



## Short communication

## A dry-inoculation method for nut kernels

Tyann Blessington<sup>a,b</sup>, Christopher G. Theofel<sup>a</sup>, Linda J. Harris<sup>a,b,\*</sup><sup>a</sup> Department of Food Science and Technology, University of California, Davis, One Shields Avenue, Davis, CA 95616-8598, USA<sup>b</sup> Western Center for Food Safety, 1477 Drew Avenue, Suite 101, Davis, CA 95618, USA

## ARTICLE INFO

## Article history:

Received 28 January 2012

Received in revised form

11 September 2012

Accepted 18 September 2012

Available online 4 October 2012

## Keywords:

Almond

Walnut

Inoculation

Dry

*Salmonella*

Kernel

## ABSTRACT

A dry-inoculation method for almonds and walnuts was developed to eliminate the need for the post-inoculation drying required for wet-inoculation methods. The survival of *Salmonella enterica* Enteritidis PT 30 on wet- and dry-inoculated almond and walnut kernels stored under ambient conditions (average: 23 °C; 41 or 47% RH) was then compared over 14 weeks. For wet inoculation, an aqueous *Salmonella* preparation was added directly to almond or walnut kernels, which were then dried under ambient conditions (3 or 7 days, respectively) to initial nut moisture levels. For the dry inoculation, liquid inoculum was mixed with sterilized sand and dried for 24 h at 40 °C. The dried inoculated sand was mixed with kernels, and the sand was removed by shaking the mixture in a sterile sieve. Mixing procedures to optimize the bacterial transfer from sand to kernel were evaluated; in general, similar levels were achieved on walnuts (4.8–5.2 log CFU/g) and almonds (4.2–5.1 log CFU/g). The decline of *Salmonella* Enteritidis populations was similar during ambient storage (98 days) for both wet- and dry-inoculation methods for both almonds and walnuts. The dry-inoculation method mimics some of the suspected routes of contamination for tree nuts and may be appropriate for some postharvest challenge studies.

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## 1. Introduction

Liquid carriers are commonly used in laboratory studies to inoculate low-moisture foods (water activity less than 0.70) (Beuchat and Mann, 2010a, 2010b, 2011a, 2011b; Blessington et al., 2012; Danyluk et al., 2005; Komitopoulou and Peñaloza, 2009; Uesugi et al., 2006). Addition of a liquid inoculum changes the moisture content and water activity of a dry substrate and typically requires a subsequent, sometimes lengthy drying step. Moisture adsorption/desorption isotherms are inherently non-equivalent; thus, the moisture and water activity of the dried, inoculated product may not be the same as the original food (Palipane and Driscoll, 1993). Some dry ingredients, such as herbs, spices or powders, may not return to their original form after wetting and, in some cases, the inoculum may be exposed to water-soluble antimicrobial compounds (Shelef, 1984).

As an alternative, some options for preparing a dry inoculum include: freeze or vacuum drying the bacterial culture (Flowers et al., 1987; Riemann, 1968); or wet inoculation of dry inert carriers (e.g., chalk, talc, or sand) followed by air drying (Beuchat

and Mann, 2010b, 2011b; De Roin et al., 2003; Hoffmans and Fung, 1993; Kattopalli et al., 2012). *Listeria monocytogenes* was successfully inoculated onto ready-to-eat meats by direct application of a dry sand inoculum (De Roin et al., 2003), and chalk was used as a carrier to inoculate pecans (Beuchat and Mann, 2011b) and dry poultry feed (Hoffmans and Fung, 1993).

The vehicle(s) for contamination of almonds and walnuts with foodborne pathogens is currently unknown; however, routes that involve both wet and dry carriers are feasible during production and processing. Aqueous contamination can occur after almonds or walnuts are shaken to the ground during harvest or during the wet-hulling of walnuts (Blessington et al., 2008; Pan et al., 2012; Uesugi et al., 2006). Dry contamination is also feasible in orchards or processing facilities; *Salmonella* has been isolated from almond orchards and hulling and processing facilities (Isaacs et al., 2005; Uesugi et al., 2007). Dusts generated in the handling of tree nuts from harvest through processing serve as a potential source of contamination (Du et al., 2010; FDA, 2009; Pan et al., 2012).

