The Influence of Postharvest Handling Practices on the Microbiota of English Walnuts (*Juglans regia* L.)

By

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**ABSTRACT**

The influence of walnut harvest and postharvest handling on indigenous microbiota (aerobic plate counts [APC] and *E. coli*/coliform counts [ECC]) on walnuts is not widely understood. Walnuts were sampled at various points during these operations including from the tree, different points during harvest, receiving at the huller, after hulling and after drying. APC and ECC were determined for inshell walnuts and walnut kernels from either visibly intact or broken shells. Several formulations of peroxyacetic acid sprays were applied to walnuts after hulling and the impact on microbial loads on walnuts and huller surfaces was evaluated. Changes in microbial loads on inshell walnuts during harvest and postharvest handling were minimal. For both a thin and a hard shell variety of walnuts, significant increases in APC and ECC were observed on kernels from visibly intact shells after hulling. Walnut kernels with broken shells had significantly higher populations of APC and ECC than walnut kernels from visibly intact shells. Walnut shells had a higher level of breakage after drying than any point prior to drying, independent of cultivar and shell type. Antimicrobial sprays had minimal efficacy on inshell walnuts; sprays did sometimes cause significant decreases of microbes on conveyor belts, though reductions ranged from 0 to 4 log CFU/100 cm². Freshly hulled walnuts were inoculated with five-strain cocktails of *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* and then dehydrated and stored under simulated commercial conditions. Populations of all three pathogens decreased by approximately 3 log CFU/nut during drying. Slow steady declines of *Salmonella* were observed over 3 months to a population of 3 log CFU/nut; the population of
Salmonella remained at this level from 4 to 7 months of storage. In contrast, E. coli O157:H7 and L. monocytogenes populations continued a rapid decline after drying and within the first week to month of storage, to 3 and 2 log CFU/nut, respectively. Over the next 3 to 5 months the population densities continued to slowly decline to near the limit of detection but by 7 months all samples were still positive by plate count or enrichment of samples. A survey was also undertaken to determine the natural contamination of inshell walnuts with Salmonella and E. coli O157:H7. Salmonella was detected in three out of 1,904 375-g walnut samples that represented California walnut production during the 2011 and 2012 harvest (average prevalence = 0.16%); E. coli was not detected in any sample. The findings of these studies can be applied to the development of food safety programs for walnut handlers and to develop a risk assessment model for the walnut industry.
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ABSTRACT

INTRODUCTION

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Chapter 1: The microbiological safety of nut and nut pastes

1.1 Nuts and Nut Products

Nuts are an important agricultural commodity and have been a significant part of the human diet for at least 780,000 years (Goren-Inbar, 2002). Nuts are botanically defined as “a hard, indehiscent, one-seeded pericarp generally resulting from a compound ovary, as the chestnut or acorn” or filbert (Rosengarten, 1984). The word “nut” however is commonly used in a much broader sense to include drupes (almonds, pecans, pistachios, and walnuts), legumes (peanuts), and seeds (Brazil nuts, cashews, flax, sunflower seeds, sesame seeds and pine nuts), which have a similar composition and structure to botanical nuts, but are not nuts in the strictest sense (Rosengarten, 1984) (Table 1.1). Though botanically diverse, nuts have generally shared characteristics, namely a relatively hard, inedible outer shell with a softer, edible inner nut (also called the meat or kernel). Drupes also have a fleshy outer coating often called the hull, which is removed in processing. The hull may be discarded as waste (e.g., walnuts and pistachios) or may be used as animal feed (e.g., almonds); shells may also be used in a wide range of products or purposes from animal bedding to biofuel. The hull corresponds to the flesh of other drupes, like nectarines and peaches, and the nut corresponds to the stone of these fruits. Peanuts are legumes, like peas and other beans, in which the nut consist of pods made of a single folded carpel surrounding two seeds. The pod of a peanut corresponds to the shell of a true nut. Seeds, in the sense used here, represent a variety of different plant seeds, which are consumed as nuts but do not fall into a single group.

Nuts are grown in a wide variety of different climates and regions of the world. The top three producing countries for various nuts are summarized in Table 1.2. The dynamics of
production are ever changing but there has been a trend of increased nut production worldwide. Since nuts are a diverse group of agricultural products encompassing a variety of assorted plants, they have different requirements for growth. As a consequence, nuts are grown in virtually every region of the world depending on the growth requirements for each product. Almonds are grown extensively in the moderate climates of the central valley of California in the United States with other significant production in Australia, Spain and other countries on the Mediterranean coast. Walnuts are adapted to Mediterranean-like climate zones (CWB, 2012) and are grown widely in China, Iran and the US. California produces 99% of the US walnut supply, the majority of which are grown in the central valley. Other nuts are adapted to grow in more tropical climates such as macadamia nuts grown in Australia or Hawaii or Brazil nuts grown in Bolivia and Brazil and cashews grown in Africa (Nigeria, Côte d'Ivoire), India, Asia (Vietnam, Indonesia) and Brazil. Hazelnuts are widely grown in Turkey, Europe, Caucasus, the U.S. and Iran. Iran is the largest producer of pistachios with significant and growing production in the U.S. This assortment of growing climates creates unique challenges for the microbial safety of the nuts grown in each distinct region of the world due to variations in temperature, humidity, local production practices, harvest conditions, and postharvest handling.

All nuts go through various initial mechanical sorting steps that facilitate removal of debris such as sticks, rocks, leaves and loose dirt. Most tree nuts have an outer fleshy hull that is removed during hulling. Hulling may occur before or after drying, depending on the nut. Almonds dry on the ground after harvest and the dry almond hulls are removed using a series of sheer rollers – shells are sometimes removed at the same time. Other tree nuts (e.g., pistachios and walnuts) are more typically hulled shortly after harvest, usually with mechanical abrasion combined with water spays; inshell nuts are subsequently dried with forced, heated air.
Nuts are sold both in-the-shell and shelled (kernel only), with the exception of cashews, which are only sold shelled due to the highly toxic nature of the tissue between the shell and kernel (Menninger, 1977). Shelling is usually a dry mechanical process but in some countries nuts are still shelled by hand. Pecans are dried for sale inshell or are conditioned with hot water prior to shelling to make the kernels pliable and prevent breakage; the nutmeat is then dried alone (Beuchat and Mann, 2011). Some nuts, like pecans and walnuts, are stored in-the-shell for up to a year at ambient or under cool conditions and then shelled on an as-needed basis.

Nuts can be consumed out-of-hand as a snack but they are also extensively used as food ingredients in baked goods, confectionary products, and snack foods. Shelled nuts may be further processed including sorting for size and quality. The kernel pellicle (skin) may be removed by a dry process (e.g., dry blanching of peanuts) or by application of hot water or steam (e.g., wet blanching of almonds). Kernels can be used whole or transformed into many different forms, halved, chopped in various sizes, sliced, slivered, or may be ground into nut meals, flours, or pastes. There are many methods to roast nuts that may include introduction of salt or other flavorings. In some cases treatments may be applied to specifically reduce microbial loads without changing sensory properties.

Nut or seed pastes can be ground into fine particles to a paste-like consistency. Nut pastes may be made solely of ground nuts or may have other ingredients added such as salt, sugar or other seasonings, and/or hydrogenated vegetable oils to prevent separation. Often these products are called “butters” (i.e. peanut or almond butter). Peanut butter is one of the most common and easily recognizable nut pastes and accounted for 60% (~2.5 billion pounds) of peanut use in the United States in 2010 (ERS USDA, 2012). Increasingly, other nuts are also made into butters for consumers seeking different flavor or nutrient profiles and those with peanut allergies. Butters
are commonly made from almonds, cashews, hazelnuts, macadamias, pistachios, sunflower seeds, and sesame seeds (tahini) (Mangels, 2001). Also popular are flavored spreads that combine nut butters with other ingredients such as chocolate. Nuts may also be used to create confectionary products, such as marzipan (a mixture of ground almonds, sugar or honey and flavoring) or halva (made by mixing the sesame seed paste (tahini) with sugar and other ingredients).

Nuts tend to be high in fat and low in moisture. With the exception of seasonal specialty products such as fresh undried almonds or pistachios, nuts are typically dried to a water activity below $a_w$ 0.70 (between 0.50 and 0.65) (Kader and Thompson, 2002), which is lower than the minimum required for bacteria and most fungi to grow.

Worldwide nut production and consumption has expanded rapidly in recent years. In 2012 production of peanuts and tree nuts were 80 and 7.7 billion pounds, respectively, an increase of 2% and 5.5% from 2011, respectively (INC, 2012). Rapid growth has been coupled with an increase in reported outbreaks of foodborne illness linked to the consumption of nuts. Thus it is crucial for the nut industry to implement food safety programs that are adequate to handle current and anticipated increased production. This chapter will discuss ways nuts can become contaminated with foodborne pathogenic bacteria and how the bacteria survive in the nut production and processing environments.

1.2  Foodborne pathogens

Nuts and nut products have long been considered low risk for microbial food safety because their water activity is typically below 0.70, which prevents microbial growth (Beuchat, 1978). However, foodborne pathogens are able to survive at low levels in low-$a_w$ foods; these
products including nuts and their products are increasingly recognized as important contributors to outbreaks of foodborne illness (Beuchat et al., 2013; Podolak et al., 2010; Scott et al., 2009).

