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Survival of *Salmonella, Escherichia coli* O157:H7, and *Listeria monocytogenes* on Inoculated Almonds and Pistachios Stored at −19, 4, and 24°C

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ABSTRACT

The survival of *Salmonella, Escherichia coli* O157:H7, and *Listeria monocytogenes* was determined on almonds and pistachios held at typical storage temperatures. Almond kernels and inshell pistachios were inoculated with four- to six-strain cocktails of nalidixic acid–resistant *Salmonella, E. coli* O157:H7, or *L. monocytogenes* at 6 log CFU/g and then dried for 72 h. After drying, inoculated nuts were stored at −19, 4, or 24°C for up to 12 months. During the initial drying period after inoculation, levels of all pathogens declined by 1 to 2 log CFU/g on both almonds and pistachios. During storage, moisture content (4.8%) and water activity (0.4) of the almonds and pistachios were consistent at −19°C; increased slowly to 6% and 0.6, respectively, at 4°C; and fluctuated from 4 to 5% and 0.3 to 0.5 at 24°C, respectively. Every 1 or 2 months, levels of each pathogen were enumerated by plating; samples were enriched when levels fell below the limit of detection. No reduction in population level was observed at −19 or 4°C for either pathogen, with the exception of *E. coli* O157:H7–inoculated almonds stored at 4°C (decline of 0.09 log CFU/g/month). At 24°C, initial rates of decline were 0.20, 0.60, and 0.71 log CFU/g/month on almonds and 0.15, 0.35, and 0.86 log CFU/g/month on pistachios for *Salmonella, E. coli* O157:H7, and *L. monocytogenes,* respectively, but distinct tailing of the survival curves was noted for both *E. coli* O157:H7 and *L. monocytogenes*.

The shelf life of low-moisture foods like nuts may be a year or more. Thus, outbreaks associated with these products often span many months. Cases of salmonellosis associated with consumption of raw almonds were reported over periods of 8 and 9 months *(24, 25),* and peanut butter–associated outbreaks were reported over 5 and 9 months *(11, 12)*. The duration of these outbreaks supports laboratory evidence that *Salmonella* can survive for long periods of time in almonds, pecans, peanut butter, and walnuts *(5, 6, 8, 32, 37)*. *Escherichia coli* O157:H7 illnesses have been epidemiologically associated with consumption of inshell hazelnuts *(14)* and walnut kernels *(9)*; these outbreaks occurred months after the product was harvested.

Almond and pistachio handlers may store untreated nuts for 12 months or longer in controlled environments (between 4 and 20°C) or at ambient temperatures. Ambient temperatures are common during shipping and retail handling *(15, 28)*. After purchase, consumers may store nuts for up to an additional 12 months in the freezer, refrigerator, or at ambient temperatures *(29)*. In general, microbial populations on nuts or in nut products remain unchanged at refrigerator or freezer temperatures, whereas storage at room temperature and above leads to slow but steady declines of *Salmonella (5, 6, 8, 37)*. Although survival of *Salmonella* during storage has been investigated on several nut types, no survival data are available for pistachios. Survival of *E. coli* O157:H7 and *Listeria monocytogenes* has been described in walnut kernels *(6),* but no data are available for these pathogens on other nut kernels, including almonds and pistachios.

The objectives of this study were (i) to examine the impact of inoculation of almond kernels and inshell pistachios on moisture and water activity (a_w) during drying and storage and (ii) to evaluate the survival of cocktails of *Salmonella enterica, E. coli* O157:H7, and *L. monocytogenes* on almond kernels and inshell pistachios during storage at −19, 4, and 24°C.

MATERIALS AND METHODS

Almonds and pistachios. Almonds (*Prunus dulcis*) used for this study were untreated (raw) Nonpareil variety (size 25/27: 25 to 27 kernels per 28 g) obtained from Blue Diamond Growers (Sacramento, CA). The raw inshell pistachios (*Pistacia vera*) were medium-sized U.S. Fancy grade, obtained from the Administrative Committee for Pistachios (Fresno, CA).

Bacterial cultures. For safety reasons, nonpathogenic *E. coli* K-12 (ATCC 10798) was used in experiments monitoring the moisture content and a_w of nuts during the inoculum-drying period and for preliminary recovery studies on pistachios (inshell and kernels).

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Pathogens used in this study were as follows: *Salmonella enterica* Enteritidis PT 9c (RM4635), a clinical isolate from the 2004 outbreak associated with raw almonds *(25)* (provided by Dr. Robert Mandrell, U.S. Department of Agriculture, Agricultural Research Service); *S. enterica* Enteritidis PT 30 (ATCC BAA-1045), isolated from raw almonds associated with the 2000 to 2001 outbreak *(24)*; *S. enterica* Tennessee (K4643), a clinical isolate from the 2006 to 2007 outbreak associated with peanut butter *(11)*; *S. enterica* Oranienburg (1839), isolated from pecans (provided by Dr. Larry Beuchat, University of Georgia, Griffin); *S. enterica* Anatum, isolated from an almond survey *(16)*; *S. enterica* Montevideo (GRC1), isolated from pistachios *(13)* (provided by the U.S. Food and Drug Administration [FDA]); *E. coli* O157:H7 (Odwalla strain 223), a clinical isolate from an outbreak associated with apple juice *(10)*; *E. coli* O157:H7 (CDC 658), a clinical isolate from an outbreak associated with cantaloupe *(7)*; *E. coli* O157:H7 (H1730), a clinical isolate from an outbreak associated with lettuce (provided by Dr. L. Beuchat); *E. coli* O157:H7 (F4546), a clinical isolate from an outbreak associated with alfalfa sprouts (provided by Dr. L. Beuchat); *E. coli* O157:H7 (EC4042), a clinical isolate from an outbreak associated with spinach *(27)*; *L. monocytogenes* serotype 4b (LCDC81-861), isolated from raw cabbage associated with an outbreak *(34)*; *L. monocytogenes* serotype 4b (Scott A), a clinical isolate from an outbreak associated with milk *(21)*; *L. monocytogenes* serotype 1/2 (V7), isolated from milk from an outbreak associated with milk; and *L. monocytogenes* serotype 4b (101M), isolated from beef from an outbreak associated with beef. (All *L. monocytogenes* strains were provided by Dr. L. Beuchat.)

