Research Paper

Growth of *Salmonella* and Other Foodborne Pathogens on Inoculated Inshell Pistachios during Simulated Delays between Hulling and Drying

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ABSTRACT

During harvest, pistachios are hulled, separated in water into floater and sinker streams (in large part on the basis of nut density), and then dried before storage. Higher prevalence and levels of *Salmonella* were previously observed in floater pistachios, but contributing factors are unclear. To examine the behavior of pathogens on hulled pistachios during simulated drying delays, floater and sinker pistachios collected from commercial processors were inoculated at 1 or 3 log CFU/g with cocktails of *Salmonella* and in some cases *Escherichia coli* O157:H7 or *Listeria monocytogenes* and incubated for up to 30 h at 37°C and 90% relative humidity. Populations were measured by plating onto tryptic soy agar and appropriate selective agars. In most cases, no significant growth (P > 0.05) of *Salmonella* was observed in the first 3 h after inoculation in hulled floaters and sinkers. Growth of *Salmonella* was greater on floater pistachios than on corresponding sinkers and on floater pistachios with ≥25% hull adhering to the shell surface than on corresponding floaters with <25% adhering hull. Maximum *Salmonella* populations (2 to 7 log CFU/g) were ~2-log higher on floaters than on corresponding sinkers. The growth of *E. coli* O157:H7 and *Salmonella* on hulled pistachios was similar, but a longer lag time (approximately 11 h) and significantly lower maximum populations (4 versus 5 to 6 log CFU/g; P < 0.05) were predicted for *L. monocytogenes*. Significant growth of pathogens on hulled pistachios is possible when delays between hulling and drying are longer than 3 h, and pathogen growth is enhanced in the presence of adhering hull material.

HIGHLIGHTS

- Foodborne pathogens multiplied on undried inshell pistachios.
- Pathogen growth was greater when hull material was present.
- Drying delays of >3 h led to significant increases in pathogen populations.
- Managing drying delays will reduce the risk for growth of foodborne pathogens.

Key words: *Escherichia coli* O157:H7; Harvest; *Listeria monocytogenes*; Pistachio; Postharvest; *Salmonella*

The United States is a global leader in commercial production of pistachios (*Pistacia vera*) (3), and California produces 99% of the total U.S. crop (4). Over the past two decades, pistachio production in California has grown rapidly, from 81 million kg in 1997 to a record crop of over 408 million kg in 2016 (2). In California, pistachios are harvested from late August to early October (3), as described previously in more detail (14, 27).

The pistachio fruits are shaken from the trees and transported in bins or bottom dump trailers directly to hulling facilities. Once unloaded, the pistachios are precleaned to remove leaves, sticks, and other debris, and the hulls are removed by abrasion and then discarded. The hulled inshell pistachios are rinsed to remove remaining loosely adhering hull and then deposited into a tank of water (float tank) that separates mature heavier “sinkers” (fully developed nuts) from lighter “floaters” (i.e., nuts that are underdeveloped, blank, with adhering hull, or with insect or other damage) (14).

Floaters and sinkers are dried separately to moisture levels of about 8 to 15% (<15% moisture, water activity [aw] of <0.85) (14, 27). The dried nuts are transferred to large storage silos (500,000- to 750,000-kg capacity) that have perforated floors. In these silos, pistachios are exposed to forced warm dry ambient air over several days to further reduce equilibrated moisture levels to <7% (aw of <0.70). When drying is complete, pistachios are fumigated to control insects, and the silo is sealed. Adequately dried pistachios can be stored in silos for up to 18 months (1).
Tree nuts have been associated with outbreaks of salmonellosis and enterohemorrhagic *Escherichia coli* gastroenteritis (16) and recalls due to the presence of enterohemorrhagic *E. coli*, *Listeria monocytogenes*, and *Salmonella*. In the United States, recalls of pistachios have been initiated due to the presence of *Salmonella* (31), and the consumption of raw or roasted pistachios was linked to salmonellosis cases in 2009, 2013, and 2016 (10, 12, 30).

*Salmonella* and other pathogens cannot grow on dry pistachios but will survive for extended periods under conditions of ambient storage (17). Raw inshell pistachios collected from California storage silos shortly after harvest (2010 through 2012) had a 3-year average prevalence of *Salmonella* of 2.0% (95% confidence interval [CI], 1.3 to 3.1%; 1,032 samples) for floaters and 0.37% (95% CI, 0.21 to 0.67%; 2,934 samples) for sinkers (13). Geometric mean most-probable-number (MPN) values for *Salmonella* were higher in floaters (0.66 MPN/100 g; 95% CI, 0.14 to 5.3 MPN/100 g) than in sinkers (0.18 MPN/100 g; 95% CI, 0.10 to 0.62 MPN/100 g). Factors contributing to the higher prevalence and levels of *Salmonella* in floater pistachios are unclear. The predicted mean probability of salmonellosis per individual serving associated with consuming pistachios was an order of magnitude higher for floater pistachios than for sinkers (20, 25).

The moisture of harvested pistachios ranges from 40 to 50% on a fresh weight basis (27), and respiration can result in rapid elevation of relative humidity (RH) and temperature (28) in the harvest trailers. During harvest the pistachios remain in trailers for times that are impacted by the rate the trailer is filled, the duration of transport from the orchard to the hulling facility, and the hold time between receiving and unloading the trailer (14). In a previous study, the fate of *Salmonella* on inshell pistachios was evaluated under simulated postharvest conditions from the time of shaking the tree to just before hulling. Significant growth of *Salmonella* on inshell pistachios was observed at 23, 35, and 37°C and at both 50 and 90% RH when incubation times exceeded 3 to 6 h (21).