1.2.1 Outbreaks of foodborne illness

Outbreaks associated with consumption of nuts and nut butters have been primarily caused by *Salmonella* which accounted for 78% (18) of the 23 reported outbreaks (Table 1.3). Other outbreaks have been linked to *E. coli* O157:H7 gastroenteritis (inshell hazelnuts and walnut kernels) and in unusual cases *Clostridium botulinum* intoxication (peanut butter and canned peanuts). As with other low-\(a_w\) foods, outbreaks linked to nuts tend to be spread over many months and over wide geographic areas and as such they are challenging to investigate. It is possible that nut-associated outbreaks involving strains with common serotypes or fingerprints have gone unrecognized.

Consumption of raw almonds was associated with North American outbreaks in 2000/2001 and 2003/2004. A total of 168 cases reported in the US and Canada from October 2000 to July 2001 was epidemiologically linked to consumption of raw California almonds that were sold in bulk (Isaacs et al., 2005). The outbreak was identified, in part, by association with a very rare strain, *Salmonella* Enteritidis phagetype (PT) 30. Ultimately the same organism was isolated from case patients, almond samples collected from homes, the retailer, distributors and warehouses implicated in the outbreak. Traceback investigations led to a processing facility where the nuts were packed and ultimately to the hulling and shelling facility where the implicated lots of almonds were handled. The same strain of *Salmonella* Enteritidis PT30 was isolated from environmental swabs collected at both the huller and processing facility (Isaacs et al., 2005). This was significant because at the time of the investigation the huller had not been in operation for several months. Likewise, drag swabs of the implicated orchards collected nearly 9
months after the outbreak almonds had been harvested were positive for *Salmonella* Enteritidis PT30 (Isaccs et al., 2005). This strain continued to be isolated from the implicated orchards for five additional years (Uesugi et al., 2007). Although many potential sources of contamination were investigated the ultimate source of the orchard contamination was never determined. However, it was concluded that the almonds most likely were contaminated in the orchard during harvest and that this contamination spread during post-harvest handling.

The 2004 almond outbreak was associated with an equally rare *Salmonella* Enteritidis PT9c with 47 cases reported in the US and Canada from September 2003 to April 2004. Raw almond kernels recovered from a consumer’s house and samples collected at the almond processor were negative for *Salmonella*; however, the outbreak strain was isolated from one environmental sample collected at the processor and from three samples from two huller-shellers that supplied almonds to the primary implicated processor (Keady et al., 2004). The source of the *Salmonella* was not identified.

The almond industry in California reacted to these outbreaks and implemented a food safety action plan that included funding for research and the promulgation of regulations. Since September of 2007, these regulations require all California almonds sold in North America to be treated by a process that is validated to achieve a 4-log reduction in *Salmonella* (Federal Register, 2007). The process criterion was based on an initial risk assessment that predicted that this level of reduction was sufficient to prevent illness (Danyluk et al., 2006). The process criterion was later supported by second risk assessment that included significant amounts of additional data (Lambertini et al., 2012).
Peanut butter was first linked to an outbreak of salmonellosis in 1996 in Australia (Scheil, 1998). Fifteen cases were identified in total; the outbreak strain was isolated in peanut butter from the consumers households, from unopened jars collected at retail outlets and the processor. The levels of *Salmonella* in the peanut butter were determined to be less than three organisms per gram. The source was ultimately determined to be contaminated roasted peanuts received from a peanut roasting facility. This was the first time *Salmonella* was isolated in peanut butter and recommendations were to increase the focus on measures to prevent contamination in the processing environment.

A decade later two large outbreaks associated with peanut butter occurred in the US and Canada (2006-07 and 2008-09) that were associated with 628 and 714 confirmed cases, respectively, with the latter being attributed to eight deaths. In both of these outbreaks, contamination of the product with *Salmonella* most likely occurred within the processing environment after the peanuts were roasted (CDC, 2007a, 2007b, 2009a, 2009b; FDA, 2009a). In both cases, ingress of water into the facility was thought to contribute to contamination by providing an opportunity for multiplication of the organism in the environment. *Salmonella* is capable of multiplication in nut dusts that are combined with small amounts of water (Du et al., 2010). In the 2009 outbreak, the peanut butter was produced in bulk for use as an ingredient. As a consequence, the outbreak led to the recall of several thousand products (FDA, 2009b).

After an inspection of the production facilities implicated in the 2009 outbreak, a number of issues were indicated as sources of contamination risk, including but not limited to: failure to store product in a manner that protects it from contamination (cross-contamination between treated and untreated product); disrepair in the processing facility (water leakage though roof and skylights); and failure to prevent contamination of food and food-contact surfaces due to poor
ventilation (FDA, 2009c). It was also determined that the company was testing for *Salmonella* and had shipped product which originally tested positive, but retested negative. This was a deliberate action on the part of the company involved and has spurred a huge backlash for the company, which has gone bankrupt and is now facing criminal charges.

*Clostridium botulinum* is not considered an issue in low-a$_w$ foods because the organism cannot grow and produce toxin below an a$_w$ of 0.93 (Baird-Parker and Freame, 1967). However, three unusual outbreaks of botulism in nut products have been reported (Chou et al., 1988; O’Mahony et al., 1990; Sheppard et al., 2012). Canned peanuts processed in an unlicensed facility were implicated in a botulism outbreak in Taiwan in 1986 among workers who ate in their factory cafeteria (Chou et al., 1988). The dried, shell peanuts were boiled, placed into glass jars with the cooking liquid and the jars were steamed for about an hour. Immune compromised adult patients experienced intestinal toxemia after ingestion of peanut butter (Sheppard et al., 2012) containing the *C. botulinum* spores. As with infant botulism (Sobel, 2005), spores of *C. botulinum* can grow in the intestinal tract of persons with Crohn’s disease or other intestinal complications (Sobel, 2005). An outbreak of botulism in June 1989 was linked to hazelnut yogurt in the UK; the toxin was detected in a can of the hazelnut conserve used to flavor the yogurt (O’Mahony et al., 1990). The cans of the low acid hazelnut conserve did not receive sufficient thermal processing allowing *C. botulinum* to survive, grow and produce toxin. These cases indicate that botulism linked to nut products is rare but can occur in products with elevated a$_w$ in the absence of appropriate controls.

### 1.2.2 Recalls of nuts

Nuts and nut products are often recalled for undeclared allergens, presence of foreign material, and elevated levels of aflatoxin (FDA, 2013). Nuts (almonds, hazelnuts, macadamias,
peanuts, pine nuts, pistachios and walnuts) and nut pastes (peanut butter, cashew butter, and
tahini) have been associated with a number of Class I recalls in the U.S. and Canada due to
isolation of *Salmonella*, and to a lesser extent *Escherichia coli* O157:H7 or *Listeria
monocytogenes* (Palumbo et al., 2012a). Recently, nuts, seeds, and their products have been the
predominant low-aw food category implicated in recalls and market withdrawals in the US and
Canada associated with *Salmonella* in low-aw products (Beuchat et al., 2013).

1.2.2 Prevalence and levels of foodborne pathogens

A limited number of retail surveys have screened nuts and edible seeds for the presence
of *L. monocytogenes*, *E. coli* O157:H7, *Staphylococcus aureus* and *Bacillus cereus* (Palumbo et
al., 2012b). Most of the surveys have focused exclusively on *Salmonella* (Table 1.4) due to the
association of this organism with outbreaks in nuts and related nut products. The majority of the
published surveys have not detected *Salmonella* in many samples; many of these surveys have
evaluated a small number of samples of individual nuts collected at retail and analyzed 25-g
units (Brockmann et al., 2004; Willis et al., 2009; NSW Food Authority, 2012). Some of these
samples have been roasted which would decrease the likelihood of finding pathogens (Little et
al., 2009, 2010).

A survey of ready-to-eat nut products (915 samples) collected from retailers,
manufacturers and growers was performed in Australia by the New South Wales (NSW) Food
Authority in 2011 (NSW Food Authority, 2012). This study examined almonds, Brazil nuts,
cashews, hazelnuts, macadamias, mixed nuts, peanuts, pecans, pistachios and walnuts. Only a
single sample (macadamias; one out of 76 25-g samples) was found to contain *Salmonella*. Other
retail surveys have been performed in the UK and Brazil (Freire and Offord, 2002; Kajs et al.,
1976; Little et al., 2009 and 2010; Willis et al., 2009); *Salmonella* was isolated in two out of 469
25-g samples of Brazil nuts (Little et al., 2010), an unreported number of subsamples from a 2-kg sample of Brazil nuts (Freire and Offord, 2002), and one out of 284 25-g samples of flax seed (Willis et al., 2009).