To enable pathogen enumeration in the presence of relatively high background microbial populations on the raw nuts, a stepwise procedure *(31)* was used to isolate mutants that were able to grow in media supplemented with nalidixic acid (Sigma, St. Louis, MO) at 50 μg/ml. The wild-type and mutant strains of *Salmonella* had similar growth characteristics and exhibited a similar ability to survive low-moisture storage *(22)*.

Preparation of inocula. Unless otherwise specified, all media were BD Difco, obtained from BD (Franklin Lakes, NJ). The inocula were prepared as described previously *(18)*. Individual strains were separately grown at 37°C for 24 h in tryptic soy broth supplemented with nalidixic acid (TSBN) at 50 μg/ml. Cultures were transferred twice consecutively at 24-h intervals, and then 1 ml of each culture was spread onto tryptic soy agar (TSA) supplemented with nalidixic acid (TSAN) in large-format petri plates (150 by 15 mm; Fisher Scientific, Fair Lawn, NJ). Plates were incubated at 37°C for 24 ± 2 h. To collect the resulting bacterial lawn, 9 ml of a 0.1% peptone solution was added to each plate, and then the lawn was loosened with a sterile plate spreader.

Three separate four- to six-strain cocktails were prepared, one for each organism. Equal volumes of cell suspensions for each strain were pooled into their respective *Salmonella, E. coli* O157:H7, or *L. monocytogenes* mixtures. Pathogen populations for each individual strain, as well as for the three pooled cocktails, were determined by performing serial dilutions in 0.1% peptone, followed by plating onto both selective and nonselective agars. All three cocktails were plated on TSAN supplemented with cycloheximide (CYC) at 50 μg/ml to inhibit molds (TSAN+CYC) and were incubated at 37°C for either 24 h (*Salmonella* and *E. coli* O157:H7) or 48 h (*L. monocytogenes*) before enumeration.

Inoculation and storage of almonds and pistachios. Almond kernels and inshell pistachios were inoculated as described by Uesugi et al. *(37)* for almonds; a ratio of 25 ml of liquid inoculum was added to 400 g of nuts. Inshell pistachios rather than kernels were selected for study because the majority of these nuts are stored and sold in-the-shell. Inoculation with each pathogen cocktail was performed in two separate trials, 4 weeks apart ($n = 3$ for each). For each of the three organisms during each of the trials, 1,600 g of almonds or pistachios was combined with 100 ml of inoculum cocktail in large plastic bags (30.5 by 30.5 cm; Bitran, Com-Pac International, Carbondale, IL). The bag was shaken continuously for 1 min to ensure even coating of the nuts with the inoculum; the nuts were then spread out on a double layer of filter paper (Qualitative P8 Grade, Fisher Scientific) on a metal rack in a plastic tub with the lid ajar to dry for 3 days at ambient conditions (temperature, ~24°C; relative humidity (RH), 35 to 40%) in the laboratory. After drying, the inoculated nuts were pooled, mixed with a sterile scoop (Sterileware, Fisher Scientific), separated into three approximately equal aliquots, and stored in sealed 3.8-liter plastic zipper bags at ambient conditions for another 4 days to allow equilibration to ambient conditions. The sealed bags were then stored at freezer (−19°C), refrigerator (4°C), or ambient (24°C) temperatures for up to 12 months. Uninoculated, untreated control samples were similarly stored. Temperature data loggers (TempTale 4, Sensitech Inc., Beverly, MA) were used to record temperature and RH at each storage location.

Moisture content and a_w. In preliminary experiments, moisture content and a_w were determined for uninoculated almond kernels and inshell pistachios and nuts that had been inoculated with *E. coli* K-12. For trials 1 and 2, the moisture content and a_w were measured for uninoculated nuts held in sealed plastic zipper bags at room temperature or under refrigerated or frozen storage.

The moisture content and a_w of kernels were determined as previously described *(18)*. Kernels (50 g) were ground for 20 s in a 2.5-qt commercial food processor (Waring, Torrington, CT) and manually shaken through a U.S. standard #12 testing sieve (1.7-mm openings; Fisher Scientific). The a_w of the sieved samples was measured in triplicate with a water activity meter (Aqualab model 4TE, Decagon Devices, Pullman, WA). Percent moisture of sieved samples (4 g) was determined with a moisture analyzer (model HG63, Mettler-Toledo, Columbus, OH).

Distribution of inoculum between shell and kernel. Because of product handling during shelling, for safety reasons, *E. coli* K-12 was used to determine the distribution of inoculated organisms between the shell and the kernel. Inshell pistachios (200 g) and kernels (200 g) were inoculated with nonpathogenic *E. coli* K-12 to a level of approximately 6 log CFU/g after drying. On days 0 (predrying), 1, and 3, the following samples were evaluated in triplicate: kernels, whole inshells, shells removed from inshells, and kernels removed from inshells (kernels of the inoculated inshell pistachios were aseptically removed from the shells with sterile tweezers). Each sample (10 g) was added to 20 ml of 0.1% peptone in a 200-ml Whirl-Pak bag (Nasco, Modesto, CA), shaken for 30 s, rubbed by hand for 15 s, and then shaken for an additional 30 s. Samples were diluted in 0.1% peptone and then plated onto TSA. Plates were incubated at 37°C for 24 h before enumeration.

Enumeration of *Salmonella, E. coli* O157:H7, and *L. monocytogenes*. Inoculated almond kernels or inshell pistachios (10 g in triplicate) were added to 20 ml of 0.1% peptone in a 200-ml Whirl-Pak bag. Almond samples were mixed in a mechanical stomacher (model 400, Seward, Bohemia, NY) at high speed for 2 min. Pistachio samples were shaken for 30 s, rubbed by hand for 15 s, and then shaken for an additional 30 s. Serial dilutions of the

liquid portion of the homogenized slurry were made in 0.1% peptone.

Appropriate dilutions were plated onto TSAN+CYC and incubated at 37°C for either 24 h (*Salmonella* and *E. coli* O157:H7) or 48 h (*L. monocytogenes*). Dilutions of the *Salmonella*-inoculated nuts were plated onto bismuth sulfite agar (BSA) supplemented with nalidixic acid and CYC (BSAN+CYC) and incubated at 37°C for 48 h before colony enumeration; *E. coli* O157:H7–inoculated samples were diluted and plated onto sorbitol MacConkey agar (SMAC) supplemented with nalidixic acid and CYC (SMACN+CYC) and incubated at 37°C for 24 h; and *L. monocytogenes*–inoculated samples were diluted and plated onto modified Oxford medium (Oxford medium base with modified Oxford antimicrobic supplement) with CYC (MOX+CYC) and incubated at 37°C for 48 h. Levels of each pathogen on almonds and pistachios were determined on days 0, 3, and 7 as well as every 1 or 2 months thereafter for up to 12 months. At each sampling time, the background populations on uninoculated control samples were determined in duplicate by plating onto TSAN+CYC, BSAN+CYC, SMACN+CYC, and MOX+CYC.