After hulling, rinsing, and then separation in the float tank, pistachios are conveyed to a column dryer and dried for 6 to 24 h using forced air at initial temperatures of 80 to 105°C and final temperatures of about 70°C. The dryers work most efficiently when full. Filling the dryer can take longer at the beginning or the end of harvest when the volume of incoming product is lower and the receipt of harvest loads is more sporadic, which is a greater issue for small-volume hullers. Delays also are more likely for the floater pistachio stream because floaters make up a smaller portion of the incoming crop (typical floater portion is 15%) (1). Filling also can be delayed because of equipment failure or interrupted power supply. When the dryer is at less-than-full capacity, to prevent quality defects the heater may be temporarily turned down or off and ambient air applied. The objective of this study was to examine the fate of *Salmonella enterica* on hulled post-float tank floater and sinker pistachios during simulated drying delays and to compare the fate of *S. enterica* with that of *E. coli* O157:H7 and *L. monocytogenes* under selected conditions.

### MATERIALS AND METHODS

**Pistachios.** Freshly hulled floater and sinker pistachios were collected during three commercial harvests (2014, 2015, and 2016) from conveyor belts after the float tank at four processors in the Central Valley of California. Samples were collected during the early season (week 1 or 2), midseason (week 3 or 4), or late season (week 5) of the harvest. The seasonal collection times were established in a concurrent study on in hull pistachios (21). As in that study, the pistachios were placed into polyethylene bags (30.5 by 30.5 cm; Bitran, Com-Pac International, Carbondale, IL), which were sealed and then transported (~4 h) on ice to the laboratory. The sealed bags were then transferred to lidded plastic tubs and stored at 4°C for up to 12 h before inoculation.

**Bacterial cultures.** *Salmonella* strains used in this study were described in detail by Moussavi et al. (21). Rifampin-resistant variants of the following strains were used: *Salmonella enteritidis* phage type (PT) 30, ATCC BAA-1045; *Salmonella enteritidis* PT 9c, RM4635 (provided by Dr. Robert Mandrell, U.S. Department of Agriculture, Agricultural Research Service); *Salmonella Anatum*, LJH1242; *Salmonella Tennessee*, K4643 (provided by Dr. Larry R. Beuchat, University of Georgia, Griffin); and *Salmonella Montevideo*, GRCl (provided by the U.S. Food and Drug Administration [FDA]). The following strains were also used: *E. coli* O157:H7, Odwalla strain 223 (a clinical isolate from an apple juice–associated outbreak) (9); *E. coli* O157:H7, CDC 658 (a clinical isolate from a cantaloupe-associated outbreak, provided by Dr. Larry R. Beuchat) (6); *E. coli* O157:H7, EC4042 (a clinical isolate from a spinach-associated outbreak, provided by the FDA) (18); *E. coli* O157:H7, EC173 (isolated from prepackaged raw cookie dough, provided by the FDA) (24); *E. coli* O157:H7 PT 4, Health Canada NML 11-1865 (a clinical isolate from a walnut-associated outbreak, provided by Dr. Alex Gill, Health Canada) (8); *L. monocytogenes* 4b, LCDC81-861 (isolated from raw cabbage associated with an outbreak, provided by Dr. Larry R. Beuchat) (26); *L. monocytogenes* 4b, LJH552 (isolated from tomatoes); *L. monocytogenes*, LIS0234 (isolated from raw, diced yellow onions associated with a 2012 onion recall, provided by the FDA) (29); *L. monocytogenes*, LIS0133 (an environmental isolate from a celery processing facility associated with an outbreak, provided by the FDA) (13); and *L. monocytogenes*, PTVS 308 (isolated from cantaloupe associated with an outbreak, provided by Dr. Trevor Suslow, University of California, Davis) (11). Isolates were stored at −80°C in tryptic soy broth (TSB) supplemented with 15% glycerol. Unless otherwise specified, all media were obtained from BD (Franklin Lakes, NJ).

**Preparation of inocula.** The preparation of inocula and inoculation procedure were based on the method described previously (21). Frozen stock cultures of each strain were streaked onto tryptic soy agar (TSA) supplemented with rifampin (75 to 100 μg/mL; TSAR) and incubated at 37 ± 2°C for 24 ± 4 h. A single isolated colony from each plate was transferred into 10 mL of TSB and incubated at 37 ± 2°C for 24 ± 4 h. A loopful (~10 μL) of culture was then transferred into TSB and incubated at 37 ± 2°C overnight. An aliquot (1 mL) of the overnight culture was spread over large TSAR plates (150 by 15 mm; Fisher Scientific, Pittsburgh, PA)—one plate each for *Salmonella* and *E. coli* O157:H7 culture and two plates for *L. monocytogenes*—and incubated at 37 ± 2°C for 24 ± 1 h. After incubation, the resulting bacterial lawn was collected by adding 9 mL (for *Salmonella* and *E. coli* O157:H7) or 6 mL (for *L. monocytogenes*).
of 0.1% peptone to each TSAR plate and then scraping the slurry on the plate surface with a sterile spreader.

Three multistrain cocktails were prepared by combining equal volumes of the cell suspensions of each strain, one each for *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*, resulting in levels of 11 log CFU/mL. Sterile ultrapure water (Milli-Q Advantage A10, MilliporeSigma, Burlington, MA) was used to dilute the cocktail to the desired final target inoculum level.