There have been several larger surveys of specific raw nuts that have determined a prevalence from 0.1 to 2.3% (Table 1.4). An 8-year survey in California analyzed 13,972 100-g samples of raw almond kernels and found a prevalence of $0.98 \pm 0.32\%$ (Bansal et al., 2010; Danyluk et al., 2007; Lambertini et al., 2012; Harris, unpublished). In contrast, the prevalence of *Salmonella* in lots of almonds associated with an outbreak was 65% (Danyluk et al., 2007). A similar prevalence was detected in the same study for inshell almonds sampled over 2 years (1.5%; seven positive out of 455 100-g samples) (Bansal et al., 2010); the prevalence in California inshell walnuts was 0.16%; three of 1904 375-g samples). Raw peanut kernels were sampled over 3 years (2008 to 2010); 22 of 944 375-g samples were positive for *Salmonella* (2.3% prevalence) (Calhoun et al., 2013). Sesame seeds, sampled from imported shipments entering the U.S. were contaminated with *Salmonella* at 11% of 750-g samples from 177 shipments (Van Doren et al., 2013a) and 9.9% of 1500-g samples from 233 shipments (Van Doren et al., 2013b).

Because the prevalence is low, levels of *Salmonella* in positive lots can be challenging to determine. Over 4 years, the most probable number (MPN) was determined for 99 initially positive raw almond samples with estimated levels between 0.0044 and 0.15 MPN/g of *Salmonella* (Lambertini et al., 2012). Quantifiable levels of *Salmonella* (0.09 and 0.23 MPN/g) were reported in two samples of Brazil nuts (Little et al., 2010). In 22 samples of sesame seeds levels of *Salmonella* were $6 \times 10^{-4}$ to 0.04 MPN/g (Van Doren et al., 2013b). Levels of *Salmonella* in outbreak-associated almonds were estimated to be 1.2 MPN/g (Lambertini et al.,
and <0.03 to 2 MPN/g in inshell peanuts associated with an outbreak (Kirk et al., 2004). These levels are relatively low, even in outbreak situations contributing to the difficulty in detecting the pathogen in nuts. Routine testing should not be expected to identify contaminated lots.

Over an 8-year survey a total of 151 *Salmonella* was isolated from almonds representing 49 different serovars (Bansal et al., 2010; Danyluk et al., 2007; Bansal and Harris, unpublished); 13 different serovars were identified from the 22 *Salmonella* isolated from peanuts over 3 years (Calhoun et al., 2013). A survey of imported spices, including sesame seeds, found a total of 94 different serotypes in 187 positive samples (Van Doren et al., 2013a); sesame seed alone was found to have 18 different serotypes of *Salmonella* present in 20 positive samples. The diversity of *Salmonella* serotypes that have been identified in nut and seed surveys may be a reflection of a wide range of contamination sources that might include soil, water, wildlife and domestic animals.

### 1.2.3 Routes of contamination

While there are many similarities in harvest and postharvest handling, each nut type usually employs different specialized harvest and processing methods. Figure 1.1 outlines the harvest and processing steps that would be typical for almonds, walnuts, pistachios, and peanuts grown in the U.S. In other regions of the world there may be a greater reliance on manual labor. In many cases tree nuts and peanuts are mechanically harvested. Trees are shaken to release the nuts either to the ground (e.g., almonds, walnuts, hazelnuts) or onto catch frames (North American pistachios). In some regions (e.g., California) almonds dry on the ground for 7 to 10 days after shaking the tree. Nuts that are shaken to the ground are then mechanically swept into windrows and then harvested by sweeping into harvest containers or trailers. Most nuts are
further processed shortly after harvesting but almonds may be stockpiled for days to months. Stockpiled almonds are covered with tarps and fumigated to control insects.

Peanuts grow in the soil and are mechanically harvested by lifting the plant out of the ground so that the pods are exposed to the air and can dry in the sun for 2 to 3 days. The peanuts are then threshed from the vine and delivered to buying stations for curing, cleaning, and grading before storage in warehouses. Peanut shelling operations consist of cleaning to remove dirt, rocks and other foreign material followed by shelling to remove shells from kernels. Gravity separation then removes all but the peanut kernels which are sorted, sized, packaged, graded, and stored.

The kernel inside an intact shell was once thought to be virtually sterile (Chipley et al., 1971; Kajs et al., 1976; Meyer and Vaughn, 1969), however, there is substantial evidence the shell provides variable levels of protection from contamination. Walnut kernels have low populations of bacteria when extracted from inshell walnuts removed directly from the tree (Chapter 2). Total aerobic plate and E. coli/colliform counts on kernels increase significantly as the walnuts move through hulling and drying (Blessington, 2011; Chapter 2). When the kernel becomes exposed to the environment, the probability of microbial contamination increases due to the possibility for dirt, water, and other carriers of microorganisms to encounter the kernel. King et al. (1970) showed that almonds harvested onto canvas had lower counts than almonds harvested to the ground, indicating contamination from the soil has a significant influence on kernel microbial populations. For instance, shell breakage can expose the kernel within and lead to contamination (Beuchat and Mann, 2010; King et al., 1970; Chapter 2). Suture wetting can also allow for microbial infiltration of the nut shell and subsequent contamination of the kernel.
(Marcus and Amling, 1973). Drying may also influence shell integrity allowing contamination
(King et al., 1970; Meyer et al, 1969).

The different steps in harvest and processing of nuts provide various opportunities for
contamination of the nutmeat (Fig. 1.1). Kernels may be contaminated through wet or dry means.
Wet or dry contamination may occur in the field, in the processing equipment, or in the post-
processing environment. Wet contamination can occur when harvesting nuts to the ground, float
tanks or rinse waters, or by unintentional re-wetting of dried nuts. Danyluk et al. (2008) showed
that Salmonella can infiltrate almond shells when exposed to an aqueous environment for 24 h.
This is a feasible scenario which may be encountered by almonds in the orchard, thus leading to
contamination of the kernels. Dry contamination may also occur when harvesting to the ground,
but also through dust present during shelling or in storage facilities. Since different nuts have
different harvest, processing, and handling methods, the risks for each vary accordingly. For
instance, pistachios are harvested onto catch frames and never touch the ground, which reduces
risk of contamination from orchard soil. Uesugi et al. (2007) showed that Salmonella was able to
persist in the soil of one almond orchard for up to 5 years. This could lead to multiple outbreak
scenarios over multiple harvest seasons if proper control is not taken to prevent contamination of
the product.

1.2.4 Survival of pathogens in nuts and nut-processing environments

Once a pathogen is introduced into nuts, nut pastes, or the environment where they are
processed, it is possible for the organism to persist for extended periods of time (Beuchat and
Heaton, 1975; Beuchat and Mann, 2010; Blessington et al., 2012 and 2013; Burnett et al., 2000;
Kimber et al., 2012; Uesugi et al., 2006). Salmonella, E. coli O157:H7 and L. monocytogenes on
inoculated pistachios and almonds and stored at ambient temperature (24°C) all displayed
different rates of decline (Kimber et al., 2012), which were not always linear. These decline curves tend to have a fairly rapid die off after inoculation followed by long-term persistence. Though all three pathogens decrease over time, all three can persist in low levels for over 2 years (Fig. 1.2).

Survival of pathogens also increases at lower temperature; virtually no decline is seen at freezing or refrigeration temperatures for many different nut commodities including almonds (Uesugi et al., 2006, Kimber et al., 2012), peanut butter (Burnett et al., 2000), pecans (Beuchat and Heaton, 1975; Beuchat and Mann, 2010), pistachios (Kimber et al. 2012) and walnuts (Blessington et al., 2012 and 2013; Chapter 3). Though lower temperatures reduce the rate of oxidation of the fats within the nuts, the same conditions are beneficial to the survival of human pathogens.

In some cases, the inoculation level of *Salmonella* does not influence the rate of decline, such as in almonds (Uesugi et al., 2006). However, *Salmonella* has been shown to decline faster at lower inoculation levels in walnuts (Blessington et al., 2012 and 2013). Declines in *Salmonella* on almonds were calculated to be linear and ranged from 0.16 to 0.32 log CFU/g/month (Lambertini et al., 2012)
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S. Hurt, M. Poulson, R. Pallipamu, J. Wicklund, C.Braden, J. Lockett, S. Van Duyne, A. 

M. Schwieger, P. J. Sanderson, I. S. T. Fisher, P. S. Mead, O. N. Gill, C. L. R. Bartlett, and 
B. Rowe. 1996. International epidemiological and microbiological study of outbreak of 
Salmonella Agona infection from a ready to eat savoury snack-I: England and Wales and the 