When the colony counts on selective agar approached the limit of detection and colony counts on TSAN+CYC were significantly higher than those on selective agar, replica plating was performed. An autoclaved piece of velvet was pressed onto the surface of the TSAN+CYC spread plate. The inoculated velvet was then pressed onto the surface of the appropriate selective agar plate: either BSAN+CYC for *Salmonella,* CHROMagar O157 (CHROMagar, Paris, France) for *E. coli* O157:H7, or MOX+CYC for *L. monocytogenes*. These plates were incubated as previously described, and colonies were enumerated. Counts for TSAN were adjusted as appropriate.

Enrichment. When counts were expected to be close to the limit of detection (0.3 log CFU/g), samples were enriched according to FDA *Bacteriological Analytical Manual* enrichment protocols *(38)*. The enrichment procedure was carried to completion only when colonies were absent on plating media. For *Salmonella,* 20 ml of double-strength lactose broth was added to the sample and incubated at 37°C for 24 h; 1 ml was then added to 9 ml of tetrathionate broth and incubated at 37°C for 24 h, and 0.1 ml was added to 9.9 ml of Rappaport-Vassiliadis broth and incubated at 42°C for 48 h. The enrichment broths were streaked onto xylose lysine deoxycholate agar (XLD), Hektoen enteric agar (HE), and BSAN+CYC and incubated at 35°C for 24 h (XLD and HE) or 48 h (BSAN+CYC); plates were then examined for growth of typical colonies (red with a black center on XLD, dark green with a black center on HE, and black on BSA).

For *E. coli* O157:H7, samples were enriched by adding 20 ml of double-strength modified buffered peptone water with pyruvate (Acumedia, Lansing, MI) and incubating at 37°C for 24 h. These enrichments were then streaked onto SMACN+CYC and incubated at 37°C for 24 h before plates were examined for growth of typical colonies (colorless or pale pink).

For *L. monocytogenes,* a modified FDA *Bacteriological Analytical Manual* enrichment method was used in which 20 ml of UVM-modified *Listeria* enrichment broth was added to the sample and incubated at 30°C for 48 h *(23)*. This enrichment was streaked onto MOX+CYC and incubated at 35°C for 48 h before plates were examined for growth of typical colonies (dark with zone of clearing).

Distribution of *Salmonella* serovars in stored samples. After the end of trial 2 (13.5 and 14.5 months for almonds and pistachios, respectively), *Salmonella*-inoculated samples stored at −19 or 24°C were plated onto TSAN+CYC. For each nut type, a total of 100 colonies were purified from samples stored at each temperature. For samples stored at −19°C, well-separated colonies were randomly selected from multiple plates. Because counts were very low for samples stored at 24°C, aliquots of the lowest dilution were distributed over multiple plates. Colonies appearing on the plates were streaked onto XLD and HE and, after confirmation for typical *Salmonella* colonies on these media, were further processed. Of the cocktail strains, only *Salmonella* Anatum or *Salmonella* Oranienburg were able to grow on agar supplemented with 100 μg/ml ampicillin (Sigma) or 50 μg/ml chloramphenicol (Sigma), respectively. Thus all isolates were streaked onto TSA supplemented with 100 μg/ml ampicillin or 50 μg/ml chloramphenicol to identify *Salmonella* Anatum or *Salmonella* Oranienburg, respectively. All remaining isolates were screened for *Salmonella* Enteritidis by PCR, using Enteritidis-specific primers *(35)*. *Salmonella* Enteritidis PT 9c was then separated from PT 30 using primers (S1–S4 set) from Soumet et al. *(36)*. GoTaq Flexi DNA polymerase (Promega, Madison, WI) was used, and reactions were conducted following the manufacturer's instructions. The final concentration of each primer in a PCR reaction was 1 μM. Pulsed-field gel electrophoresis profiles using *Xba*I (New England Biolabs, Ipswich, MA) were generated for isolates that failed to be identified by either antibiotic resistance or PCR (*Salmonella* Montevideo and *Salmonella* Tennessee) following standard PulseNet procedures *(33)*.

Modeling microbial decline and statistical analysis. All experiments were replicated twice (trials 1 and 2), with triplicate samples within each replicate. Values were compiled from the two data sets ($n = 6$) and were analyzed by analysis of variance (ANOVA) with the JMP 8 software package (SAS Institute, Cary, NC) to determine whether time had a significant influence on microbial decline ($P < 0.05$). Best-fit models (Baranyi, Gompertz, or linear regression) were chosen based on R^2 value and shape as determined with the aid of DMFit *(2)* and JMP 8. One-way ANOVA, means comparisons, and Tukey-Kramer honestly significant difference tests were performed on various data sets with the JMP 8 software. Differences between the mean values were considered significant at $P < 0.05$.

RESULTS

Influence of wet inoculation on nut moisture and a_w. The moisture and a_w of *E. coli* K-12–inoculated nuts after inoculation, drying, and holding were compared with uninoculated nuts (Table 1). Over 7 days, the moisture of the uninoculated almonds ranged from 3.9 to 4.3% and the a_w ranged from 0.36 to 0.45. During the holding period the a_w was not significantly different ($P > 0.05$) for inoculated and uninoculated almonds, and the moisture levels in inoculated almonds were 0.2 to 0.5% higher than for uninoculated almonds. In contrast, the moisture levels in inoculated pistachios were consistently lower by 0.1 to 0.6% than in the uninoculated pistachios during the holding period. The a_w values also were consistently and significantly lower by 0.04 to 0.09 for the uninoculated pistachios. Because the differences in a_w and moisture content between inoculated and uninoculated nuts were small, uninoculated nuts were used to monitor moisture and a_w throughout the year-long storage study.