**Inoculation procedure.** Before inoculation, floater and sinker pistachios were removed from refrigerated storage and held at room temperature for ~1 h. In one case, late season floater pistachios were visually sorted into product that had ~25% or more adhering hull (designated as with hull) from those pistachios that had <25% adhering hull (designated as without hull). Floater and sinker pistachio samples (600 to 800 g) were weighed separately into polyethylene bags (30.5 by 30.5 cm; Bitran), and 37.5 to 50 mL (25 mL of inoculum into 400 g of pistachios) of the *Salmonella*, *E. coli* O157:H7, or *L. monocytogenes* inoculum was added. Each bag was sealed and then shaken continuously by hand for 2 min to ensure an even coating of the nuts with the inoculum. The inoculated nuts were then spread onto two sheets of filter paper (46 by 57 cm; Qualitative P5, Fisher Scientific) that were folded in half and placed in a biosafety cabinet for 30 min to dry.

After drying, each sample of inoculated pistachios was transferred to a separate metal tray inside a plastic bin, and the open bins were placed in an environmental chamber (Percival, Geneva Scientific, Fontana, WI) and incubated for up to 30 h at 37°C and 90% RH. Uninoculated control pistachios were held under the same conditions. During the incubation period, temperature and RH were monitored and recorded using data loggers (TempTale 4, Sensitech, Beverly, MA).

**Enumeration of background microbiota and inoculated cells.** At each sampling time, a random sample of sinkers or floaters (20 g) was combined with 40 mL of 0.1% peptone in a separate two-chamber filter bag (Nasco, Modesto, CA). Each sample was processed by shaking for 30 s, rubbing for 15 s, and shaking for an additional 30 s. The liquid portion in each bag was serially diluted in 0.1% peptone. Appropriate dilutions were plated in duplicate on nonselective TSA (uninoculated controls), TSAR supplemented with cycloheximide (50 μg/mL) to inhibit the growth of molds, and appropriate selective media using spot plating (20 μL), spread plating (100 μL per plate), or a spiral plater (Autoplate, Advanced Instruments, Norwood, MA). The selective media—CHROMagar Salmonella, CHROMagar O157, and CHROMagar Listeria (CHROMagar, Paris, France) for *Salmonella, E. coli* O157:H7, and *L. monocytogenes*, respectively—were all supplemented with rifampin (75 μg/mL). Samples were incubated at 37 ± 2°C for 24 ± 2 h (TSA, TSAR, CHROMagar Salmonella, and CHROMagar O157) or 48 ± 2 h (CHROMagar Listeria). Colonies were counted manually or with a colony counter (ProtoCOL 2, Symbiosis, Frederick, MD).

The background microbiota for uninoculated pistachios was determined by plating appropriate dilutions onto TSA for aerobic plate counts (APCs) and, for 2016 samples only, onto CHROMagar ECC for *E. coli*–coliform counts (ECCs) and incubating at 37 ± 2°C for 24 ± 2 h. Samples also were plated onto appropriate pathogen-selective media and incubated as described above. All colonies visible on TSA (APCs) and all pink (presumptive coliform) and all blue (presumptive *E. coli*) colonies on CHROMagar ECC were counted.

**Characterization of pistachio samples.** In some cases, floater and sinker pistachios, as obtained from the hulling facility, were characterized on the basis of weight as a fraction of a ~1-kg sample. Each sample was weighed, placed in a tray, and then sorted into six fractions: sticks, loose material (leaves, hulls, and unidentified debris), with hull (unhulled pistachios), inshell hulled (hull-free inshell pistachios), shells (loose), and kernels (loose). Each fraction was weighed, and the weight percentage for each component was calculated.

**Moisture content and *a*<sub>w</sub>.** Moisture content and *a*<sub>w</sub> were determined for kernels at the beginning and end of the incubation period. Floater and sinker pistachios were shelled, and the kernels (40 g) were processed for 20 s in a commercial food processor (Waring, Torrington, CT). The *a*<sub>w</sub> and percent moisture were determined for triplicate subsamples of the ground material with an *a*<sub>w</sub> meter (Aqualab model 4TE, Decagon Devices, Pullman, WA) and moisture analyzer (3.6- to 4.0-g samples; model HG63, Mettler-Toledo, Columbus, OH), respectively.

**Statistical analysis.** Pistachios collected from a single processor on a single day were considered an independent lot. Two 600-g sublots or one 800-g sublot of pistachios were inoculated with a single inoculum preparation. At each sampling time the inoculated pistachios were mixed, and three samples from each of the two 600-g sublots or four samples from the one 800-g sublot were randomly selected. A single uninoculated control sublot (500 g) was held under the same conditions as the inoculated sublots. Two random samples from the uninoculated sublot were plated onto selective and nonselective agars at each sampling time, and three random samples were used to determine moisture content and *a*<sub>w</sub> at the beginning and end of the incubation period. Data were analyzed using Excel 2010 (Microsoft, Redmond, WA) and Prism 6 software (GraphPad Software, La Jolla, CA). An analysis of variance, Tukey’s multiple comparisons test, and a *t* test were performed. Differences between mean values were considered significant at *P* < 0.05. Individual growth curves for inoculated floater and sinker pistachios were fit using the DMFit version 3.5 add-in for Microsoft Excel (http://www.combase.cc/) (5). The lag time, growth rate, and maximum population changes (difference between the initial inoculated and highest levels) and time to maximum population were determined.