Wet-inoculation methods have been commonly used for laboratory contamination of tree nuts (Beuchat and Heaton, 1975; Beuchat and Mann, 2010a,b; Blessington et al., 2012; Kimber et al., 2012; Komitopoulou and Peñaloza, 2009; Uesugi et al., 2006). After wet inoculation, the water activity of almonds returned to pre-inoculation levels in 1–3 days at low ambient relative humidity (RH) (Kimber et al., 2012; Uesugi et al., 2006). When this method

\* Corresponding author. Department of Food Science and Technology, University of California, Davis, One Shields Avenue, Davis, CA 95616-8598, USA. Tel.: +1 530 754 9485; fax: +1 530 752 4759.

E-mail address: [ljharris@ucdavis.edu](mailto:ljharris@ucdavis.edu) (L.J. Harris).

was applied to walnut kernels a higher proportion of inoculum was needed to uniformly coat the kernels, contributing to a significantly greater drying time (7 days) (Blessington et al., 2012).

Wet-inoculation methods have been used successfully for nuts, but some inherent problems are associated with this approach. Ambient temperatures and RH during drying may impact post-inoculation drying times. Application of the inoculum slurry may alter the properties of the kernel surface. It is unknown if application of wet inoculum modifies the kernel surface in a manner that would influence the viability of the inoculated bacteria during subsequent storage and processing. The objectives of this study were to develop an inoculation method to mimic dry contamination of almonds and walnuts, and to compare the survival of *Salmonella enterica* Enteritidis PT 30 on dry- and wet-inoculated almond and walnut kernels.

## 2. Materials and methods

### 2.1. Almond and walnut samples

Almond kernels (Nonpareil variety) from a commercial processor in California and walnut kernels (Chandler variety) from a California grower were used in this study. Kernels were stored at ambient conditions ( $23 \pm 0.4$  °C;  $41\% \pm 6.7\%$  RH for most of the experiments and  $23 \pm 0.4$  °C;  $47\% \pm 6.4\%$  RH for Trial 2) for less than 6 months before use.

### 2.2. Cultures and growth conditions

*Salmonella* Enteritidis PT 30 (ATCC BAA-1045), isolated from raw almonds linked to an outbreak, was used to study bacterial survival. In preliminary studies an attenuated *Salmonella* Typhimurium (Hassan and Curtiss 3rd, 1994) was also used. Mutants of *Salmonella* that were able to grow in medium supplemented with rifampicin at 50 µg/ml were isolated. No differences in survival of the *Salmonella* Enteritidis strain were observed during drying of the antibiotic-resistant mutant and parent strain (data not shown). *Escherichia coli* K12 was used as a pathogen substitute on kernel and sand samples monitored for moisture content and water activity before, during, and after inoculation. All isolates were stored at  $-80$  °C in tryptic soy broth (TSB) supplemented with 15% glycerol (Fisher Scientific, Fair Lawn, NJ). Culture media were obtained from BD (Franklin Lakes, NJ).

### 2.3. Inoculum preparation

*Salmonella* and *E. coli* inocula were prepared as described by Uesugi et al. (2006). The frozen stock culture was streaked onto tryptic soy agar (TSA: tryptic soy broth plus 1.5% granulated agar) and incubated at  $37 \pm 2$  °C for  $24 \pm 3$  h. A single colony was transferred into TSB; after overnight incubation at  $37 \pm 2$  °C, a 10-µl loop of the culture was transferred into TSB and incubated at  $37 \pm 2$  °C. The second overnight culture (1 ml) was spread over large TSA plates (150 × 15 mm) and incubated at  $37 \pm 2$  °C for  $24 \pm 3$  h. The resulting bacterial lawn was collected by adding 9 ml of 0.1% peptone to each plate, loosening the lawn with a sterile spreader (Lazy-L Spreader, Andwin Scientific, Tryon, NC), and pipetting the suspension into a sterile container. The culture slurry was diluted in 0.1% peptone and mixed by vortexing for 30 s. Bacterial populations in the individual and final diluted inocula were determined by serial dilution in Butterfield's phosphate buffer (BPB) and plating onto TSA and bismuth sulfite agar (BSA) supplemented with rifampicin (50 µg/ml). Cultures on TSA and BSA were incubated at  $37 \pm 2$  °C for  $24 \pm 3$  h and  $48 \pm 3$  h, respectively. After incubation, plates were enumerated as described below.

### 2.4. Wet-inoculation procedure for almonds and walnuts

Almonds and walnuts were wet inoculated using methods described by Uesugi et al. (2006) and Blessington et al. (2012). The inoculum was combined with kernels at a liquid-to-nut ratio of 25 ml per 400 g of almonds or 33 ml per 200 g of walnuts. Inoculated almond or walnut kernels were spread over filter paper within a partially-closed container to dry at ambient conditions for 3 or 7 days, respectively. Inoculum levels were determined on kernels immediately after inoculation and after drying, as described below.