Table 1.1. Classification of common nuts by category and botanical family

<table>
<thead>
<tr>
<th>Category</th>
<th>Common name</th>
<th>Scientific name</th>
<th>Botanical family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achene</td>
<td>Sunflower seed</td>
<td><em>Helianthus annuus</em></td>
<td>Compositae</td>
</tr>
<tr>
<td>Capsule</td>
<td>Brazil nut</td>
<td><em>Bertholletia excelsa</em></td>
<td>Lecythidaceae</td>
</tr>
<tr>
<td></td>
<td>Sesame seed</td>
<td><em>Sesamum indicum</em></td>
<td>Pedaliaceae</td>
</tr>
<tr>
<td>Drupes</td>
<td>Almond</td>
<td><em>Prunus amygdalus</em></td>
<td>Rosaceae</td>
</tr>
<tr>
<td></td>
<td>Cashew</td>
<td><em>Anacardium occidentale</em></td>
<td>Anacardiaceae</td>
</tr>
<tr>
<td></td>
<td>Coconut</td>
<td><em>Cocos nucifera</em></td>
<td>Palmae</td>
</tr>
<tr>
<td></td>
<td>Hickory</td>
<td><em>Carya spp.</em></td>
<td>Juglandacae</td>
</tr>
<tr>
<td></td>
<td>Macadamia</td>
<td><em>Macadamia integrifolia</em></td>
<td>Proteaceae</td>
</tr>
<tr>
<td></td>
<td>Pecan</td>
<td><em>Carya illinoinsensis</em></td>
<td>Juglandacae</td>
</tr>
<tr>
<td></td>
<td>Pistachio</td>
<td><em>Pistacia vera</em></td>
<td>Anacardiaceae</td>
</tr>
<tr>
<td></td>
<td>Walnut</td>
<td><em>Juglans regia</em></td>
<td>Juglandacae</td>
</tr>
<tr>
<td>Nuts</td>
<td>Acorn</td>
<td><em>Quercus alba</em></td>
<td>Fagaceae</td>
</tr>
<tr>
<td></td>
<td>Chestnut</td>
<td><em>Castanea dentata</em></td>
<td>Fagaceae</td>
</tr>
<tr>
<td></td>
<td>Filbert (Hazelnut)</td>
<td><em>Corylus avellana</em></td>
<td>Corylaceae</td>
</tr>
<tr>
<td>Legumes</td>
<td>Peanut</td>
<td><em>Arachis hypogaea</em></td>
<td>Leguminosae</td>
</tr>
<tr>
<td>Nut</td>
<td>1st Country</td>
<td>1st Production (1,000 tons)</td>
<td>2nd Country</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------</td>
<td>-----------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Almond, with shell</td>
<td>USA</td>
<td>731</td>
<td>Spain</td>
</tr>
<tr>
<td>Brazil nut, with shell</td>
<td>Bolivia</td>
<td>48.5</td>
<td>Brazil</td>
</tr>
<tr>
<td>Cashew, with shell</td>
<td>Vietnam</td>
<td>1,270</td>
<td>Nigeria</td>
</tr>
<tr>
<td>Coconut</td>
<td>Indonesia</td>
<td>17,500</td>
<td>Philippines</td>
</tr>
<tr>
<td>Hazelnut, with shell</td>
<td>Turkey</td>
<td>430</td>
<td>Italy</td>
</tr>
<tr>
<td>Peanut, with shell</td>
<td>China</td>
<td>16,100</td>
<td>India</td>
</tr>
<tr>
<td>Pistachio</td>
<td>Iran</td>
<td>472</td>
<td>USA</td>
</tr>
<tr>
<td>Sesame seed</td>
<td>Myanmar</td>
<td>862</td>
<td>India</td>
</tr>
<tr>
<td>Sunflower seed</td>
<td>Russia</td>
<td>9,700</td>
<td>Ukraine</td>
</tr>
<tr>
<td>Walnuts, with shell</td>
<td>China</td>
<td>1,660</td>
<td>Iran</td>
</tr>
<tr>
<td>Other Nuts(^1)</td>
<td>China</td>
<td>132</td>
<td>USA</td>
</tr>
</tbody>
</table>

\(^1\) Includes pecan, butternut, pili nut, Java almond, Chinese olives, paradise nut, macadamia nut and pignolia (pine) nut
<table>
<thead>
<tr>
<th>Type</th>
<th>Product</th>
<th>Pathogen</th>
<th>Year</th>
<th>Number of confirmed cases</th>
<th>Outbreak Location(s)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuts</td>
<td>Almond</td>
<td>Raw whole</td>
<td>S. enteritidis PT 30</td>
<td>2000-01</td>
<td>168</td>
<td>Canada, USA</td>
</tr>
<tr>
<td>Nuts</td>
<td>Raw whole</td>
<td>S. Enteritidis PT 9c</td>
<td>2004</td>
<td>47</td>
<td>Canada, USA</td>
<td>Keady et al., 2004; CDPH, 2004</td>
</tr>
<tr>
<td>Nuts</td>
<td>Raw whole</td>
<td>S. Enteritidis</td>
<td>2005-06</td>
<td>15</td>
<td>Sweden</td>
<td>Le det Muller et al., 2007</td>
</tr>
<tr>
<td>Nuts</td>
<td>Raw whole</td>
<td>Serovar not given</td>
<td>2012</td>
<td>27</td>
<td>Australia</td>
<td>FSANZ, 2012</td>
</tr>
<tr>
<td>Coconut</td>
<td>Desiccated</td>
<td>S. typhi, S. Senftenberg and possibly others</td>
<td>1953</td>
<td>&gt;50 (est. from epi curve)</td>
<td>Australia</td>
<td>Wilson and Mackenzie, 1955</td>
</tr>
<tr>
<td>Hazelnut</td>
<td>In-shell</td>
<td>E. coli O157:H7</td>
<td>2011</td>
<td>7</td>
<td>USA</td>
<td>CDC, 2011a, Miller et al., 2012</td>
</tr>
<tr>
<td>Peanut</td>
<td>Canned</td>
<td>C. botulinum (type A)</td>
<td>1986</td>
<td>9</td>
<td>Taiwan</td>
<td>Chou et al., 1988</td>
</tr>
<tr>
<td>Savory snack</td>
<td></td>
<td>S. Agona PT 15</td>
<td>1994-95</td>
<td>71</td>
<td>United Kingdom, Israel, USA</td>
<td>Killelea, 1996; Shohat, 1996; Threlfall et al., 1996</td>
</tr>
<tr>
<td>Nut</td>
<td>Type/Description</td>
<td>Species</td>
<td>Year</td>
<td>Reference(s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------</td>
<td>------------------</td>
<td>------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pine nut</td>
<td>Whole, bulk</td>
<td>S. Enteritidis</td>
<td>2011</td>
<td>USA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Boiled</td>
<td>S. Thompson</td>
<td>2006</td>
<td>USA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walnut</td>
<td>Raw shelled halves, pieces, crumbles</td>
<td>E. coli O157:H7</td>
<td>2011</td>
<td>USA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nut</td>
<td>Paste</td>
<td>C. botulinum</td>
<td>1989</td>
<td>United Kingdom</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazelnut</td>
<td>Yogurt</td>
<td>(type B)</td>
<td></td>
<td>O'Mahony et al., 1990</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut</td>
<td>Butter</td>
<td>S. Mbandaka</td>
<td>1996</td>
<td>Australia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nut</td>
<td>Paste</td>
<td>S. Tennessen</td>
<td>2006-07</td>
<td>USA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut</td>
<td>Butter</td>
<td>C. botulinum (types A and B)</td>
<td>2006-08</td>
<td>Canada</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut</td>
<td>Butter, butter-containing products</td>
<td>S. Typhimurium</td>
<td>2008-09</td>
<td>USA, one case in Canada</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut</td>
<td>Butter</td>
<td>S. Bredeney</td>
<td>2012</td>
<td>USA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sesame</td>
<td>Halva</td>
<td>S. Typhimurium</td>
<td>2001</td>
<td>Australia, Sweden, Norway, United Kingdom, Germany</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seed</td>
<td></td>
<td>DT 104</td>
<td></td>
<td>O'Grady, 2001; de Jong, 2001; Brockmann, 2001; Little, 2001;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Intestinal toxemia botulism, which is very rare. Two of three patients studied had a history of Crohn’s disease and bowel surgery.
Table 1.4. *Salmonella* prevalence in naturally-contaminated nuts and nut pastes