Nut moisture content and a_w during storage. The temperature and RH of each storage environment were

TABLE 1. *Moisture content and water activity (a_w) of uninoculated and* E. coli *K-12–inoculated almonds and pistachios during drying (3 days) and holding (4 days) at 24°C*[a]

Nut	Treatment	Day	Moisture content (%)		Water activity	
			Uninoculated	Inoculated	Uninoculated	Inoculated
Almonds	Inoculation	0	4.1 ± 0.17		0.41 ± 0.01	
	Drying	1	4.0 A[b] ± 0.05	4.5 B ± 0.27	0.38 a ± 0.05	0.42 a ± 0.07
		2	4.2 A ± 0.12	4.7 B ± 0.07	0.41 a ± 0.02	0.45 b ± 0.02
		3	4.2 A ± 0.25	4.5 B ± 0.07	0.41 a ± 0.02	0.43 a ± 0.03
	Holding	4	4.2 A ± 0.21	4.5 B ± 0.08	0.42 a ± 0.01	0.42 a ± 0.03
		5	4.3 A ± 0.28	4.5 A ± 0.15	0.45 a ± 0.02	0.45 a ± 0.02
		6	3.9 A ± 0.29	4.4 A ± 0.10	0.40 a ± 0.01	0.46 a ± 0.02
		7	4.0 A ± 0.20	4.4 B ± 0.06	0.36 a ± 0.02	0.37 a ± 0.00
Pistachios	Inoculation	0	4.5 ± 0.18		0.46 ± 0.02	
	Drying	1	4.3 A ± 0.49	4.5 A ± 0.74	0.43 a ± 0.07	0.45 a ± 0.09
		2	4.4 A ± 0.31	4.1 A ± 0.21	0.44 a ± 0.03	0.40 a ± 0.04
		3	4.5 A ± 0.16	4.3 B ± 0.13	0.46 a ± 0.04	0.41 a ± 0.03
	Holding	4	4.3 A ± 0.38	4.3 A ± 0.24	0.44 a ± 0.05	0.40 a ± 0.04
		5	4.7 A ± 0.06	4.3 B ± 0.22	0.49 a ± 0.00	0.43 b ± 0.01
		6	4.6 A ± 0.05	4.0 B ± 0.26	0.49 a ± 0.01	0.40 b ± 0.04
		7	4.4 A ± 0.14	4.0 B ± 0.12	0.40 a ± 0.01	0.36 b ± 0.01

[a] Values are the mean ± standard deviation, $n = 6$.

[b] Within nut type and within rows, mean moisture (%) values with different uppercase letters are significantly different ($P < 0.05$), and mean a_w values with different lowercase letters are significantly different ($P < 0.05$).

monitored using data loggers. The median temperature and RH recorded in trials 1 and 2 were as follows: for ambient storage, 24°C (38% RH) and 24°C (39% RH), respectively; for refrigerated storage, 4.3°C (89% RH) and 4.1°C (90% RH), respectively; and for frozen storage, −19°C (55% RH) and −18°C (43% RH), respectively. Greater fluctuations in humidity than in temperature were noted at each storage condition. At ambient storage, the laboratory humidity was impacted by climate, particularly rainfall, and occasionally increased to as high as 60% for short periods of time (Fig. 1). Samples in refrigerated and frozen storage were subjected to humidity fluctuations when doors were opened or when cooling cycles were activated.

On day 0 of storage, the average moisture content and a_w values for the uninoculated almonds were 4.0% ± 0.20% and 0.36 ± 0.02, respectively (Fig. 2). At all storage temperatures, the moisture and a_w of the almonds increased during the first 2 months by 0.4 to 0.6% and 0.11 to 0.17, respectively. There was little subsequent change from month 2 (day 49) through month 7 (day 203). After 7 months, the moisture and a_w of the almonds stored at 4°C increased slightly (to 5.8% moisture and 0.55 a_w, respectively); at −19°C, values remained steady (at 4.9% moisture and 0.45 a_w, respectively); and at 24°C, values declined slightly (to 4.2% moisture and 0.33 a_w, respectively). For pistachios, the moisture and a_w values at all three storage temperatures remained very similar until about month 7, at which time the values began to diverge (Fig. 2). From months 7 through 12, the values for the pistachios followed the same general trend as for the almonds but with less fluctuation: average moisture contents at the storage temperatures 24, 4, and −19°C were 4.2, 5.9, and 5.1%, respectively, and average a_w values were 0.34, 0.55, and 0.46, respectively.

Background microbiota. Background populations were determined on TSA, BSA, SMAC, and MOX without antibiotic as well as on corresponding media supplemented with nalidixic acid (data not shown). Large spreading colonies observed on TSA were inhibited by the addition of nalidixic acid. The background microbiota were completely inhibited on SMACN but not on TSAN or BSAN. However, in every case, the colonies that grew on TSAN were molds and the colonies that grew on BSAN were not typical of *Salmonella*. The addition of CYC to all media completely eliminated background molds that otherwise interfered with colony counting of inoculated strains.

Pistachio inoculum recovery. Previous studies from this laboratory have validated the methods used to prepare *Salmonella*-inoculated almond kernels *(17, 37),* and other researchers have adapted these methods for use in pecans *(4, 5)* and walnuts *(6).* However, there are no publications describing methods to inoculate pistachios. The method used for almonds was adapted for inshell nuts because the majority of pistachios are stored and consumed in-the-shell. Whole inshell pistachios and pistachio kernels were inoculated with *E. coli* K-12 and sampled on day 0 (wet) and 1 and 3 days postinoculation. *E. coli* K-12 was used for safety reasons because this experiment required extensive handling of the inoculated pistachios. The shell was aseptically removed from some of the inshell pistachios, and the shells and kernels were processed separately. No significant difference ($P > 0.05$) in the populations of *E. coli* K-12 was seen among the four samples after 3 days of drying at ambient conditions, although greater variability was observed on the separated kernels and shells. Therefore, for further studies, inshell pistachios were used.