**RESULTS AND DISCUSSION**

Limited published data are available on the drying delay times, temperatures, humidities, and overall volumes of hulled pistachios that might be exposed to less-than-optimally drying conditions. Pistachio dryers are located outdoors; thus, pistachios are exposed to ambient conditions when the heaters are not engaged. During the approximately 45-day harvests in 2014, 2015, and 2016, daytime high temperatures in the region were 30 to 40°C for 82, 80, and 64% of the time, respectively (7). Across the three harvest years, the mean RH was 39 to 40%, with mean minimums of 23 to 25% and mean maximums of 60 to 65% (7). The experiments with hulled pistachios were conducted at 37°C and 90% RH to simulate a large volume of wet pistachios exposed to warm ambient temperatures.

For early and midseason harvested pistachios, paired samples (collected at the same time from the same facility) of commercially hulled floaters and sinkers were collected for the three harvest years (2014, 2015, and 2016), and for
early season floater pistachios samples were collected on the same day in 2015 from three geographically separate pistachio processors (P1, P2, and P3) in California’s Central Valley.

**Characterization of floater and sinker pistachios.**

The goal of the hulling step is to remove the outer hull, leaving an intact inshell pistachio, ideally without any adhering hull material. Hulls are removed by abrasion and concurrent and subsequent spraying with water. However, the degree to which the hull is successfully removed can depend on a number of factors, including the growing conditions for that harvest year, degree of senescence at time of harvest, and various forms of damage (e.g., insect or mechanical). The float tank serves to separate the fully developed heavier sinker nuts from the lighter floaters. Floaters may have underdeveloped or no kernel, or they may have loosely or tightly adhering hull that may be a result of insect or other types of damage.

For the current study, the portion of fully hulled inshell pistachios was lower and more variable among floater samples (mean ± standard deviation, 48.4% ± 26.1%) and consistently high among corresponding sinker samples (93.8% ± 2.1%; Fig. 1). The proportion of inshell pistachios with adhering hull was significantly higher and more variable in floaters (49.1% ± 27.5%) than in sinkers (3.8% ± 1.4%). Other components (loose material, sticks, kernels, and shells) made up less than 6.2% of the weight in all samples (Fig. 1).

For early season floater pistachios collected from processors P1, P2, and P3, 50.1, 76.3, and 80.4%, respectively, had adhering hull, and most of the remaining material was hulled inshell pistachios. Other components (loose material, sticks, kernels, and shells) of pistachios made up less than 1% in samples from all three pistachio processors. The difference observed in the amount of hull attached to floater pistachios collected from the three processors on the same day could be due to differences in equipment hulling efficiencies and/or differences in the degree of senescence of the harvested crop handled by the three processors on that day.

**Moisture content and \(a_w\).** In 2014, 2015, and 2016, early and midseason harvest sinkers and floaters collected from the same facility at the same time were compared. In most cases, initially and after 30 h of incubation, floaters had a higher moisture content (25.6% ± 0.36 to 47.6% ± 0.44%) but similar \(a_w\) (0.95 to 0.98) compared with the paired sinker moisture content (24.6% ± 0.27 to 45.7% ± 0.18%) or \(a_w\) (0.95 to 0.99) (Table 1). In most cases, the initial moisture content was lower for pistachios collected midseason than for those collected early in the season. In general, moisture content significantly declined after 30 h of incubation; little or no decline was observed for \(a_w\). The reduction in moisture content over 30 h of incubation differed significantly among samples. A decline of 22% was observed for early season floaters harvested in 2016, whereas a decline of 1.8% was observed for midseason floaters harvested in 2014.

Only floaters were used in the studies comparing processors, three pathogens, and the presence or absence of a hull. For these floaters, maximum decreases in moisture content (13.9%) and \(a_w\) (0.07) were observed for the experiment in which the growth of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* was compared (Table 1). The early season floater pistachios from processors P2 and P3 had significantly higher moisture content and \(a_w\) at both the beginning and end of incubation time than did pistachios from P1. Pistachios from P1 also had the greatest decline in moisture content (9.1%) and \(a_w\) (0.02) after 30 h of incubation (Table 1). Moisture content and \(a_w\) of all floater samples in this experiment decreased significantly after 30 h of incubation. Moisture content of the late season pistachios with hull increased significantly at 30 h of incubation, whereas the \(a_w\) did not change (Table 1). In contrast, both the initial moisture content and \(a_w\) of the floaters without hull had decreased significantly at 30 h.

**Background microbiota.**

The initial APCs for early season and midseason uninoculated pistachios were 4.50 ± 0.18 to 6.50 ± 0.53 log CFU/g (floaters) and 5.45 ± 0.21 to 6.65 ± 0.03 log CFU/g (sinkers) (Figs. 2 through 5). APCs for floaters increased significantly by 2.59 to 4.74 log CFU/g within the first 12 to 16 h. APCs for sinkers increased by a maximum of 0.72 to 2.90 log CFU/g within the first 12 h. Thereafter, in most cases, populations either reached a plateau or decreased significantly. Maximum APCs were 8.80 ± 0.01 to 9.91 ± 0.04 log CFU/g for floaters and 7.77 ± 0.14 to 8.75 ± 0.54 log CFU/g for sinkers (Figs. 2 through 5).

The initial APCs for late season uninoculated floaters and sinkers were similar (5.54 ± 0.18 and 5.34 ± 0.06 log CFU/g, respectively), and these levels did not change significantly for the first 8 h after inoculation (5.40 ± 0.05 and 5.47 ± 0.01 log CFU/g, respectively) (Fig. 6B). Thereafter, the APC was significantly higher on the pistachios with hull at all sampling times. At 30 h, the APCs on floaters with and without hull were 9.26 ± 0.15 and 7.37 ± 0.41 log CFU/g, respectively.