<table>
<thead>
<tr>
<th>Nut Type</th>
<th>Where collected</th>
<th>Sample size (g)</th>
<th>No. of samples tested (n)</th>
<th>No. positive for <em>Salmonella</em> (n+)</th>
<th>Percent positive (if n&gt;50)</th>
<th><em>Salmonella</em> serotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Almond, raw kernel</td>
<td>Receiving, California</td>
<td>100</td>
<td>13,972</td>
<td>137</td>
<td>0.98 ± 0.32 (for 2001–7 and 2010)</td>
<td>Montevideo, Thompson, Enteritidis, Typhimurium, Senftenberg, and 30 others</td>
<td>Bansal et al., 2010; Danyluk et al., 2007; Lambertini et al., 2012; Harris, unpublished</td>
</tr>
<tr>
<td>Almond, raw inshell</td>
<td>Receiving, California</td>
<td>100</td>
<td>455</td>
<td>7</td>
<td>1.5 (for 2006–7)</td>
<td>Muenchen, Typhimurium, Newport, Thompson, Give, IIIa:18::z32</td>
<td>Bansal et al., 2010</td>
</tr>
<tr>
<td>Almond, raw kernel</td>
<td>Receiving, Australia</td>
<td>25</td>
<td>60</td>
<td>1</td>
<td>1.7</td>
<td>Fremanltle subsp. II</td>
<td>Eglezos, Huang, and Stuttard, 2008</td>
</tr>
<tr>
<td>Almond, treated</td>
<td>RTE packages, Australia</td>
<td>25</td>
<td>42</td>
<td>0</td>
<td></td>
<td></td>
<td>Eglezos, 2010</td>
</tr>
<tr>
<td>Brazil nut, shelled and whole in-shell</td>
<td>Processor</td>
<td>50</td>
<td>20</td>
<td>0</td>
<td></td>
<td></td>
<td>Arrus et al., 2005</td>
</tr>
<tr>
<td>Brazil nut</td>
<td>Receiving, Australia</td>
<td>25</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td></td>
<td>Eglezos, Huang, and Stuttard, 2008</td>
</tr>
<tr>
<td>Brazil nut</td>
<td>RTE packages, Australia</td>
<td>25</td>
<td>40</td>
<td>0</td>
<td></td>
<td></td>
<td>Eglezos, 2010</td>
</tr>
<tr>
<td>Cashew</td>
<td>Receiving, Australia</td>
<td>25</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
<td>Eglezos, Huang, and Stuttard, 2008</td>
</tr>
<tr>
<td>Cashew</td>
<td>RTE packages, Australia</td>
<td>25</td>
<td>45</td>
<td>0</td>
<td></td>
<td></td>
<td>Eglezos, 2010</td>
</tr>
<tr>
<td>Hazelnut</td>
<td>Receiving, Australia</td>
<td>25</td>
<td>48</td>
<td>0</td>
<td></td>
<td></td>
<td>Eglezos, Huang, and Stuttard, 2008</td>
</tr>
<tr>
<td>Hazelnut</td>
<td>RTE packages, Australia</td>
<td>25</td>
<td>51</td>
<td>0</td>
<td></td>
<td></td>
<td>Eglezos, 2010</td>
</tr>
<tr>
<td>Peanut</td>
<td>Receiving, Australia</td>
<td>25</td>
<td>653</td>
<td>0</td>
<td>0</td>
<td></td>
<td>Eglezos, Huang, and Stuttard, 2008</td>
</tr>
<tr>
<td>Peanut</td>
<td>RTE packages, Australia</td>
<td>25</td>
<td>343</td>
<td>0</td>
<td>0</td>
<td></td>
<td>Eglezos, 2010</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------------</td>
<td>-------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Peanut Processor, USA</td>
<td></td>
<td>375</td>
<td>944</td>
<td>22</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sesame seed Importer, USA</td>
<td></td>
<td>375</td>
<td>177</td>
<td>20</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sesame seed Importer, USA</td>
<td>1,500 (composite samples)</td>
<td></td>
<td>233</td>
<td>23</td>
<td>9.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walnut, raw inshell Processor, California</td>
<td>100</td>
<td>935</td>
<td>0</td>
<td>0 (2010)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walnut, raw inshell Processor, California</td>
<td>375</td>
<td>1,904</td>
<td>3</td>
<td>0.16 (average 2011, 2012)</td>
<td></td>
<td></td>
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</table>
Figure 1.1. Process flow diagram for four different nuts. Shaded boxes indicate sites of potential wet contamination; dark outlined boxes indicate sites of potential dry contamination; dashed boxes indicate sites of potential growth. *Almonds are typically hulled and shelled at the same time.
Figure 1.2. Survival of *Salmonella* (triangles), *E. coli* O157:H7 (squares) and *Listeria monocytogenes* (circles) on inoculated almonds (A) and pistachios (B) stored at 23°C; asterisk (*) indicates six of six replicates were positive via enrichment of 10-g samples (modified from Kimber et al., 2012).
Chapter 2: Evaluation of the Natural Microbial Loads and Effect of Antimicrobial Sprays in Postharvest Handling of California Walnuts

ABSTRACT

The changes in concentrations of aerobic plate counts (APC) and *E. coli* / coliform (ECC) counts of inshell walnuts and walnut kernels were evaluated during harvest and postharvest handling of walnuts at a commercial hulling-dehydration facility. The APC and ECC for inshell walnuts collected from the tree were 6 and 4 log CFU/nut, respectively. These counts increased by 1 log CFU/nut during harvest and hulling and decreased by 1 log CFU/nut during drying. The APC and ECC for kernels extracted from visibly intact nuts were 2.1 and 1.7 log CFU/nut, respectively, for walnuts collected from the tree. These counts increased significantly upon receipt at the huller (APC) or after hulling (ECC) but did not decline after drying. Microbial loads on kernels from visibly intact shells did not increase between shaking from the tree and receiving at the huller but did increase significantly after the hulling step; similar results were observed for both a thin and a hard shell walnut cultivar. Kernels extracted from walnuts with broken shells had significantly higher populations than kernels extracted from walnuts with visibly intact shells at the steps prior to drying but not afterwards. A decrease in visible shell integrity was evident after the drying process, with approximately 4 and 47% of walnuts having broken shells before and after drying, respectively. Microbial loads in the float tank filled with fresh water were between 6.5 and 7 log CFU/ml shortly after hulling began. Loads on conveyor belts increased significantly within the first 30 min of use. Application of four PAA formulations to the walnuts after hulling at 100 and 200 ppm reduced both APC and ECC on walnuts by less than 1 log CFU/nut; counts were not significantly different for the water control. APC and ECC
on conveyor belts ranged from 0 to 4.4 log CFU/100 cm²; greater reductions were generally observed with application of 200 ppm PAA, but no formulation was consistently more effective than water. APC and ECC on both the shell and kernels of stored inshell walnuts decreased during the first 4 months of storage and then leveled off. Declines were not significantly influenced by treatment with antimicrobial sprays. A better understanding of how microbial populations are affected by postharvest handling practices will allow the walnut industry to develop scientifically-based food safety programs.

**INTRODUCTION**

English walnuts (*Julans regia* L.) are primarily grown in the United States and China; in 2012 each country produced an estimated 195 million kg (430 million lbs) or about 38% of worldwide walnut production (INC, 2012). California produces 99% of all walnuts grown in the U.S.; walnuts were the ninth top grossing agricultural crop in the U.S. in 2011 with a value of $1.3 billion (USDA, 2012a).

Walnuts are drupes consisting of a fleshy green fruit, called the hull, surrounding a hard-shelled seed that is commonly called the nut or kernel. When walnuts mature the hulls dehisce or split open, releasing the nut. At peak maturity, walnuts are mechanically harvested by shaking the tree, which drops the fruit to the ground. At this point the nuts may completely release from the hull or they may be fully or partially covered by the hull. The walnuts are then mechanically collected from the ground by sweeping into windrows, loaded into trailers, and transported to a huller-dehydrator (Kader and Thompson, 2002). At the huller-dehydrator, walnuts go through a number of operations to remove debris (sticks, leaves, rocks, etc.) including passage through a tank of water called the float or rock tank, which separates the floating walnuts from sinking
rocks. Immediately after the float tank, the remaining walnut hulls are removed by mechanical abrasion leaving the inshell nut. Hulling is typically followed by a water rinse applied by spray bars over a conveyer or in a “squirrel cage” that rotates the nuts while spraying with a high volume water rinse to remove remaining dirt, debris, and adhering hull material before hand sorting and mechanical drying. Walnuts may be dried in metal pallet bins called “pothole dryers” or in large false-bottom stationary bin dryers. Forced air at temperatures between 32 and 43°C (90 to 110°F) is used to bring the walnuts from about 35% moisture to a final total moisture content (shells and kernels) of about 8% (Thompson et al., 1998). Walnuts are stored in-the-shell in bins or in large silos for up to a year. As needed, walnuts are removed from storage and sorted, sized, and cracked as appropriate to yield inshell whole walnuts and kernels (halves and various sizes of pieces). Inshell walnuts are seasonally popular for out-of-hand consumption; walnut kernels are widely used as an ingredient in many foods and may also be eaten as a snack.

During harvest and postharvest handling, the shell and kernels may become contaminated with microorganisms by both wet and dry mechanisms. Nut kernels within an intact shell are thought to be protected from microbial contamination (Chipley et al., 1971; Kajs et al., 1976; Meyer and Vaughn, 1969), but microbial infiltration can occur even when nut shells are apparently intact (Beuchat and Heaton, 1975; Beuchat and Mann, 2010; Danyluk et al., 2008; Meyer and Vaughn, 1969). Shell breakage occurs to varying degrees during various harvest and processing steps including drying, thereby exposing the kernel within and leading to enhanced potential for contamination (Beuchat and Mann, 2010; King et al., 1970; Meyer and Vaughn, 1969).

Wet conditions are usually undesirable in the orchard during harvest but irrigation water or rainfall may facilitate the infiltration of microbes through nut shells (Danyluk et al., 2008;
Marcus and Amling, 1973). Water in the float tank may also facilitate movement of microorganisms among nuts and infiltration through the shell. Meyer and Vaughn (1969) correlated *Escherichia coli*-contaminated water sampled during hulling of black walnuts (*Juglans nigra*) with a small sample of highly contaminated nuts, emphasizing the potential for cross contamination at this step. Dry routes of contamination include contact with soil or with dust or organic material on harvest equipment, during post-dehydration transport or storage, and during hulling or shelling (Du et al., 2007; King et al., 1970). *Salmonella Enteritidis* PT30 was isolated from outbreak-associated almond orchards for 5 years (Uesugi et al., 2007); contact of nuts with the ground may be one source of contamination during harvest. Although walnut orchards may differ in this respect since almonds are left to dry on the orchard floor while walnuts are picked up immediately after shaking, contact between nuts and the ground may still be one source of contamination during harvest.