Inoculum level. Cocktails of *Salmonella, E. coli* O157:H7, and *L. monocytogenes* were inoculated at two

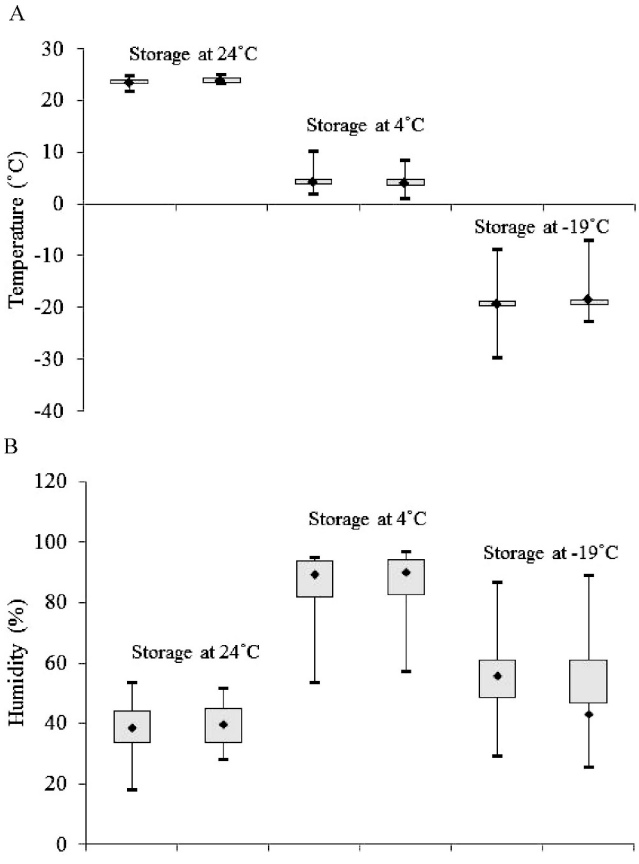

FIGURE 1. *(A) Temperature (°C) and (B) humidity (%) recorded by data loggers in each storage condition for trials 1 and 2 over a period of 1 year. Maximum and minimum are denoted by whiskers, 75th and 25th percentile are denoted by box, and median is denoted by diamond.*

or three different initial levels onto almonds and pistachios and held for 1 month of storage at 24°C. The greatest population declines for *Salmonella* occurred after inoculation during the 3-day drying period. At inoculum levels of 10, 6, and 4 log CFU/g, the populations of *Salmonella* decreased by 0.8, 1.6, and 1.3 log CFU/g on almonds, respectively, and by 0.6, 1.1, and 1.0 log CFU/g on pistachios, respectively. After the 3-day drying period, there was no significant difference ($P > 0.05$) in the declines for all three inoculum levels. Counts on both TSAN and BSAN after 30 days were not significantly different ($P > 0.05$).

At inoculum levels of 6 and 4 log CFU/g, the *E. coli* O157:H7 populations enumerated on TSAN decreased by 2.5 and 2.4 log CFU/g on almonds, respectively, and by 1.2 log CFU/g on pistachios (for both inoculum levels). Counts on TSAN and SMACN were not significantly different ($P > 0.05$). For *L. monocytogenes* inoculated at 6 and 4 log CFU/g, reductions were 1.3 and 1.6 log CFU/g on almonds, respectively, and 1.3 log CFU/g on pistachios (for both inoculum levels). Reductions observed on MOX were not significantly different for both inoculum levels when compared with the respective reductions on TSAN. Because reductions during and after drying at the initial inoculum levels of 6 and 4 log CFU/g were similar within organism and nut type, an inoculation level of 6 log CFU/g was selected for further study.

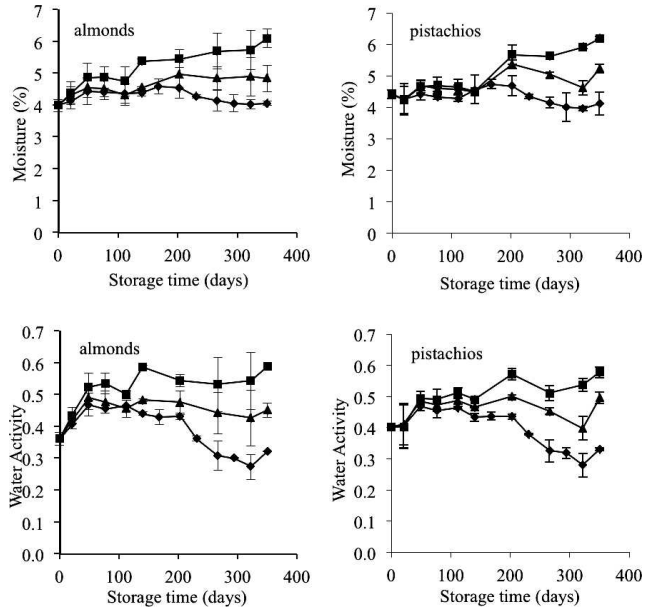

FIGURE 2. *Moisture (%) and a_w of almonds and pistachios during 12 months of storage at 24°C (diamond), 4°C (square), and −19°C (triangle). Error bars indicate standard deviation* (n = 6).

Survival of foodborne pathogens during drying. Almonds and pistachios were inoculated at a target of 6 log CFU/g, dried for 3 days, and then held at ambient temperature for 4 days before storage at −19, 4, or 24°C. Reductions on almonds inoculated with *Salmonella, E. coli* O157:H7, and *L. monocytogenes* were 1.7, 2.1, and 1.6 log CFU/g, respectively, after the 3-day drying period. Reductions on inoculated pistachios were not significantly different ($P > 0.05$) among the three pathogens; reductions of 1.2, 1.4, and 1.4 log CFU/g after 3 days were observed for *Salmonella, E. coli* O157:H7, and *L. monocytogenes,* respectively.

Reduction of pathogens during storage. *Salmonella* counts on TSAN+CYC and BSAN+CYC were not significantly different at any temperature or time on either almonds or pistachios (Fig. 3). Counts did not decline when the nuts were stored at either −19 or 4°C. At 24°C, in both cases, slow declines were observed, but levels of *Salmonella* were above the limit of detection at the end of the study. In contrast, for all storage conditions, *E. coli* O157:H7 counts on the selective (SMACN+CYC) agar were at least 0.4 log CFU/g lower than on the nonselective (TSAN+CYC) agar (Fig. 4). After 3 months (77 days) of storage at 24°C, *E. coli* counts on almonds were consistently below the limit of detection on SMACN+CYC (Fig. 4) but not on TSAN replica plated onto SMACN. *E. coli* counts determined on TSAN+CYC were below the limit of detection at month 7 (203 days) for almonds but remained above the limit of detection for pistachios throughout 12 months of storage. For the *E. coli*–inoculated almonds and pistachios stored at −19 and 4°C, there was no significant difference between colony counts on the two agars.

