almond hull and shell slurries and survival in drying almond hulls. *J. Food Prot.* 69:712–718.
41. U.S. Food and Drug Administration. 2010. Tropical Nut & Fruit of Charlotte, NC announces a nationwide voluntary recall of walnut products because of possible health risk. Available at: <http://www.fda.gov/Safety/Recalls/ucm236656.htm>. Accessed 11 November 2013.
42. Weinzirl, J. 1929. Nuts as a possible source of *Escherichia coli* found in candy. *Am. J. Epidemiol.* 10:265–268.