Walnuts have been recalled due to the detection of *Salmonella* (CDPH, 2010; FDA 2010a, 2010b, 2011; Mojave Food Corporation, 2010), *E. coli* O157:H7 (CFIA, 2011a, 2011b) and *Listeria monocytogenes* (FDA, 2009). An *E. coli* outbreak was epidemiologically linked to the consumption of walnuts in Canada, although the pathogen was not detected in the remaining product (CFIA, 2011c; Health and Safety Watch, 2011; PHAC, 2011). The California Walnut Board has surveyed California inshell walnuts (2011 and 2012 harvests) collected from throughout the state for the presence of *Salmonella* and *E. coli* O157:H7. A low prevalence (0.16%; 3 out of 1,904 375-g samples) of *Salmonella* was detected; *E. coli* O157:H7 was not isolated from any of the 1,904 375-g samples (Chapter 3).

The current study explores the microbiological food safety of California walnuts from harvest through storage. Walnuts were sampled during two harvest seasons (2011 and 2012) to
define microbial populations associated with walnuts during harvest, hulling, drying, and storage. Microbial loads on walnut shells and kernels were examined to determine if harvest and/or processing operations provide opportunities for contamination of the nut meat. The role of shell integrity on the potential for kernel contamination was explored by comparing the microbial loads of walnut kernels from intact and broken shells, and attempts were made to quantify shell breakage to identify operations that may increase shell breakage and, in turn, increase the potential for kernel contamination. Antimicrobial sprays were evaluated for efficacy in reducing microbial loads on inshell walnuts as well as on huller equipment as a potential intervention step that could be incorporated into food safety programs at walnut hulling operations. Natural microbial populations were measured on treated and untreated walnuts during storage to determine the effect of storage on population size and to evaluate if application of antimicrobials had a residual impact on these populations.

**MATERIALS AND METHODS**

**Collaborating growers and huller-dehydrators.** Two different huller-dehydrator facilities near Stockton, California, were sampled during two harvest years (2011 and 2012). The facility sampled in 2011 (trial 1; Fig. 2.1) was a small, pilot-scale operation (processing approx. 4,500 kg/h [5 tons/h]) and the facility sampled in 2012 (trial 2; Fig. 2.2) was a full-scale operation (processing approx. 45,000 kg/h [50 tons/h]) owned by the same company. All samples were evaluated in an on-site laboratory located at the smaller huller-dehydrator facility. Orchard samples were collected from two walnut orchards within a 16-km (10-mile) radius of this laboratory.
Walnuts. Walnuts (*J. regia* L.) were collected from commercial orchards or within the commercial huller-dehydrator facilities. In the orchards, inshell walnuts that could be easily extracted from fruit with fully split hulls were aseptically removed directly from the tree; walnuts that were free of the hull were collected from the ground after the trees were shaken, and from windrows after walnuts were swept up. Samples were aseptically collected using gloved hands covered in an inverted, new plastic bag. For each cultivar, corresponding samples were collected over a 3-day period: on day 1 from the trees before shaking and from the ground after shaking, on day 2 from the windrows, and on day 3 from the huller-dehydrator facility as described below.

For trial 1, inshell walnuts free of hull material were collected from the receiving pit, float tank, sort table, and dryer bins; ‘Chandler’ and ‘Hartley’ cultivar walnuts were sampled for comparison. For trial 2, samples were pulled from the receiving pit, sort table, and dryer bins; the walnut cultivars ‘Chandler’, ‘Howard’, ‘Tulare’, and ‘Vina’ were sampled but the effect of variety was not measured. Most samples were retrieved using a sterile scoop (SterileWare, Bel-Art Products, Wayne, NJ). To collect walnuts from the float tank and after the squirrel cage, a metal mesh strainer was used that allowed the water to drain from the sample. The strainer was sprayed with 70% ethanol and allowed to dry for 15 min between uses.

At each sampling point, enough walnuts (approximately 500–1000 g) were collected to half fill a 30.5 × 30.5 cm zippered polyethylene bag (Bitran, Com-Pac International, Carbondale, IL). All samples were held on ice for no more than 4 h before sample preparation and plating.

Sampling of conveyor belts. In trial 2, two separate conveyor belts were sampled to evaluate the microbial loads in these areas: the sort table conveyor (conveyor A) and a cross-
conveyor leading from the sort table to the dryer bins (conveyor B) (Fig. 2.2). Samples were collected when the facility was in operation and while either water (control) or antimicrobial spray(s) was being applied to the walnuts. The conveyors were sampled by lightly pressing a sterile cellulose sponge pre-moistened with 10 ml of Dey-Engley neutralizing broth (Solar Biologicals Inc., Ogdensburg, NY) to the moving belt for 5 s. The belt speeds were determined using a tachymeter and used to calculate the area of conveyor belt sampled.

**Float tank water.** Water from the float tank was sampled in trial 2. Samples were collected with sterile 250-ml water samplers (Sterilin, Stafford, UK) at the beginning of the day at the time of equipment startup, at 30 min, and then at 1, 2, and 3 h. Water samples were held on ice no more than 2 h before analysis. Antimicrobials were not used in the water in the facility during this time.

**Antimicrobial treatments.** Spray systems for antimicrobial treatments were installed in both hulling facilities. Several different PAA formulations were evaluated (Table 2.1); each differed in the relative concentrations of PAA and hydrogen peroxide (H₂O₂). In the 2011 trial, lauric arginate ethyl ester (LAE) was applied as an additional spray immediately after the PAA spray. Table 2.2 summarizes the different treatments evaluated, and provides the total PAA concentration for each treatment or treatment combination.

PAA-containing products were applied with spray nozzles: 46500A-1-PP-VI, ProMax Clip Eyelet with QPTA6505 ProMax Quick VeeJet nozzles (Spraying Systems Co., Wheaton, IL) at a spray rate of 1.9 liters/min/nozzle (0.5 gal/min/nozzle). In trial 1, a total of eight overhead nozzles (total flow rate = 15.2 liters/min [4 gal/min]) was used over 2 m of an upward sloping metal mesh belt (Fig. 2.3A). In trial 2, a total of 18 overhead nozzles (total flow rate =
34.2 liters/min [9 gal/min]) was used, with 13 nozzles that were distributed over a 2-m mesh belt (Fig. 2.4A) followed by a shaker table leading to a 1-m mesh belt with an additional five overhead nozzles (Fig. 2.4B). The PAA dosage was controlled by a Dosatron water-powered dosing meter (Dosatron, Clearwater, FL) in trial 1 and a ProMinent disinfection controller (ProMinent Fluid Controls, Inc., Pittsburgh, PA) in trial 2.

The LAE-containing product (CytoGuard LA, A&B Ingredients, Fairfield, NJ) was applied with a separate spray system that consisted of six Sanitary PulsaJet air atomizing spray nozzles (part number 1/4JCO-SS+SU13A-SS, Spraying Systems Co.) in two consecutive spray bars with three overhead nozzles on each (Fig. 2.3B). LAE spray bars were located after the PAA spray bars and immediately before the sort table to give the walnuts a coating with the LAE product as they tumbled off the first conveyor onto the second conveyor. Sprays were applied at 69 kPa (10 psi) liquid pressure and 140 kPa (20 psi) air pressure (flow = 2.46 liters/h/nozzle = 3.26 ml/kg [0.05 fl. oz/lb] walnuts).

On each sampling day, water was applied through the spray system used for application of PAA but before any antimicrobial was added. Hulling equipment was allowed to run at normal speeds with walnuts for at least 30 min before collecting control (water sprayed) samples. After switching from the water spray, antimicrobial sprays were applied for at least 15 min before collecting test samples to allow systems to become saturated with the solutions. In all but one case a single treatment (product and concentration combination) was tested on each sampling day. For the BioSide HS 15% in trial 2, the same treatment was applied twice in the same day at least 3 h apart with water applied between the two replicates.
**Microbial populations during storage.** In trial 1, both control and PAA-treated ‘Chandler’ inshell walnuts were dried and stored on-site under commercial storage conditions (ambient warehouse conditions for the first 5 months (October to February), followed by an additional 4 months (March to June) in a commercial cold storage unit). Samples were collected monthly and analyzed to determine total aerobic plate count and coliform counts. Temperature and relative humidity were monitored for 2 months of ambient storage (January to February) and the 4 months of cold storage (March to June) with programmable temperature data loggers (TempTale 4, Sensitech Inc., Beverly, MA) that were placed in the bins along with the walnuts being sampled.

**Quantifying walnut shell breakage.** A scoring system was developed to quantify the amount of shell breakage. Samples of inshell walnuts were sorted by degree of shell breakage and classified into six categories with breakage levels from 0 to 5, with 0 representing no visible shell breakage and 5 representing the most severe breakage. A scoring guide was developed that included a description of breakage characteristics (Fig. 2.5). This scoring system was then used to determine the proportion of breakage for walnut samples that were collected in the field after tree shaking and at the receiving pit, sort table, and dryer bins. Samples were drawn by randomly filling a 9.5-liter bucket with walnuts (approximately 200–300 nuts) at each sampling point.