### 2.5. Preparation of dry inoculum

Prepared aqueous inoculum was mixed with sand (fine white silicon dioxide, Fisher Scientific) or chalk (calcium carbonate powder, Fisher) at ratios of 17.5–31.2 ml per 100 g in a new zippered polyethylene bag (30.5 × 30.5 cm) (Bitran, Com-Pac International, Inc., Carbondale, IL). Each bag was sealed and the inoculated sand or chalk was massaged by hand for 1 min. The inoculated mixtures were held in open bags, or were transferred onto filter paper sheets placed on a metal drying rack (Uesugi et al., 2006) or into standard size white basket-shaped paper coffee filters (Melitta USA, Clearwater, FL) placed within 500- or 1900-ml jars (Nalgene, Rochester, NY) or directly onto metal baking pans (46 × 66 cm). Mixtures were dried at ambient conditions or in a laboratory incubator set to 40 °C. After drying, inoculated carriers were transferred to a zippered polyethylene bag and mixed by hand or pulverized with a mortar and pestle from the outside of the bag to break up any clumps.

The dried inoculated carriers were stored at 4 °C in a closed plastic container for up to 6 days before inoculation of kernels. Levels of *Salmonella* were determined on the carriers before storage and also before inoculation onto kernels, as described below.

### 2.6. Dry inoculation of almonds or walnuts with sand or chalk carriers

Dried inoculated sand (5, 25, or 50 g) or chalk (14 g) was added to 200 g of kernels in a zippered polyethylene bag (30.5 × 30.5 cm), and the sealed bag was manually mixed for 2 min. Mixing consisted of either shaking (in a 10-cm arc), rubbing (vigorously between two hands), rubbing and shaking, or rolling (similar to using a rolling pin). The majority of the visible sand was then separated from the nuts by shaking in a sterile U.S. standard #12 testing sieve (1.7-mm openings; Fisher Scientific) for 1 min. The same sieving technique was used for chalk-inoculated nuts, but considerable chalk remained on the kernels. For some experiments the weight of the nuts was determined before addition of sand or chalk and after sieving. Inoculated kernels were pooled in zippered polyethylene bags (30.5 × 30.5 cm), manually mixed, and stored in a closed bin at ambient conditions for up to 14 weeks. Inoculum levels were determined on the sand immediately before and on the kernels immediately after inoculation; bacterial levels were recovered and enumerated as stated below.

### 2.7. Enumeration

*Salmonella*-inoculated kernels (5 g) were added to 10 ml of 0.1% peptone in a sterile 532-ml (18-oz) Whirl-Pak bag (Nasco, Modesto, CA) and mixed by stomaching (Stomacher 400 Circulator, Seward Laboratory Systems Inc., Bohemia, NY) for 4 min at 230 rpm. Inoculated carrier (2–5 g) was added to 10 ml of 0.1% peptone in a sterile 50-ml tube and mixed by vortexing for 30 s (Blessington et al., 2012; Uesugi et al., 2006). Subsequent serial dilutions of

the liquid portion of each slurry or carrier solutions were made in BPB. Appropriate dilutions were plated onto TSA and bismuth sulfite agar (BSA) supplemented with rifampicin (50 µg/ml). TSA and BSA plates were incubated at  $37 \pm 2$  °C for  $24 \pm 3$  h and  $48 \pm 3$  h, respectively. Colonies were counted and bacterial populations were determined. Neither the kernels nor the carriers were liquefied during mixing. Therefore, the calculated CFU per millimeter of the plated solution multiplied by the ratio of the volume of BPB to weight of kernel or carrier was considered to be equivalent to the CFU per gram of kernel or carrier (Blessington et al., 2012; Uesugi et al., 2006).

### 2.8. Moisture content and water activity determination

The moisture content and water activity of the sand, chalk, and kernels (uninoculated, wet inoculated and dry inoculated with *E. coli* K12) were determined before and after the inoculation and drying steps. Kernels (~40 g) were ground in a 2.8-L food processor (Waring, Pro Food Processor, Torrington, CT) and then shaken through a U.S. standard #12 sieve. Sieved kernel or carrier samples (4 g) were placed in individual foil pans (0.6 × 10.2 cm), and the percent moisture was determined with a moisture analyzer (HG63 Halogen Moisture Analyzer, Mettler-Toledo, Columbus, OH). The water activity of the sieved samples was measured with a water activity meter (AquaLab model CX2, Decagon Devices, Pullman, WA).