In 2016, presumptive coliform counts also were determined for uninoculated floater and sinker pistachios (Fig. 3). *E. coli* colonies were not detected on CHROMagar ECC in any of the eight samples evaluated. In most cases, at each sampling time, no significant differences were found for APCs and presumptive coliform counts. Initial presumptive coliform populations for early season and midseason pistachios were 4.61 ± 0.18 to 5.71 ± 0.03 log CFU/g, and counts increased by 0.73 to 4.06 log CFU/g within 12 h. Thereafter, in most cases, populations did not change. For floaters, maximum APCs and coliform counts were 9.40 ± 0.19 and 9.14 ± 0.27 log CFU/g, respectively, for the early season samples and 9.54 ± 0.03 and 9.48 ± 0.19 log CFU/g, respectively, for the midseason. For sinkers, maximum APCs and coliform counts were 8.03 ± 0.61 and 8.09 log CFU/g, respectively, for the early season and 8.25 ± 0.53 and 8.10 ± 0.49 log CFU/g, respectively, for the midseason (Fig. 3).
Fate of *Salmonella* on inoculated hulled floaters or sinkers at two initial inoculum levels. The fate of *Salmonella* inoculated onto paired floater and sinker pistachios was evaluated on samples collected during three harvest years. Because the growth curves were different among the samples collected in different years, at different times, and from different places, the data were treated separately. *Salmonella* counts on TSAR and CHROMagar Salmonella were not significantly different at any time ($P > 0.05$); thus, only data from TSAR are presented. No background populations on uninoculated hulled floaters or sinkers were detected on TSAR or CHROMagar Salmonella at any time.

Floater and sinker pistachios were inoculated with *Salmonella* at $2.31 \pm 0.18$ to $3.09 \pm 0.04$ log CFU/g (high level; Figs. 2 and 3) or at $0.46 \pm 0.22$ to $1.71 \pm 0.09$ log CFU/g (low level; Fig. 3). In 2014 (Fig. 2) and 2016 (Fig. 3), no significant growth of *Salmonella* was observed during the first 3 h after inoculation on either floaters or sinkers at either high or low inoculum levels. In 2015, small but significantly higher populations of *Salmonella* were measured at 3 h on floaters (0.35- and 0.47-log increase for early and midseason harvest, respectively) and sinkers (0.29- and 0.45-log increase for early and midseason harvest, respectively) (Fig. 2). In 2016, significantly lower *Salmonella* counts (0.24- and 0.27-log decrease, respectively) were
TABLE 1. Moisture content and water activity of kernels from sinker and floater pistachios collected at different times during commercial harvests and stored at 37°C and 90% RH

<table>
<thead>
<tr>
<th>Pistachio type and harvest season</th>
<th>Harvest yr</th>
<th>Moisture (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Water activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h</td>
<td>30 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 h</td>
<td>30 h</td>
</tr>
<tr>
<td>SINKERS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (wk 1)</td>
<td>2015</td>
<td>45.7 ± 0.18 CD a</td>
<td>35.7 ± 0.17 F b</td>
</tr>
<tr>
<td>Early (wk 2)</td>
<td>2014</td>
<td>39.7 ± 0.32 G a</td>
<td>24.6 ± 0.27 K b</td>
</tr>
<tr>
<td>Early (wk 2)</td>
<td>2016</td>
<td>45.4 ± 0.30 CD a</td>
<td>25.6 ± 0.35 J b</td>
</tr>
<tr>
<td>Mid (wk 3)</td>
<td>2015</td>
<td>38.8 ± 0.49 G a</td>
<td>30.6 ± 0.63 H b</td>
</tr>
<tr>
<td>Mid (wk 4)</td>
<td>2014</td>
<td>43.0 ± 0.55 E a</td>
<td>38.2 ± 0.30 E b</td>
</tr>
<tr>
<td>Mid (wk 4)</td>
<td>2016</td>
<td>44.7 ± 0.52 D a</td>
<td>37.5 ± 0.69 E b</td>
</tr>
<tr>
<td>FLOTTERS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (wk 1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2015</td>
<td>47.3 ± 0.11 AB a</td>
<td>39.3 ± 0.56 CDE b</td>
</tr>
<tr>
<td>P1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2015</td>
<td>35.0 ± 0.22 H a</td>
<td>25.9 ± 0.35 J b</td>
</tr>
<tr>
<td>P2</td>
<td>2015</td>
<td>46.1 ± 0.64 BC a</td>
<td>39.0 ± 0.39 P b</td>
</tr>
<tr>
<td>P3</td>
<td>2015</td>
<td>45.8 ± 0.52 CD a</td>
<td>40.3 ± 0.31 C b</td>
</tr>
<tr>
<td>Early (wk 2)</td>
<td>2014</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Early (wk 2)</td>
<td>2016</td>
<td>47.6 ± 0.44 A a</td>
<td>25.6 ± 0.36 I b</td>
</tr>
<tr>
<td>Mid (wk 3)</td>
<td>2015</td>
<td>41.7 ± 0.29 F a</td>
<td>32.0 ± 0.99 H b</td>
</tr>
<tr>
<td>Mid (wk 4)</td>
<td>2014</td>
<td>45.0 ± 0.47 CD a</td>
<td>43.2 ± 0.30 B b</td>
</tr>
<tr>
<td>Mid (wk 4)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2015</td>
<td>35.6 ± 0.72 H a</td>
<td>21.7 ± 0.29 L b</td>
</tr>
<tr>
<td>Mid (wk 4)</td>
<td>2016</td>
<td>39.6 ± 0.36 G b</td>
<td>45.2 ± 0.38 A a</td>
</tr>
<tr>
<td>Late (wk 5), with hull</td>
<td>2015</td>
<td>31.4 ± 0.54 J b</td>
<td>34.4 ± 0.36 G a</td>
</tr>
<tr>
<td>Late (wk 5), without hull</td>
<td>2015</td>
<td>35.2 ± 0.72 H a</td>
<td>29.3 ± 0.04 I b</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± standard deviations, n = 3. Within columns, mean values with different uppercase letters are significantly different (P < 0.05); within rows, mean values with different lowercase letters are significantly different (P < 0.05).