**Preparation of samples for analysis.** Walnut samples were initially scored for shell breakage. Walnuts were separated using a sterile scoop (SterileWare) into those with visibly intact shells (breakage score 0 and 1) and those with visibly cracked, broken, or missing shells (breakage score 2–5). Microbial populations were determined for both inshell walnuts and for kernels that were aseptically removed from visibly intact or visibly cracked walnuts as described below.
For analysis of inshell walnuts, five randomly selected walnuts were placed into a 710-ml (24-oz) Whirl-Pak bag (Nasco, Modesto, CA) with 50 ml of Dey-Engley (DE) neutralizing buffer (Difco brand, BD, Franklin Lakes, NJ) or 0.1% peptone and the bags were alternately shaken and rubbed for 2 min. The DE was used to inactivate the residual test antimicrobials in treated samples. Either DE or 0.1% peptone was used as the wash buffer for the water controls.

Before kernels were extracted for microbial analysis, the shells of the nuts were thoroughly wiped with an alcohol wipe (70% isopropyl; Bio-Pure, Diversified Biotech, Dedham, MA) to reduce the potential for cross contamination from the shell to the kernel during cracking. Walnuts were aseptically cracked using sterile nut crackers and the kernels were extracted aseptically using sterile forceps. Nut crackers and forceps were sterilized by soaking in 70% ethanol and air drying between uses. Each kernel sample consisted of nut meats extracted from three inshell walnuts. Kernels were placed into a 200-ml (7-oz) Whirl-Pak filtering bag with 30 ml of DE or 0.1% peptone and the samples were then homogenized for 30 s at high speed with a Smasher blender-homogenizer (AES Chemunex, Combourg, France). The DE was used to inactivate any residual test antimicrobial in the treated samples. Either DE or 0.1% peptone was used as the wash buffer for the water controls.

Sponges used to sample the conveyors were each placed into a 532-ml (18-oz) Whirl-Pak bag with 20 ml of DE and the samples were homogenized for 30 s at high speed.

**Microbial analysis.** Ten-fold serial dilutions of the prepared samples were made in 9 ml of Butterfield’s phosphate buffer (BPB) (Hardy Diagnostics, Santa Maria, CA). Total aerobic plate count (APC) was determined by plating appropriate dilutions onto tryptic soy agar (TSA; Difco brand, BD) supplemented with cycloheximide (cyclo) (Acros Organics, Geel, Belgium) at
50 mg/liter to reduce mold growth. *E. coli/c*oliform (ECC) counts were determined on CHROMagar ECC plates (CHROMagar, Paris, France). Colonies were counted after incubation at 37°C for 24 h. All colonies that were visible on TSA after 24 h were included in the APC count and all pink (coliform) and blue (presumptive *E. coli*) were counted on CHROMagar ECC.

**Statistical analysis.** Orchard samples (from tree to dehydrator) were obtained by following walnuts in a single orchard from harvest through to hulling and dehydration; six random samples were analyzed from the bags of walnuts collected at each point. All antimicrobial treatments, except BioSide HS 15% in trial 2, were evaluated over two applications that occurred on separate days or in some cases twice on the same day with a 3-h gap between samplings; three 50-g walnut samples and three single sponge samples per location were analyzed during each antimicrobial application (*n* = 6). In trial 2, three samples were collected after a single treatment with BioSide HS 15% at 100 and 200 ppm (*n* = 3). The change in microbial levels in the float tank water and on the conveyor belts for the first 3 h after startup was determined by collecting three samples from each location at each time point (*n* = 3). All statistical analyses were performed using the JMP 10 software package (SAS Institute, Cary, NC) including analysis of variance and *t*-tests. Differences between means were considered significant at *P* < 0.05.

**RESULTS**

**Microbial loads of walnuts through harvest, hulling and drying.** Walnuts were sampled during two harvest seasons (2011, trial 1; 2012, trial 2) to quantify microbial populations during harvest, hulling, drying, and storage. The change in microbial loads from the tree through hulling and drying was determined for visibly intact inshell ‘Chandler’ walnuts in
trial 1. Levels of APC and ECC on inshell walnuts were 6 and 4 log CFU/nut, respectively, when collected directly from the tree before shaking (Fig. 2.6). These levels generally increased by approximately 1 log CFU/nut from receiving through hulling (collected at the float tank) and sorting, and then decreased by approximately the same amount during drying (sampled approximately 12 h later after drying was complete) to 6 and 5 log CFU/nut, respectively.

Microbial levels were also determined for kernels extracted from corresponding walnuts with visibly intact shells and, in some cases, from walnuts with visibly broken shells. Levels of APC and ECC on kernels extracted from walnuts with intact shells retrieved from the tree were 2.1 and 1.7 log CFU/nut, respectively (limit of detection [LOD] was 50 CFU/nut or 1.7 log CFU/nut). APC and ECC levels increased by 2 and 1 log CFU/nut, respectively, at receipt (after shaking, sweeping, transport, and unloading) for walnuts with visibly intact shells (Fig. 2.7). Thereafter, levels were not significantly different (APC) or increased by about 1 log (ECC) during hulling, sorting, and drying. In contrast, APC and ECC on kernels extracted from broken shells at the sort table were 2.2 and 2.5 log CFU/nut, respectively, which was higher than the corresponding levels on kernels from intact shells (Fig 2.7). However, after drying, levels of APC (5.3 or 5.6 log CFU/nut, respectively) and ECC (4.2 log CFU/nut) on kernels from intact or broken shells were not significantly different.

To further explore the importance of shell integrity on contamination of walnut kernels, a second study was undertaken in trial 2 to compare ‘Hartley’ (a hard-shell cultivar) to ‘Chandler’ (a thin-shell cultivar) walnuts. Samples were collected before harvest from the tree and after shaking to the ground, after sweeping into windrows, at huller receipt, and after hulling (sort table). The microbial levels on the shells ranged from 5.4 to 7.9 log CFU/nut (APC) and 4.7 to 7.1 log CFU/nut (ECC) for both the ‘Hartley’ and ‘Chandler’ varieties. Although there was a
significant difference between the two varieties and at some sample locations within a variety there was no apparent trend in the data (Fig. 2.8). In general, APC and ECC counts on inshell walnuts collected from the tree in trial 2 were at least 1 log higher than observed in trial 1; counts for walnuts collected at the sort table were similar in both trials (6 to 7 log CFU/nut for APC and 6 log CFU/nut for ECC).

Before hulling, the microbial levels of kernels extracted from visibly intact ‘Hartley’ and ‘Chandler’ walnuts were similar ranging from 1.6 to 2.7 log CFU/nut (APC) and 1.3 to 2.1 log CFU/nut (ECC) (Fig. 2.9). APC and ECC were not significantly different among samples collected from the tree through to huller receipt (Fig. 2.9). APC and ECC levels on the kernels increased significantly after hulling for both walnut cultivars: on ‘Hartley’ kernels the populations increased to 3.6 and 3.1 log CFU/nut, respectively and on ‘Chandler’ kernels the populations increased to 4.1 and 3.4 log CFU/nut, respectively. There was no significant difference between the two cultivars for either APC or ECC levels on kernels at any collection point.

**Shell breakage during processing.** Walnuts are exposed to significant physical force from harvest to drying. To determine the degree of shell breakage between harvesting and drying, walnut shell breakage scores were determined for inshell ‘Chandler’ walnuts sampled from the orchard floor, at huller receipt, after hulling and sorting, and after drying. ‘Hartley’, ‘Tulare’, and ‘Vina’ walnuts were also sampled and breakage scores determined at one to three of these locations (Appendix Table A.1, Fig. A.1). ‘Chandler’ and ‘Tulare’ are thin shell varieties (primarily used for sale as kernels) and ‘Hartley’ and ‘Vina’ are hard shell varieties (primarily used for sale inshell). Walnut shells of all cultivars had a lower level of breakage when sampled at points before drying (orchard, receiving pit, and sort table) than after drying.
Before drying, 96–99% of ‘Chandler’ walnuts were visibly intact (breakage level 0). After drying, 23% of ‘Chandler’ walnuts were intact, 47% had minor breakage (level 1), and 30% had more significant damage (level 2–5). At receipt, 97–99% of ‘Tulare’ and ‘Vina’ walnuts were intact; after drying, 68% of ‘Tulare’ walnuts were intact, 13% had minor breakage, and 20% had more significant damage; 70% of ‘Vina’ walnuts were intact, 15% had minor breakage, and 14% had more significant damage. ‘Hartley’ variety walnuts were only sampled after drying; 25% of sampled nuts were intact, 9% had minor breakage, and the remaining 66% had significant damage. The breakage from levels 2 to 5 for ‘Hartley’ was approximately evenly distributed, with 14–19% in each category.

**Microbial loads on huller equipment over time without antimicrobial sprays.**

Microbial loads of the conveyors and the float tank were monitored during a 3-h processing period to better understand how the populations on the equipment change as the day progresses. At the end of the day the hulling equipment was sprayed down with water to remove visible debris and then allowed to dry overnight. The float tank also was emptied and rinsed at the end of the day and refilled with approximately 1500 liters (400 gallons) of well water the next morning before the hulling began. Baseline microbial loads were determined for conveyor belts that were sampled first thing in the morning before running any walnuts. The equipment was then run at standard speed with walnuts that were sprayed with water after the hulling step. The initial APC levels were 2.8 and 4.1 log CFU/100 cm² for conveyer belts A and B, respectively; these levels increased to 6.8 and 6.5 log CFU/100 cm², respectively (Fig. 2.11) after the facility had been hulling walnuts for 30 min. Similar increases were observed for the ECC levels, which were initially 1.9 and 3.9 log CFU/100 cm² and then increased to 6.1 and 6.3 log CFU/100 cm², for belts A and B, respectively. From 0.5 to 3 h, the microbial loads on the conveyors fluctuated.
by approximately 0.5 log CFU/100 cm². APC and ECC levels in the float tank were 6.5 and 6.0 log CFU/ml before running walnuts and increased 0.8 to 1.2 log CFU/ml, respectively, over 3 h of processing (Fig. 2.11). The maximum level measured for the float tank water, for both APC and ECC, was 7.3 log CFU/ml.