### 2.9. Experiment design, calculations, and statistical analysis

To determine survival of *Salmonella* Enteritidis on inoculated dry sand, microbial levels were analyzed at the initiation (day 0) and 2, 6, 13, 30, 70, and 170 days of ambient and refrigerated (4 °C) storage. Microbial levels on kernels were analyzed at the beginning of storage either immediately after inoculation (dry inoculation) or after wet inoculation and drying for 3 days (almonds) or 7 days (walnuts). Six samples (unless otherwise stated) were used to estimate the population at each sampling time, and three replicates were used to estimate moisture content and water activity at each sampling time. Bacterial survival during ambient storage was directly compared between wet- and dry-inoculated almonds and, separately, between wet- and dry-inoculated walnuts at the initiation and 1, 3, 7, 14, 28, 42, 56, 70, 84, and 98 days of storage. Analyses of variance and *t*-tests were performed with the JMP 9 software package (SAS Institute, Cary, NC). Differences between the mean values were considered significant at  $P < 0.05$ . Rates of bacterial decline during storage were converted from log CFU per gram per day to log CFU per gram per month by multiplying by 30.4 (average days in a month). Bacterial levels on both BSA and TSA were similar; unless otherwise stated, bacterial levels reported are those from BSA.

## 3. Results and discussion

### 3.1. Identification and evaluation of a dry carrier

Standard inert substances such as chalk or sand (as purchased from a chemical supply company) have been used to dry inoculate a range of foods (Beuchat and Mann, 2010b, 2011b; De Roin et al., 2003; Hoffmans and Fung, 1993; Riemann, 1968). For this study we considered the use of a non-standardized carrier such as orchard soil or dust collected from a nut processing facility because both are potential routes for natural contamination of tree nuts (Pan et al., 2012). Our earlier research demonstrated the survival of *Salmonella* in both dry soil and dust, and growth in the wetted samples (Danyluk et al., 2008; Du et al., 2010). Neither orchard soil

nor nut processing dust is of standard composition or readily available. Therefore, sand and chalk were selected as potential carriers given their history of previous use (Beuchat and Mann, 2010b, 2011b; De Roin et al., 2003; Hoffmans and Fung, 1993).

Initial studies using non-pathogenic *E. coli* or attenuated *Salmonella* Typhimurium were conducted to determine appropriate inoculum-to-carrier ratios and to identify a simple and reproducible drying method. Sand was uniformly coated with inoculum at a liquid-to-carrier ratio of 35 ml per 200 g; a greater amount of liquid (62.5 ml per 200 g) was needed to completely coat the chalk. Transfer of the wet chalk from the zippered bag to the container for drying was difficult because the chalk formed a sticky paste; the recovery volume of the dried, inoculated chalk was lower than for the dried, inoculated sand. Once dry, the inoculated chalk formed hard clumps that had to be pulverized with a mortar and pestle before further use. Preliminary results from inoculation trials indicated that the chalk was more difficult to remove from nut kernel surfaces than the sand (Table 1). We subsequently chose to use sand as the primary carrier in the remaining experiments.

### 3.2. Drying the inoculated carrier

Inoculated sand was dried under ambient conditions or in an incubator set to 40 °C. The inoculated sand was transferred onto filter paper placed in metal trays and also into coffee filters placed in jars or on metal pans. Transferring the inoculated sand into 500-ml jars lined with four paper coffee filters to dry was chosen as the preferred method because the jars were large enough to hold the inoculated carrier during and after drying, thereby eliminating the need to transfer the dry inoculum to another container for storage. The filters absorbed some of the moisture from the inoculum facilitating drying. After inoculation, the sand returned to initial moisture levels within 24 h when dried at 40 °C in an incubator, but not always when dried at ambient conditions (data not shown).

To optimize the method, sand was inoculated at a ratio of 35 ml per 200 g, and quantities of 50, 100, or 200 g were placed in the filter-lined jars and dried at 40 °C. All three quantities of sand dried to similar preinoculated moisture levels within 24 h. There was a small (0.04%) difference ( $P = 0.0083$ ) between the moisture content of the 50-g and 200-g samples after drying (0.11 and 0.15% moisture, respectively); the 200-g samples formed a hard upper layer. The 100-g quantity was chosen for further study.

The distribution and homogeneity of *Salmonella* in a 100-g quantity of dried inoculated sand was determined for 2-g samples ( $n = 3$ ) collected at the surface, in the middle, and at the bottom of a single jar of the sand before mixing and a composite sample was collected after the standard mixing procedure. No significant difference ( $P = 0.14$ ) in microbial counts was observed among the sand samples from the various sections in the container and the mixed sand samples.