Over 12 months of storage, for almonds and pistachios stored at −19 and 4°C, the *L. monocytogenes* counts on

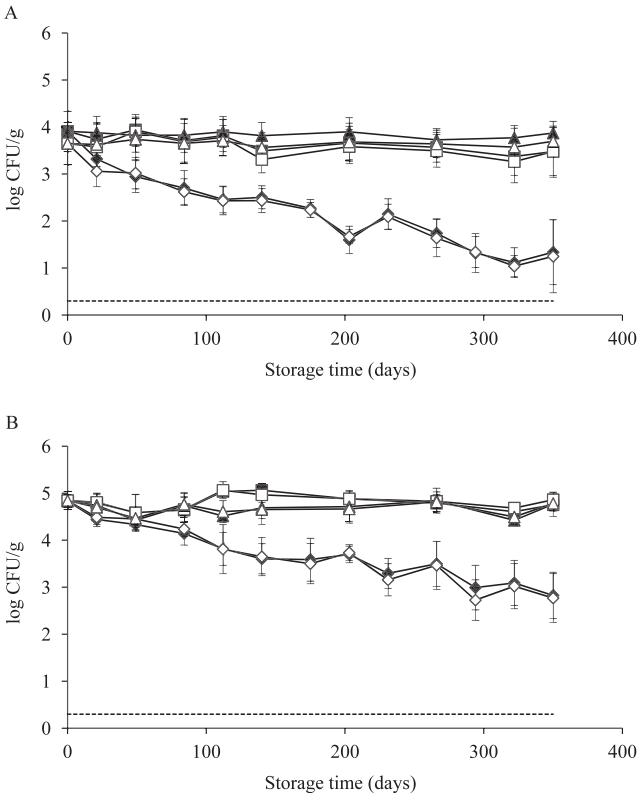

FIGURE 3. *Survival of* Salmonella *on (A) inoculated almond kernels and (B) inshell pistachios stored at 24°C (diamond), 4°C (square), and −19°C (triangle). Counts were determined on TSAN+CYC (closed symbol) and BSAN+CYC (open symbol). Values are the average of triplicate samples from each of two experiments* (n = 6)*, with standard deviation shown. Limit of detection was 0.3 log CFU/g (dashed line).*

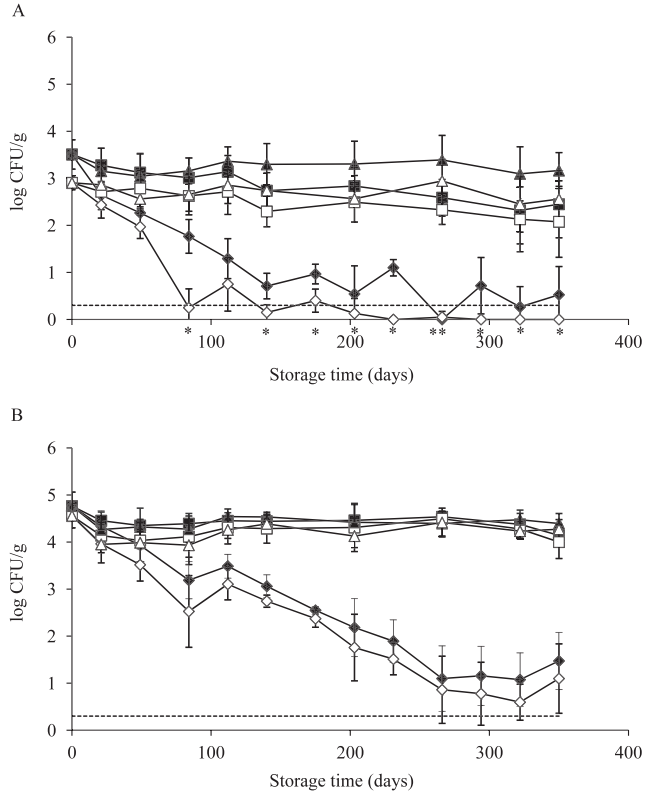

FIGURE 4. *Survival of* E. coli *O157:H7 on (A) inoculated almond kernels and (B) inshell pistachios stored at 24°C (diamond), 4°C (square), and −19°C (triangle). Counts were determined on TSAN+CYC (closed symbol) and SMACN+CYC (open symbol). Values are the average of triplicate samples from each of two experiments* (n = 6)*, with standard deviation shown. Limit of detection was 0.3 log CFU/g (dashed line); asterisk (*) indicates all six replicates were positive via enrichment of a 10-g sample.*

TSAN+CYC and MOX+CYC were not significantly different ($P > 0.05$) (Fig. 5). For *L. monocytogenes*–inoculated nuts held at 24°C, the MOX+CYC counts were 0.2 to 0.6 log CFU/g lower than on TSAN+CYC for almonds and up to 0.3 log CFU/g lower on MOX+CYC in some instances for pistachios.

For each of the survival curves generated over 12 months of storage (Figs. 3 to 5), best-fit models were determined by comparing R^2 values. Additionally, ANOVA tests were performed for each temperature to determine whether time had a significant influence on pathogen reduction. With the exception of *E. coli* O157:H7 on almonds stored at 4°C, time did not have a significant influence ($P > 0.05$) on the survival of any pathogen for either almonds or pistachios stored at −19 or 4°C (Table 2). *E. coli* O157:H7 levels on almonds declined slightly but significantly by 0.09 log CFU/g/month over 12 months of storage ($P < 0.0001$). The counts for the six samples evaluated on day 0 ranged from 3.3 to 4.0 log CFU/g (average 3.5 ± 0.31 log CFU/g) and on day 350 from 1.6 to 3.2 log CFU/g (average 2.4 ± 0.71 log CFU/g).

All three pathogens declined over time on both pistachios and almonds stored at 24°C. The linear rate of decline for *Salmonella* was 0.20 and 0.15 log CFU/g/month for almonds and pistachios, respectively (Table 2). *E. coli* O157:H7 and *L. monocytogenes* declined more rapidly than *Salmonella* on both pistachios and almonds stored at 24°C. For *E. coli* O157:H7 on almonds and *L. monocytogenes* on both nut types, faster reductions were observed over the first 100 days of storage, followed by distinct tailing. The Baranyi model *(2)* provided the best fit for these curves.