<sup>b</sup> Data refer to early season floater pistachios collected from three pistachio processors (P1, P2, and P3).

<sup>c</sup> ND, not determined.

<sup>d</sup> Data refer to midseason floater pistachios used for comparing growth of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*.

FIGURE 2. Populations of *Salmonella* (top panels; n = 6) on inoculated and APCs (bottom panels; n = 2) on un inoculated paired floaters (circles) and sinkers (squares) from early season and midseason during the 2014 (closed symbols) and 2015 (open symbols) commercial harvest years. Error bars indicate standard deviations. All samples were incubated at 37°C and 90% RH.
FIGURE 3. Populations of Salmonella (top panels; n = 4) on inoculated (closed symbols, high inoculum level; open symbols, low inoculum level) and APCs (bottom panels, closed symbols; n = 2), and coliform counts (bottom panels, open symbols; n = 2) on uninoculated floater and sinker pistachios from early season and midseason commercial harvest in 2016. Error bars indicate standard deviations. All samples were incubated at 37°C and 90% RH.

FIGURE 4. Populations of inoculated Salmonella (A) and APCs (B) on early season floater pistachios collected from processors P1 (triangles), P2 (squares), and P3 (circles) (n = 4). Samples were collected on the same day during early season commercial harvest in 2015. Error bars indicate standard deviations. All samples were incubated at 37°C and 90% RH.

FIGURE 5. Populations of inoculated Salmonella (circles), E. coli O157:H7 (squares), and L. monocytogenes (triangles) (A) and APCs (B) on floater pistachios (n = 4). Samples were collected during midseason commercial harvest in 2015. Error bars indicate standard deviations. All samples were incubated at 37°C and 90% RH.
observed at 3 h for midseason sinkers at the high inoculum level and for midseason floaters at the low initial inoculum level (Fig. 3). After 3 h, in most cases, Salmonella grew on both floaters and sinkers, but the magnitude and duration of the increase differed among the samples (Figs. 2 and 3). Populations of Salmonella were almost always significantly greater on floaters than on the corresponding sinkers at any time point, possibly because of greater amounts of hull material (and corresponding nutrients; Fig. 1) and higher moisture levels (Table 1) on the floater pistachios.

In the current study, the high and low inoculum levels of ~3 and ~1 log CFU/g, respectively, might represent a localized contamination event during harvest, cross-contamination during hulling, or cross-contamination within the float tank. As demonstrated for inhull pistachios in a concurrent study (21), lower initial inoculum levels resulted in lower maximum population sizes. Among the paired 2014 and 2015 floater and sinker samples at the high inoculum level, Salmonella populations increased by 1.57 to 4.44 log CFU/g to maximums of 4.61 ± 0.26 to 7.40 ± 0.22 log CFU/g in floaters (Fig. 2) and by 0.64 to 2.41 log CFU/g to maximums of 3.25 ± 0.45 to 5.30 ± 0.22 log CFU/g in sinkers. In some cases, population declines were observed for sinkers after an initial increase (Fig. 2). The growth curves were similar for both low and high inoculum levels (Fig. 3). At most time points, Salmonella populations remained ~1- to 2-log lower on pistachios at the low inoculum level (Fig. 3). Maximum increases were similar at both high and low inoculum levels, respectively: floaters, early season, 3.33 and 2.94 log CFU/g; floaters, midseason, 2.21 and 1.80 log CFU/g; sinkers, early season, 1.27 and 1.13 log CFU/g; sinkers, midseason, 0.96 and 1.28 log CFU/g. The higher maximum populations of Salmonella in floaters observed in the current study correspond to the higher geometric mean Salmonella levels (0.66 versus 0.18 MPN/100 g for floaters versus sinkers) and wider range (0.14 to 5.3 versus 0.10 to 0.62 MPN/100 g for floaters versus sinkers) previously reported in floater pistachios collected from storage silos (15).

No significant growth of Salmonella was observed during incubation in the first 3 h after inoculation of early season floaters collected on the same day from three processors (P1, P2, and P3; Fig. 4A). Thereafter, significant differences (P < 0.05) in the growth of Salmonella were observed among floaters from the three processors; maximum levels were 5.00 ± 0.27 to 6.52 ± 0.19 log CFU/g (Fig. 4A). The highest growth rate and maximum Salmonella populations were observed on pistachios from P3.