For all further experiments, the dry inoculum was prepared by mixing 100 g of sand and 17.5 ml of aqueous inoculum, placing the

**Table 1**  
Weight of almonds and walnuts before and after inoculation.

Dry inoculum	Kernel type	Weight before inoculation (g)	Weight after sieving (g)	Difference in weight (g)
None	Almond	200.12 ± 0.14 a <sup>a</sup>	200.04 ± 0.16 a	-0.08
	Walnut	200.45 ± 0.31 a	199.58 ± 0.48 a	-0.38
Sand (25 g)	Almond	200.31 ± 0.32 a	200.13 ± 0.26 a	-0.18
	Walnut	200.54 ± 0.29 a	198.12 ± 0.48 b	-2.43
Chalk (14 g)	Almond	200.50 ± 0.29 a	207.71 ± 0.35 b	+7.21
	Walnut	200.58 ± 0.14 a	202.17 ± 0.86 b	+1.59

<sup>a</sup> Values are means ± standard deviation ( $n = 3$ ). Within rows, means with different letters are significantly different ( $P < 0.05$ ).

mixture in 500-ml jars lined with four paper coffee filters, and drying for 24 h in an incubator set at 40 °C. An average dry sand inoculum level of  $7.5 \pm 0.3$  log CFU/g with an inoculum range between 6.7 and 7.7 log CFU/g (Table 2).

### 3.3. Dry inoculation of tree nut kernels

Mixing almond and walnut kernels with the dried inoculated sand (and subsequent sand removal) did not influence moisture levels or water activity of the almonds or walnuts. The moisture ( $4.10 \pm 0.03\%$ ) or water activity ( $0.34 \pm 0.01$ ) of the almonds was the same before or after inoculation. Small but insignificant differences in moisture ( $3.09 \pm 0.09$  or  $3.05 \pm 0.36$ ;  $P = 0.34$ ) or water activity ( $0.48 \pm 0$  or  $0.47 \pm 0.02$ ;  $P = 0.85$ ) of the walnuts was observed before and after inoculation, respectively. One advantage of a dry inoculum is that the kernels can be used for some experiments (e.g., storage studies) immediately after inoculation. For other types of challenge studies, the inoculum may require a post-inoculation humidity equilibration period.

When the dry inoculum (7.5 log CFU/g) was mixed with almond or walnut kernels (at sand-to-nut ratios of 5, 25, and 50 g per 200 g) by rubbing and shaking for 2 min, the sand covered the kernels without causing visible damage to the kernel structure or appearance. At these ratios, similar bacterial populations (up to 0.6 log CFU/g difference between nuts) were inoculated onto walnuts (4.2, 4.8, and 4.8 log CFU/g, respectively) and almonds (3.8, 4.2, and 4.7 log CFU/g). Because there was no advantage to using the highest sand-to-nut ratio, for all further experiments, 25 g of inoculated sand was used to inoculate 200 g of kernels.

Various manual techniques were compared for mixing almond kernels and dry inoculum (8.2 log CFU/g), followed by sieving for 1 min. The individual and combined techniques of rubbing and shaking resulted in greater ( $P = 0.0033$ ) inoculation concentrations (5.0 log CFU/g) than the rolling technique (4.1 log CFU/g). Since a calculated maximum *Salmonella* concentration of 7.2 log CFU/g of kernels would be possible if every cell was successfully transferred from the sand to the kernels, significant amounts of *Salmonella* were lost with removal of the sand with sieving.

The dry-inoculation method used for all subsequent experiments consisted of combining the dried inoculated sand (25 g) with kernels (200 g), mixing by rubbing and shaking for 2 min, and separating the sand from the kernels by shaking in a sterile sieve for 1 min. Among four separate experiments, *Salmonella* levels ranged from 4.2 to 5.1 log CFU/g on almonds and from 4.7 to 5.2 log CFU/g on walnuts (Table 2). The weight of kernels before inoculation was not significantly different from the almond weight after sieving

( $P = 0.38$ ), but the weight of walnuts was significantly reduced ( $P = 0.0017$ ) by 1–2 g after sieving, most likely due to the loss of small amounts of walnut kernel (Table 1).

