International Journal of Food Microbiology 166 (2013) 341–348

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Survival of foodborne pathogens on inshell walnuts ☆

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ARTICLE INFO

Article history:
Received 16 May 2013
Received in revised form 16 July 2013
Accepted 17 July 2013
Available online 24 July 2013

Keywords:
Nut
Walnut
Inshell
Salmonella
Escherichia
Listeria

ABSTRACT

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

1. Introduction

Tree nuts have been implicated in a number of foodborne outbreaks (Scott et al., 2009). Salmonellosis has been associated with consumption of nut kernels including almonds and pine nuts (CDC, 2004; Isaacs et al., 2005; Ledet Müller et al., 2007), and *Escherichia coli* O157:H7 gastroenteritis was epidemiologically linked to consumption of walnut kernels (CFIA, 2011a, 2011b). Although outbreaks with inshell nuts are less common, *E. coli* O157:H7 was isolated from inshell hazelnuts linked to a multi-state outbreak in the U.S. (CDC, 2011). Contaminants on the shell can presumably transfer to the kernel during cracking or result in cross contamination of hands or other foods.

Independent of reported illnesses, several Class I recalls initiated in the U.S. and Canada have resulted from isolation of *Salmonella* from nut kernels (hazelnuts, FDA, 2009c; macadamia, FDA, 2009a; pecans, Hitti, 2009; pine nuts, FDA, 2010a; and walnuts, FDA, 2010b) and inshell nuts (hazelnuts, CFIA, 2012a; pistachios, FDA, 2009b; walnuts, CFIA, 2012b). Walnut kernels also were recalled in 2009 after isolation of *Listeria monocytogenes* (Hughlett, 2009).

The microbiota of walnuts has not been well described in the literature. Limited surveys have isolated coliforms (Weinzirl, 1929) and *E. coli* (Entis et al., 1984; Kokal, 1965; Little et al., 2009, 2010; Meyer and Vaughn, 1969; Riyaz-Ul-Hassan et al., 2003) from walnut kernels. *Salmonella* was isolated from walnut kernels in India (10 g, $n = 50$) (Riyaz-Ul-Hassan et al., 2003) and from one pre-packed mixed nuts sample (25 g, $n = 329$) that also contained walnuts (Little et al., 2010), but was not detected in other surveys that included walnut kernels (25 g, $n = 74$ (Little et al., 2009); 25 g, $n = 441$ (Little et al., 2010); 25 g, $n = 80$ (NSW Food Authority, 2012)). In a 3-year survey

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http://dx.doi.org/10.1016/j.ijfoodmicro.2013.07.016

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

The United States is the leading exporter of the Persian or English walnut (*Juglans regia* L.); 99% of the U.S. production (470,000 metric tons projected in 2012) is grown in California (California Walnut Commission, 2012; USDA FAS, 2012; USDA NASS, 2012). Shortly after harvest, walnuts are hulled to remove the fleshy husk and then dried with forced air; dried inshell walnuts may be stored prior to packaging or shelling. In 2011, approximately 40% of edible California walnut kernels were sold in-the-shell (estimated average 44% kernel weight) and the majority of these inshell walnuts (94%) were exported (California Walnut Board, 2012); the remaining 60% were removed from storage as needed, then cracked and sold as kernels.

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

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

2. Materials and methods

2.1. Walnut samples

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

2.2. Bacterial cultures

The pathogens used in this study were as follows: *S. enterica* Enteritidis PT 30 (ATCC BAA-1045), isolated from raw almonds associated with an outbreak (Isaacs et al., 2005); *S. enterica* Enteritidis PT 9c, a clinical isolate from an outbreak associated with raw almonds (CDC, 2004); *S. enterica* Anatum (CAHFS D0307231), isolated from an almond survey (Danyluk et al., 2007); *S. enterica* Oranienburg, isolated from pecans, (provided by Dr. Larry R. Beuchat, University of Georgia); *S. enterica* Tennessee (K4643), a clinical isolate from a peanut butter-associated outbreak (CDC, 2007); *E. coli* O157:H7 (H1730), a clinical isolate from a lettuce-associated outbreak; *E. coli* O157:H7 (CDC 658), a clinical isolate from a cantaloupe-associated outbreak; *E. coli* O157:H7 (F4546), a clinical isolate from an alfalfa sprout-associated outbreak; *E. coli* O157:H7 (Odwalla strain 223), isolated from an apple juice-associated outbreak; *E. coli* O157:H7 (EC4042), a clinical isolate from a spinach-associated outbreak (Kotewicz et al., 2008); *L. monocytogenes* (4b) (LJH552), isolated from tomatoes; *L. monocytogenes* (4b) (LCDC81-861), isolated from a raw cabbage–associated outbreak; *L. monocytogenes* (4b) (Scott A), a clinical isolate from a milk-associated outbreak; *L. monocytogenes* (1/2a) (V7), isolated from milk in a milk-associated outbreak; and *L. monocytogenes* (4b) (101 M), isolated from beef in a beef-associated outbreak. *E. coli* K12 was used as a pathogen substitute, for safety reasons and to mimic similar viscosity and chemical characteristics of inoculation liquid, in experiments in which the moisture content and water activity of the walnut shells and kernels were analyzed before, during, and after inoculation.

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

2.3. Inoculum preparation

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

2.4. Inoculation procedures

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

2.5. Storage conditions

To evaluate pathogen survival on inshell walnuts, inoculated and control nuts were stored in unsealed bags within closed plastic