Effect of adhering hull on growth of Salmonella on floater pistachios. The presence of soluble sugars (including glucose and fructose), fat, and protein (3.77% ± 0.53%, 4.60% ± 0.89%, and 5.18% ± 0.65% of dry weight, respectively) has been previously reported for pistachio hull meal (19). To directly evaluate the impact of hull material on the growth of Salmonella, late season floater pistachios were separated upon visual assessment into samples with hull (≥25% hull) or without hull (<25% hull) adhering to the shell surface and then inoculated with Salmonella (3.08 ± 0.08 or 2.57 ± 0.13 log CFU/g, respectively). No significant growth of Salmonella was observed during the first 3 h of incubation after inoculation; increases between 3 and 8 h of incubation were not significantly different for floaters with or without hull (Fig. 6A). After 8 h, populations continued to increase on floaters with hull, but no significant further increase of Salmonella was observed for floaters without hull. At 30 h, the mean Salmonella population on floaters with hull was 2.38-log higher than that on floaters without hull (7.31 ± 0.40 versus 4.93 ± 0.99 log CFU/g, respectively). The amount of attached hull would affect the amounts of readily available nutrients and may explain the lower growth of Salmonella on sinker pistachios, which had significantly less adhering hull than did floater pistachios (Figs. 1 through 3 and 6A).

Growth of E. coli O157:H7, L. monocytogenes, and Salmonella on floater pistachios. Although E. coli O157:H7 and L. monocytogenes have not been associated with pistachio outbreaks or recalls, these pathogens have been implicated in outbreaks and recalls of other tree nuts and their products (16, 31) and should be considered with Salmonella in pistachio hazard assessments. Midseason floater pistachios were inoculated with cocktails of E. coli O157:H7, L. monocytogenes, or Salmonella at 2.89 ± 0.12, 2.67 ± 0.19, or 2.99 ± 0.13 log CFU/g, respectively (Fig. 5A). The growth of E. coli O157:H7 and Salmonella on floater pistachios was similar, but a longer lag time was observed for L. monocytogenes (Fig. 5A), and significantly lower populations of L. monocytogenes were observed at all
time points after 3 h. Maximum populations determined for
E. coli O157:H7, L. monocytogenes, and Salmonella were
5.24 ± 0.15, 4.26 ± 0.48, and 6.17 ± 0.26 log CFU/g, respectively.

On dried pistachios, survival of E. coli O157:H7 and L. monocytogenes was similar to Salmonella survival at −20 and 4°C (minimal reduction over 52 weeks of storage), but more rapid declines of E. coli O157:H7 and L. monocytogenes were observed at ambient storage (23°C) (17). Five strains each of E. coli O157:H7 and L. monocytogenes inoculated onto inshell pistachios were screened for resistance to heat in hot water and hot oil treatments. All strains were less heat resistant than Salmonella Enteritidis PT 30 (23).

Pathogen growth parameters. Sigmoid functions (DMFit or asymmetric sigmoidal five-parameter logistic function) were used to fit growth curves in which typically a no-growth linear phase (lag) is followed by a steep mid-phase (log) and then a stationary phase. In most cases, similar curve fits were derived from the two fit functions. However, DMFit predicted linear or inaccurate curve fits for six growth curves (2016 early harvest sinkers with low initial inoculation levels, all 2016 midseason sinkers and floaters with low and high initial inoculation levels, and 2015 midseason floaters inoculated with L. monocytogenes). In these cases, high population variability was observed among replicate samples (high standard deviations) at many of the time points. The cause of the sample variability was not specifically determined, but variability may have been due to different proportions of hull material or different levels or composition of background microbiota in individual samples. Growth of the native microbiota to high levels may deplete readily available nutrients and influence the growth of inoculated organisms. For these six growth curves, only the asymmetric sigmoidal five-parameter logistic function was used to construct fits more like a classic growth curve (Table 2 and Supplemental Figs. S1 and S2).

In harvest years 2014, 2015, and 2016, inoculated inshell pistachios were incubated under presumably optimal growth conditions of 37°C and 90% RH. In the 2014, 2015, and 2016 early and midseason harvests, when sinkers and floaters from the same lot of inshell pistachios were compared, floaters were associated with higher predicted growth rates (except in early harvest 2014), higher maximum levels, higher maximum increases, and longer time to reach the highest level (except in 2016 midseason

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Harvest yr</th>
<th>Harvest stage</th>
<th>Streama</th>
<th>Initial level (log CFU/g)b</th>
<th>Lag time (h)</th>
<th>Growth rate (log CFU/g/h)</th>
<th>Maximum level increase (log CFU/g)c</th>
<th>Time to maximum level (h)</th>
<th>R²</th>
<th>Fit functiond</th>
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<td>Salmonella</td>
<td>2014</td>
<td>Early Sinker</td>
<td>S</td>
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<td>5.67</td>
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<td>3.82</td>
<td>1.03</td>
<td>7.5</td>
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<td>2015</td>
<td>Mid Floater</td>
<td>L</td>
<td>2.79</td>
<td>0.00</td>
<td>−0.0048</td>
<td>2.64</td>
<td>−0.14</td>
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<td>ND, AS</td>
</tr>
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<td></td>
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<td>Early Floater</td>
<td>P1</td>
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<td>3.70</td>
<td>0.35</td>
<td>4.77</td>
<td>1.91</td>
<td>9.0</td>
<td>0.89 DM, AS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mid Floater</td>
<td>H</td>
<td>2.95</td>
<td>3.55</td>
<td>0.29</td>
<td>7.16</td>
<td>1.37</td>
<td>10.0</td>
<td>0.95 DM, AS</td>
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<td></td>
<td></td>
<td>Late Floater</td>
<td>N</td>
<td>2.48</td>
<td>3.24</td>
<td>0.53</td>
<td>5.01</td>
<td>0.71</td>
<td>7.7</td>
<td>0.77 DM, AS</td>
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<td>5.82</td>
<td>2.98</td>
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<td></td>
<td>Early Floater</td>
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<td>3.06</td>
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<td>0.35</td>
<td>4.77</td>
<td>1.91</td>
<td>9.0</td>
<td>0.89 DM, AS</td>
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<td></td>
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<td>5.01</td>
<td>0.71</td>
<td>7.7</td>
<td>0.77 DM, AS</td>
</tr>
</tbody>
</table>