### 3.4. Survival of *Salmonella* on sand

Preparation of dry sand inoculum takes several days and it would advantageous to be able to prepare quantities that could be stored and used over weeks to months. For this reason, the stability of *Salmonella* populations on the sand during storage was evaluated. Sand was inoculated at an initial level of 10 log CFU/g and dried at 40 °C for 24 h. The survival of *Salmonella* on the dried sand (7.5 log CFU/g) was determined at ambient and refrigerated (4 °C) conditions. After 2 and 6 days of storage, populations of *Salmonella* on the inoculated sand declined by 0.13 ( $P = 0.024$ ) and 0.28 ( $P < 0.0001$ ) log CFU/g, respectively, at ambient conditions, and increased by 0.57 ( $P < 0.0001$ ) and 0.16 ( $P = 0.067$ ) log CFU/g, respectively, at 4 °C (Table 3). After 30 and 70 days, populations fell by 1.0 and 1.4 log CFU/g at ambient conditions, and by 0.4 log CFU/g at 4 °C. However, after 170 days of storage, populations of *Salmonella* had declined by 1.8 log CFU/g under both storage conditions (Table 3). In previous studies on tree nut kernels, no decline of *Salmonella* was observed at refrigerated or frozen storage (Beuchat and Mann, 2010a; Blessington et al., 2012; Kimber et al., 2012; Uesugi et al., 2006) and it is unclear why a decline was observed on the refrigerated sand.

### 3.5. Survival of *Salmonella* on inoculated nut kernels

To directly compare *Salmonella* survival on wet- and dry-inoculated nuts, the kernels and sand carrier were inoculated with the same aqueous inoculum preparation. Almond and walnut kernels were wet inoculated and then dried for 3 and 7 days, respectively, before ambient storage. Dry-inoculated nuts were stored immediately after inoculation.

The inoculum was diluted prior to inoculation of kernels to yield target *Salmonella* concentrations of 5 log CFU/g on the almond and walnut kernels after drying. *Salmonella* levels prior to drying were 5.3 and 6.0 log CFU/g for almonds and 6.6 and 7.2 log CFU/g for walnuts for Trial 1 and 2, respectively.

Sand was inoculated at a level of 10 log CFU/g achieving populations of 7.6–7.7 log CFU/g in trials. The inoculated sand was then stored at 4 °C for 2 or 6 days after the initial 24-h drying period, corresponding to the drying times for the wet-inoculated almonds and walnuts. *Salmonella* concentrations in the sand carrier were 7.6

**Table 2**

*Salmonella* levels in liquid inoculum, on sand before and after drying, and after transfer to almond and walnut kernels. Liquid culture (17.5 ml) was mixed with 100 g of sand and inoculated sand was dried in a 40 °C incubator for 24 h. Sand (25 g) and kernels (200 g) were mixed, the mixture was sieved and the sand discarded.

Nut type	Liquid culture (log CFU/ml)		Wet sand (log CFU/g)		Dry sand before 4 °C hold (log CFU/g)		Dry sand before being mixed with kernels (log CFU/g)		Kernel (log CFU/g)	
	TSA + Rif	BSA + Rif	TSA + Rif	BSA + Rif	TSA + Rif	BSA + Rif	TSA + Rif	BSA + Rif	TSA + Rif	BSA + Rif
Almond	$10.9 \pm 0.0$ a <sup>a</sup>	$10.8 \pm 0.1$ ab	$10.0 \pm 0.1$ ab	$10.1 \pm 0.1$ a	$7.5 \pm 0.2$ b <sup>b</sup>	$7.5 \pm 0.0$ a <sup>b</sup>	$7.5 \pm 0.2$ c	$7.5 \pm 0.0$ b	$4.5 \pm 0.1$ c	$4.2 \pm 0.1$ d
	$10.8 \pm 0.0$ ab	$10.8 \pm 0.1$ ab	$9.5 \pm 0.0$ c	$9.6 \pm 0.2$ c	$7.7 \pm 0.2$ ab <sup>c</sup>	$7.6 \pm 0.2$ a <sup>c</sup>	$8.2 \pm 0.0$ a	$8.2 \pm 0.0$ a	$5.1 \pm 0.2$ ab	$5.0 \pm 0.2$ ab
	$10.9 \pm 0.0$ a	$11.0 \pm 0.1$ a	$10.1 \pm 0.1$ a	$10.1 \pm 0.1$ a	$7.7 \pm 0.1$ ab <sup>c</sup>	$7.6 \pm 0.1$ a <sup>c</sup>	$7.9 \pm 0.2$ ab	$7.6 \pm 0.2$ b	$4.2 \pm 0.2$ c	$4.2 \pm 0.2$ d
	$10.9 \pm 0.1$ a	$11.0 \pm 0.0$ a	$10.0 \pm 0.1$ a	$10.0 \pm 0.1$ ab	$7.9 \pm 0.1$ a <sup>c</sup>	$7.6 \pm 0.2$ a <sup>c</sup>	$8.2 \pm 0.1$ a	$8.1 \pm 0.1$ a	$5.1 \pm 0.2$ a	$5.1 \pm 0.1$ a
Walnut	$10.9 \pm 0.0$ a	$10.8 \pm 0.1$ ab	$10.0 \pm 0.1$ ab	$10.1 \pm 0.1$ a	$7.5 \pm 0.2$ b <sup>b</sup>	$7.5 \pm 0.0$ a <sup>b</sup>	$7.5 \pm 0.2$ c	$7.5 \pm 0.0$ b	$4.8 \pm 0.2$ b	$4.8 \pm 0.1$ bc
	$10.7 \pm 0.0$ b	$10.8 \pm 0.0$ b	$9.8 \pm 0.2$ b	$9.9 \pm 0.1$ b	$7.1 \pm 0.1$ c <sup>b</sup>	$6.7 \pm 0.2$ b <sup>b</sup>	$7.1 \pm 0.1$ d	$6.7 \pm 0.2$ c	$4.8 \pm 0.2$ b	$4.7 \pm 0.2$ c
	$10.9 \pm 0.1$ a	$10.9 \pm 0.0$ ab	$10.1 \pm 0.1$ a	$10.1 \pm 0.1$ a	$7.9 \pm 0.2$ a <sup>c</sup>	$7.7 \pm 0.1$ a <sup>c</sup>	$7.7 \pm 0.2$ bc	$7.6 \pm 0.1$ b	$5.2 \pm 0.1$ a	$5.0 \pm 0.2$ ab
	$10.9 \pm 0.1$ a	$11.0 \pm 0.0$ a	$10.0 \pm 0.1$ a	$10.0 \pm 0.1$ ab	$7.9 \pm 0.1$ a <sup>c</sup>	$7.6 \pm 0.2$ a <sup>c</sup>	$8.1 \pm 0.0$ a	$8.0 \pm 0.1$ a	$5.2 \pm 0.1$ a	$5.2 \pm 0.2$ a