The resulting decline rates depicted in Table 2 represent the initial slope. The models include data up to the point at which one or more of the six samples fell below the limit of detection (<0.3 log CFU/g). For *E. coli* O157:H7–inoculated almonds and pistachios stored at ambient temperature, rates of decline of 0.60 and 0.35 log CFU/g/month, respectively, were calculated over 6 and 8 months, respectively. Decline rates of 0.71 and 0.86 log CFU/g/month were observed for *L. monocytogenes* on almonds and pistachios, respectively, over 7 months of storage. In all cases when counts were below the limit of detection, the 10-g samples ($n = 6$ per storage time) remained positive via enrichment through the end of the study.

Distribution of *Salmonella* strains in stored samples. At the end of trial 2, samples held at −19 and 4°C were plated onto TSAN after 13.5 and 14.5 months of storage for almonds and pistachios, respectively. A total of 100 colonies were selected from each storage condition, and

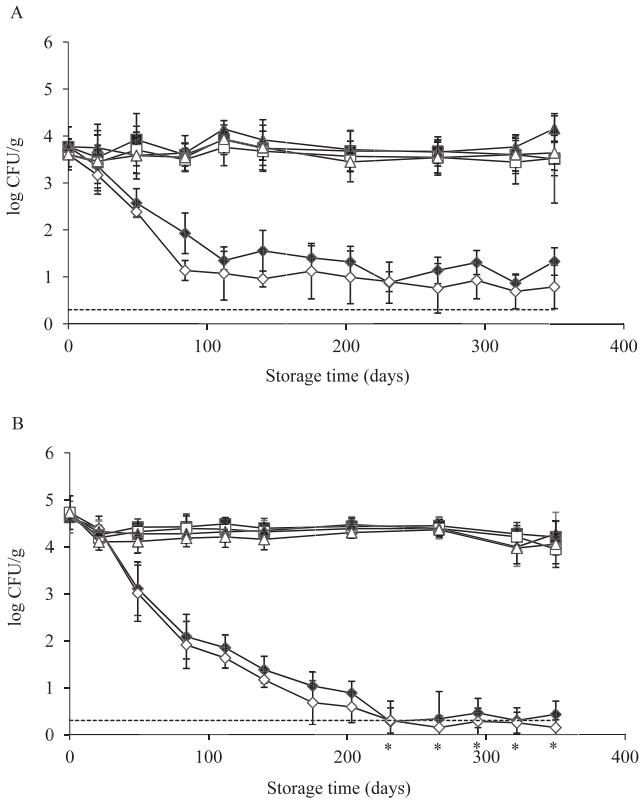

FIGURE 5. *Survival of* L. monocytogenes *on (A) inoculated almond kernels and (B) inshell pistachios stored at 24°C (diamond), 4°C (square), and −19°C (triangle). Counts were determined on TSAN+CYC (closed symbol) and MOX+CYC (open symbol). Values are the average of triplicate samples from each of two experiments (*n = 6*), with standard deviation shown. Limit of detection was 0.3 log CFU/g (dashed line); asterisk (*) indicates all six replicates were positive via enrichment of a 10-g sample.*

serotypes were determined (Table 3). No *Salmonella* Oranienburg was identified from any of the 400 characterized colonies. For pistachio samples, the relative proportions of each of the other five serotypes were similar in samples stored at −19 and 24°C. For almonds, proportions of *Salmonella* Anatum, *Salmonella* Enteritidis PT 9c, and *Salmonella* Enteritidis PT 30 were similar at both storage temperatures. In contrast, *Salmonella* Tennessee made up 0 and 15% of isolates and *Salmonella* Montevideo made up 28 and 7% of isolates from samples stored at −19 and 24°C, respectively. Comparable data from almonds and pistachios at the beginning of trial 2 were not available; however, before combining equal portions of the inoculum, the concentration of individual inoculum preparations was similar (7.8 log CFU/ml enumerated on BSA) except for levels of *Salmonella* Oranienburg, which were 0.4 log CFU/ml lower (Table 3).

DISCUSSION

A low prevalence (approximately 1%) of *Salmonella* has been documented in raw almonds and pistachios *(1, 16, 22, 28)*. Although not precisely known, it is assumed that foodborne pathogens may contaminate nuts by both wet and dry contamination routes. Almonds undergo drying both before and after shaking from the tree to the ground. However, almonds on the orchard floor can be exposed to irrigation water or rainfall or, after harvesting, by condensation that occurs on tarps used to protect the nuts during prehulling storage. In contrast, pistachios are harvested at high moisture levels and are exposed to high humidity as the nuts respire in harvest trucks. Pistachio hulls are removed in a wet process, which is followed by submersion of the inshell nuts (with naturally open shells) in a float tank prior to forced-air drying of the inshell nut. Dusts generated

TABLE 2. *Calculated rates of decline for pathogens on inoculated almond kernels or pistachio inshells during storage*

Pathogen cocktail	Nut	Storage temp (°C)	Storage time (mo)	ANOVA *P* value	Model method[a]	Rate of change (log CFU/g/day)	Rate of change (log CFU/g/mo)	R^2
Salmonella	Almonds	24	12	<0.0001	Linear	−0.0068	−0.20	0.78
		4	12	0.0447	ND[b]			
		−19	12	0.486	ND			
	Pistachios	24	12	<0.0001	Linear[c]	−0.0049	−0.15	0.59
		4	12	0.224	ND			
		−19	12	0.428	ND			
E. coli O157:H7	Almonds	24	6	<0.0001	Baranyi	−0.020	−0.60	0.88
		4	12	<0.0001	Linear	−0.0029	−0.09	0.37
		−19	12	0.520	ND			
	Pistachios	24	8	<0.0001	Linear	−0.012	−0.35	0.84
		4	12	0.653	ND			
		−19	12	0.553	ND			
L. monocytogenes	Almonds	24	7	<0.0001	Baranyi	−0.024	−0.71	0.85
		4	12	0.856	ND			
		−19	12	0.986	ND			
	Pistachios	24	7	<0.0001	Baranyi	−0.029	−0.86	0.92
		4	12	0.894	ND			
		−19	12	0.870	ND			

[a] Model (DMfit *(2)*, Gompertz, or linear regression) was chosen based on R^2 value and shape.
[b] ND, not done because time was not a significant influence on populations ($P > 0.05$).
[c] Baranyi and Gompertz models had R^2 value of 0.67 and 0.68, respectively, but were disregarded due to unreasonable shape.