* a Floater types: S, inoculated with Salmonella; P1, from processor 1; P2, from processor 2; P3, from processor 3; H, with hull; N, without hull; E, inoculated with E. coli O157:H7; L, inoculated with L. monocytogenes.
  b All samples were incubated at 37°C and 90% RH.
  c Maximum increase = maximum level − initial level.
  d DM, DMFit version 3.5 add-in for Microsoft Excel (5); AS, asymmetric sigmoidal five-parameter logistic.
  e ND, not determined.

In harvest years 2014, 2015, and 2016, inoculated inshell pistachios were incubated under presumably optimal growth conditions of 37°C and 90% RH. In the 2014, 2015, and 2016 early and midseason harvests, when sinkers and floaters from the same lot of inshell pistachios were compared, floaters were associated with higher predicted growth rates (except in early harvest 2014), higher maximum levels, higher maximum increases, and longer time to reach the highest level (except in 2016 midseason
harvest with low and high initial inoculation levels (Table 2). In 2016, low and high initial inoculum levels of Salmonella were also compared on the same lot of inshell pistachios; high inoculation levels were associated with higher predicted maximum levels and higher maximum increases (except in midseason sinkers) (Table 2).

Predicted growth parameters of Salmonella also varied among floaters collected on the same day from the three processors (P1, P2, and P3). Although floaters from P1 had the longest predicted lag time, the lowest predicted maximum level, and the shortest time to reach the maximum level, floaters from P3 were associated with highest growth rate, the highest predicted maximum level, and the highest predicted maximum increase among floaters from the three processors (Table 2). These differences might be at least partially attributed to the differences in initial and final moisture and aw observed for P1 and P3 pistachios (Table 1).

Predicted growth parameters of Salmonella on floater pistachios differed in samples with and without adhering hull. Floaters with adhering hull had a higher predicted maximum level, higher maximum increase, and longer time to reach the maximum level compared with floaters without adhering hull material (Table 2).

Growth parameters for the three pathogens were compared for 2015 midseason floaters inoculated with E. coli O157:H7 (Floaters-E), L. monocytogenes (Floaters-L), or Salmonella (Floaters-S) (Table 2 and Fig. S2, N through P). In this experiment, Salmonella was associated with the shortest lag phase, lowest growth rate, highest predicted maximum level, and the highest predicted maximum increase among the three pathogens, followed by E. coli O157:H7 and then L. monocytogenes.

Santillana Farakos et al. (25) used preliminary data (22) to model an atypical “delay in drying” scenario, which was evaluated in a quantitative microbial risk assessment for salmonellosis from pistachios. This scenario predicted significantly higher levels of Salmonella per contaminated unit and per-serving risk estimates that were orders of magnitude higher than the baseline. The authors used estimated drying delays of 6 to 48 h and assumed high humidity and temperatures conducive to growth. They estimated increases of 6.6 and 5.8 log CFU per day (0.28 and 0.24 log CFU/h) for floaters and sinkers, respectively. These estimates did not take into account the high degree of lot-to-lot variability in Salmonella growth observed in the current study; measured maximum increases were 0 to 5.25 log CFU/g and growth rates were 0 to 0.56 log CFU/g/h. Santillana Farakos et al. (25) used maximum populations of 7 log CFU/g for both floaters and sinkers. Of 22 lots evaluated in the current study, the highest predicted populations for Salmonella were 7 log CFU/g or higher in two cases (9.1%) and were less than 5 log CFU/g in 12 cases (54.5%) (Table 2); measured populations were less than 5 log CFU/g for 9 of 22 lots (40.9%; Figs. 2 through 6). Particularly relevant are the lower maximum populations consistently observed for sinkers (1.20 to 5.10 log CFU/g), especially at the lower initial inoculum levels where maximum estimated populations did not exceed 1.5 log CFU/g (1.20 and 1.49 log CFU/g). Although delays in drying of hulled pistachios could lead to increases in levels of Salmonella and should be appropriately managed, the magnitude of the risk is likely much lower than that estimated by Santillana Farakos et al. (25), and the data presented here could be used to adjust the risk model.

Salmonella can multiply on inshell pistachios under commercially relevant conditions of time, temperature, and RH that might occur with delays in the drying of hulled pistachios. Insignificant to small (<0.50 log CFU/g) increases in populations were observed during the first 3 h of incubation. The maximum populations observed were significantly different among independent replicate experiments with different lots of pistachios. However, in experiments with paired samples, growth was positively influenced by the presence of higher amounts of hull material. The lack of hull material (≤5%) may account for the significantly lower maximum populations observed on sinker pistachios and could be one reason why, in a previous survey, higher levels of naturally occurring Salmonella were present on dried floaters than on sinkers collected from preprocessing silos (15).

These findings stress the importance of managing postharvest handling of pistachios to minimize the time between hulling and drying, particularly for the floater pistachio stream or at times when high amounts of adhering hull are observed. This study focused on a single temperature and humidity. Additional information on drying delay times, temperatures, and humidities in the dryers, especially at the beginning and end of the harvest, would help to further inform the risk from such delays.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: https://doi.org/10.4315/0362-028X.JFP-18-450.s1

REFERENCES