<sup>a</sup> Values are means  $\pm$  standard deviation ( $n = 3$  or 4 for liquid culture,  $n = 6$  for sand and kernels). Within columns, means with different letters are significantly different ( $P < 0.05$ ).

<sup>b</sup> Sand was immediately used to transfer bacteria to kernels and was not stored.

<sup>c</sup> Sand was stored at 4 °C for 2–6 days before being used to transfer bacteria to kernels.

**Table 3**  
Survival of *Salmonella* on inoculated dry sand stored at 4 °C or ambient conditions.

Storage day	4 °C storage		Ambient storage	
	TSA + Rif (log CFU/g)	BSA + Rif (log CFU/g)	TSA + Rif (log CFU/g)	BSA + Rif (log CFU/g)
0	7.5 ± 0.2 <sup>a</sup>	7.5 ± 0.0	7.5 ± 0.2	7.5 ± 0.0
2	8.2 ± 0.1	8.0 ± 0.1	7.5 ± 0.0	7.4 ± 0.1
6	8.2 ± 0.0	7.6 ± 0.2	7.5 ± 0.0	7.2 ± 0.1
13	7.5 ± 0.1	7.4 ± 0.1	7.2 ± 0.2	6.9 ± 0.2
30	7.2 ± 0.1	7.1 ± 0.0	7.0 ± 0.2	6.5 ± 0.1
70	7.1 ± 0.1	7.1 ± 0.4	6.4 ± 0.0	6.1 ± 0.1
170	6.6 ± 0.2	5.7 ± 0.7	6.3 ± 0.1	5.7 ± 0.3

<sup>a</sup> Values are means ± standard deviation (n = 6).

and 8.0 to 8.1 log CFU/g for Trial 1 and 2, respectively immediately before inoculation onto kernels.

At the initiation of ambient storage, *Salmonella* concentrations on wet- and dry-inoculated nuts were 3.9 and 4.2 log CFU/g (Trial 1) and 4.3 and 5.1 log CFU/g (Trial 2), respectively, on almonds and 4.8 and 5.0 log CFU/g (Trial 1) and 5.1 and 5.2 log CFU/g (Trial 2), respectively, on walnuts (Fig. 1). The decline of *Salmonella* on wet- and dry-inoculated almond walnut kernels were similar over 98 days of ambient storage (Fig. 1) although initial counts for the dry-inoculation almonds were higher for Trial 2.