TABLE 3. *Distribution of* Salmonella *serovars among 100 colonies isolated from almond and pistachio samples after storage for 13.5 or 14.5 months, respectively, at −19 or 24°C*

	Original inocula (log CFU/ml)		Almond kernels (no. of isolates)		Pistachio kernels (no. of isolates)	
Salmonella serovar	TSA	BSA	−19°C	24°C	−19°C	24°C
Anatum	7.7 ± 0.1	7.8 ± 0.1	7	3	13	16
Enteritidis PT 9c	7.8 ± 0.1	7.8 ± 0.1	33	53	29	33
Enteritidis PT 30	7.2 ± 0.1	7.8 ± 0.1	32	22	11	12
Montevideo	7.7 ± 0.0	7.8 ± 0.1	28	7	22	20
Oranienburg	7.4 ± 0.1	7.4 ± 0.1	0	0	0	0
Tennessee	7.8 ± 0.1	7.8 ± 0.1	0	15	25	19
Total	NA[a]	NA	100	100	100	100

[a] NA, not applicable.

during hulling and shelling of almonds may contribute to cross contamination of both equipment and product *(19, 20, 32)*. Dry almonds and pistachios may be stored under conditions that could lead to dry contamination via dust, and dusts may contaminate processing equipment in the absence of well-controlled preventative programs.

Although both wet and dry contamination routes are feasible, a wet inoculum procedure previously developed for almonds *(37)* was used in the current study to apply four- to six-strain cocktails of *Salmonella, E. coli* O157:H7, and *L. monocytogenes*. Each of the six *Salmonella* serovars selected had been associated with nut outbreaks, recalls, or surveys. Similar *Salmonella* cocktails also have been used to study survival in pecans and peanut butter *(3, 4, 8)*. Previous storage studies with almonds used a single strain, *Salmonella* Enteritidis PT 30, associated with a 2000 to 2001 outbreak *(24, 28, 37)*. The *E. coli* O157:H7 and *L. monocytogenes* strains used in this study were isolated from patients involved in outbreaks associated with moist foods because, at the time when the study began, no nut isolates of *E. coli* O157:H7 or *L. monocytogenes* were available. In 2011, single outbreaks of *E. coli* O157:H7 were epidemiologically linked to hazelnuts and walnuts *(9, 14),* and in 2009, walnuts were recalled after isolation of *L. monocytogenes (30)*.

The levels of foodborne pathogens that occur naturally on raw tree nuts are largely unknown. Levels of *Salmonella* on positive almond and pistachio survey samples were estimated to most often be 1 to 3 most probable number (MPN) per 100 g; levels as high as 8 or 15 MPN/100 g were detected in 1 of 11 pistachio samples or 1 of 99 almond samples, respectively *(1, 16, 22)*. In contrast, populations on raw almonds associated with a 2001 outbreak were estimated to be in the range of 50 to 500 MPN/100 g *(16, 28)*. Although levels in outbreak-associated almonds were significantly higher than the survey data, they were still near the limit of detection for the plating methods used here (200 CFU/100 g). Significantly higher levels are needed to achieve accurate counts on plating media and to demonstrate population reductions over time. For this reason, a target inoculation level of 6 log CFU/g was chosen for this study. No difference was observed in the rate of decline of *Salmonella* Enteritidis PT 30 inoculated at different levels on almonds (approximately 8, 5, 3, and 1 log CFU per almond) stored over 6 months at 23 ± 3°C *(37)* or over 1 month at 24°C in the current study. In some cases, greater declines were observed with lower inoculum levels on pecan halves and pieces *(4)*.

The calculated decline rates determined here are within the typical shelf life of the product (12 months at ambient temperature) and under typical storage conditions (ambient, refrigerator, or freezer). Time did not significantly influence populations of *Salmonella, E. coli* O157:H7, or *L. monocytogenes* during frozen or refrigerated storage, except in the case of refrigerated *E. coli* O157:H7–inoculated almonds, in which very slow but measurable declines were determined. Similar data were documented for almonds inoculated with *Salmonella* PT 30: no reductions were observed during storage of the almonds at 4°C in either the 6-month or 18-month studies *(37)*. For *Salmonella* cocktail–inoculated pecans, microbial levels decreased by small but not always significant amounts when stored at −20 or 4°C over a period of 1 year *(4)*.

The rate of decline of *Salmonella* on almonds stored at room temperature (0.20 log CFU/g/month) was within the range of decline rates obtained from seven previous studies that evaluated *Salmonella* Enteritidis PT 30 (0.16 to 0.32 log CFU/g/month) *(28)*. The results of the current study validate the use of survival data generated for *Salmonella* Enteritidis PT 30 as broadly representative of *Salmonella* on almonds. The current and previous survival studies for *Salmonella* on nuts have either used single isolates or cocktails of strains; none have included side-by-side comparisons of the survival of individual strains. In this study, survival among *Salmonella* strains within a cocktail was compared during storage of almonds and pistachios. Because populations of *Salmonella* did not decline in samples stored at −19°C, it was assumed that the distribution of serotypes in these samples reflected the distribution that would have been observed at the initial point of storage. *Salmonella* Oranienburg was not identified in any sample, which may have been due to lower levels in the initial inoculum or greater declines of this strain during postinoculation drying. All other strains were isolated from both almonds and pistachios after 1 year of storage at room temperature, despite overall greater than 2-log CFU/g reductions in populations of *Salmonella* during this time. Each of the strains selected was originally associated with

nuts, and it is possible that survival of *Salmonella* strains from other isolation sources would have differed.

Rates of decline were slower on pistachios than on almonds for all three pathogens, although a range of rates of decline should be expected when multiple studies are compared *(28)*. On both almonds and pistachios, *Salmonella* declined more slowly during storage at ambient temperature than either *E. coli* O157:H7 or *L. monocytogenes*. Distinct tailing was observed for survival curves of *E. coli* O157:H7 and *L. monocytogenes* on both nuts. Similar tailing was previously reported for *Salmonella* on almonds and pecans stored at 35 or 37°C *(4, 37)* and on walnuts and, in some cases, pecans stored at ambient temperature *(4, 6)*. The practical significance of biphasic survival curves in tree nuts is unknown.

E. coli O157:H7 and *L. monocytogenes* have been implicated in significantly fewer recalls and foodborne outbreaks in low-moisture foods than has *Salmonella*. Aside from the current study, a study on peanut butter *(26)*, and recent work in this laboratory *(6)*, direct comparisons of the survival of these pathogens on low-moisture foods is limited, but the survival data and recent outbreaks support their inclusion in hazard assessments for these products.

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