Inoculation methods, where possible, should attempt to simulate real world conditions. Both wet and dry routes of contamination of almonds and walnuts are possible with the standard harvest and postharvest handling methods employed by these industries. In this study we developed an inoculation method intended to simulate dry contamination of almond and walnut kernels. This method prevents wetting of the kernels and eliminates the drying

period required for wet-inoculation methods. Maximum *Salmonella* levels achieved on the kernels using this method (<5 log CFU/g) were significantly lower than maximum levels achieved with wet inoculation (>8 log CFU/g) (Blessington et al., 2012; Uesugi et al., 2006) which may limit the applicability of this inoculation method. Natural contamination levels on tree nuts are thought to be very low. In almond surveys, the levels of *Salmonella* ranged from less than 0.70 log MPN/100 g (most cases) to 1.2 log MPN/100 g (Bansal et al., 2010; Danyluk et al., 2007); levels in pistachios are equally low (Harris, unpublished). In a 2001 outbreak of salmonellosis the levels of *Salmonella* were estimated to be as much as 2.1 log MPN/100 g of almonds (Lambertini et al., 2012). There is limited data on the prevalence of foodborne pathogens in walnuts but, when present, levels are expected to be no higher than those found on other nuts. Thus, lower inoculum levels achieved with dry inoculation may be very appropriate for some experiments.

Survival of *Salmonella* was similar for wet- and dry-inoculated almonds and walnuts during ambient storage and these data were comparable to previous wet-inoculation studies performed in this laboratory (Blessington et al., 2012; Kimber et al., 2012; Uesugi et al., 2006). For low-moisture foods a wet-inoculation procedure may be more appropriate if a liquid contaminant is suspected. The current study affirms the previous use of wet-inoculation procedures to evaluate storage survival of *Salmonella* on tree nuts; these data should be applicable to both wet and dry routes of contamination.

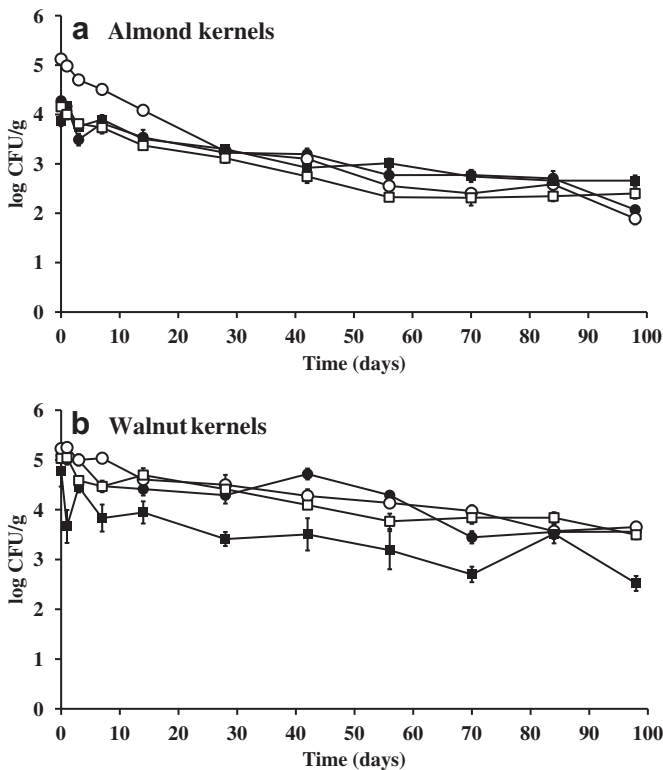
The dry-inoculation method presented here provides a viable alternative that may be useful for some challenge studies. The impact on the thermal tolerance of *Salmonella* after exposure to low moistures (0.11–0.15%) in the sand and warm temperatures (40 °C) during drying was not evaluated in the current study. Influence of the dry-inoculation method on the tolerance of *Salmonella* to environmental challenges such as heat should be evaluated before this method is used to validate commercial processes.

## Acknowledgments

Funding for this project was provided, in part, by the California Walnut Board and the U.S. Food and Drug Administration (Project No. U01-003-572). T. Blessington was supported by a fellowship from the Oak Ridge Institute for Science and Education, Center for Food Safety and Applied Nutrition, Food and Drug Administration. The authors would like to thank Susan Geiger for her assistance in the development of the methods used in this study. This project would not have been possible without the technical support of Dr. Anne-laure Moyne along with the editorial skills of Sylvia Yada.

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**Fig. 1.** Survival of *Salmonella* on almond and walnut kernels inoculated via wet-inoculation (closed symbols) or dry-inoculation (open symbols) methods and stored at ambient conditions (squares = Trial 1; circles = Trial 2). Results are mean (±standard error) plate counts on BSA + Rif (open symbol), n = 6.

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