**Effect of antimicrobials on walnut and conveyor microbial loads.** Antimicrobial sprays were evaluated for efficacy in reducing microbial loads on inshell walnuts as well as on huller equipment. In trial 1 the effect of a single formulation of PAA (i.e., Bioside 15%, 100 ppm PAA), alone and in combination with LAE, on the microbial loads of walnut shells and of kernels from both intact and broken shells was evaluated. No significant difference in microbial loads was found on inshell walnuts (Fig. 2.12) or kernels (Fig. 2.13), from either intact or broken shells, between the water-sprayed control samples and either of the antimicrobial treatments.

The effect of different PAA products at different concentrations on microbial levels on walnut shells and conveyor belts was compared in trial 2. The highest average reduction of microbial levels on walnut shells observed was 0.9 log CFU/nut and was achieved with StorOx 2.0 at 200 ppm (Figure 2.12). Greater reductions in APC and ECC counts were observed when PAA formulations were applied at 200 ppm than at 100 ppm, with the exception of BioSide HS 15%, which had similar reductions for both 100 and 200 ppm. Compared with water, only StorOx 2.0 at 200 ppm resulted in a significantly higher reduction in both APC and ECC counts on inshell walnuts; treatment with SaniDate 5.0 at 200 ppm had a significantly higher reduction in ECC counts only.

Reductions in APC and ECC achieved with use of an antimicrobial on the conveyer belts were higher than those observed on the walnut shells. A reduction of up to 4.4 log CFU/100 cm²
(ECC) was achieved with SaniDate 5.0 at 200 ppm (Figure 2.14). The microbial levels on the conveyor belts were not reduced with every antimicrobial application, but when observed the reductions ranged from 0.2 to 4.4 log CFU/100 cm². Reductions were similar for both A and B belts. Greater reductions were observed at 200 ppm than at 100 ppm for all products, though the difference was not always significant.

**Microbial loads on inshell walnuts and kernels during storage.** In trial 1, natural microbial populations were measured on treated and untreated walnuts during commercial storage to determine the effect of storage on population size and to evaluate if the PAA or PAA and LAE had a residual impact on these populations. The walnuts were placed into storage in October 2011; warehouse temperature and relative humidity were recorded beginning in January 2012. The average ambient warehouse conditions were 10–12°C and 63–65% RH for January and February 2012. The average ambient external temperature and RH was 7.3 and 9.7°C and 81 and 76% for January and February, respectively, for the Stockton area (National Climactic Data Center, 2013). Walnuts were moved to controlled-temperature storage on March 30, 2012 once ambient temperatures began to increase. Average conditions in the controlled-temperature warehouse were 9–11°C and 50–54% RH from March to June 2012.

Initial microbial loads on inshell water-sprayed walnuts after drying (t = 0 months) were 5.5 and 5.1 log CFU/nut for APC and ECC, respectively (Fig. 2.15), and decreased to 3.5 and 3.0 log CFU/nut, respectively, during ambient warehouse storage (t = 1 to 5 months). During controlled-temperature storage the APC and ECC counts remained within a 0.5 log CFU/nut range ending at 3.8 and 2.9 log CFU/nut after a total of 9 months of storage. There was no significant difference in microbial loads between walnuts that were sprayed with water and walnuts that were sprayed with either antimicrobial treatment at any sampling point.
Initial microbial loads on kernels from water-sprayed walnuts with visibly intact shells were 5.3 and 4.2 log CFU/nut for APC and ECC, respectively (Fig. 2.16). Populations on kernels from water-sprayed walnuts with visibly broken shells were initially 5.6 and 4.2 log CFU/nut for APC and ECC, respectively. APC and ECC loads on kernels from walnuts with visibly intact shells decreased to 2.0 and 1.6 log CFU/nut, respectively, during ambient warehouse storage. APC and ECC counts on kernels from walnuts with visibly broken shells decreased to 3.3 and 2.7 log CFU/nut, respectively, during ambient warehouse storage. Once in controlled-temperature storage (t = 6 to 9 months) the microbial levels on shells and kernels from both intact and broken shells were within a 0.5 log CFU/nut range for both APC and ECC. Reductions on antimicrobial-treated walnuts were similar to those on water-sprayed walnuts (Fig. 2.16).

**DISCUSSION**

**Microbial loads of walnuts through harvest, hulling and drying.** Walnuts develop inside an intact moist, green hull which is thought to protect the shell and kernel from microbial contamination (Meyer and Vaughn, 1969). Depending on maturity at harvest, the hull may remain firm, moist and green, it may have begun to decompose, or dry out and dehisce or split open (Olson et al., 1998). Both green and brown hulls can support microbial growth especially when crushed to release cell nutrients (Blessington, 2011).

Throughout this study the microbiota as determined on TSA and CHROMagar ECC media were used as global indicators of contamination and as a measure of the efficacy of antimicrobials applied post hulling. TSA is a general nutrient-rich, nonselective medium that was used to determine levels of Gram-positive or Gram-negative mesophilic bacteria capable of forming colonies under aerobic conditions. Coliform and *E. coli* populations were measured
using CHROMagar ECC, a selective and differential media. On this media, *E. coli* form blue colonies, other coliforms mauve colonies and other bacteria are either inhibited or form colorless colonies. Blue colonies were not present on most of the countable plates for most samples and as such were included in the total coliform count instead of being enumerated separately. The APC was typically higher than the ECC levels by 1 to 1.5 log CFU/nut suggesting that approximately 10% of the aerobic plate count might have been coliform bacteria.

Coliforms are sometimes used as indicators of the potential behavior of Gram-negative foodborne pathogens such as *Salmonella* and *E. coli* O157:H7. The coliform count is a subset of the total aerobic plate count that represents gram negative, non-spore forming enteric bacteria that can ferment lactose. Coliforms are commonly found on plant material and thus the presence of coliform bacteria was not unexpected.

Many bacteria are incapable of forming colonies on TSA and CHROMagar ECC under the incubation conditions used here. Molecular methods such as real-time PCR or fluorescence in situ hybridization would permit a more thorough investigation into the total bacterial amounts on the nuts than the methods employed here. However, these methods were not considered to provide an advantage over the more traditional cultural techniques in addressing the objectives of the study. They may however, be useful tools in understanding the sources of kernel contamination and should be considered in future studies.

Microbial loads on the shell of inshell walnuts collected from the tree through hulling and drying were consistent across trials. Walnuts were sampled from fruit with fully split hulls, and it is possible that the shell becomes contaminated from the decomposing hull or from insects, animals, or aerosols. The observed increase in microbial loads after the walnuts were harvested is
likely due to a combination of exposure to orchard soil and cross contamination among nuts and from equipment during harvest and hulling. The net microbial load after drying was similar to that of walnuts on the tree. Although not addressed in this study, an assessment of the microbial diversity on walnuts collected from the tree through to the dehydrated product might aid in identifying more specific sources of contamination.

Although inshell walnuts collected from the tree had APC and ECC counts of more than 5 log CFU/nut, the kernels aseptically extracted from these walnuts had counts that were several orders of magnitude lower. In most cases counts were under 10 colonies per sample, demonstrating that it was possible to extract kernels with very low-level contamination. This suggests that the shell provides reasonable protection to the kernel from microbial contamination while the nut is in the tree. However, microbial loads on the kernels increased after the walnuts were harvested even when the shell was visibly intact. *Salmonella* can infiltrate intact, inshell almonds in an aqueous suspension (Danyluk et al., 2008), demonstrating that the nutshells do not provide a perfectly impervious barrier to microorganisms. It is also possible that there were undetectable breaks in the walnut shell or along the suture or at the stem end. Meyer and Vaughn (1969) showed that dye was able to infiltrate through apparently intact black walnut shells (primarily through the suture) to the kernel when nuts were briefly submerged in an aqueous environment; contamination of these walnuts with *E. coli* also increased with decreasing shell integrity. Likewise, water uptake (and *Salmonella*) into pecans increased as the amount of shell damage increased and when pecan temperatures were warmer than water temperatures (Beuchat and Mann, 2010). Unlike pecans, which are purposely soaked for 3 to 5 min in water, walnuts typically pass through the float tank in a matter of seconds; thus temperature differential is less likely to play a role in infiltration into walnuts.
When shells are compromised the opportunity for contamination of the kernel increases. In the current study, significantly higher microbial levels were observed on walnut kernels extracted from walnuts with visibly broken or missing shells. An increase in the microbial loads of nut kernels when the shell was broken or otherwise compromised has also been observed in almonds (King et al., 1970), black walnuts (Meyer and Vaughn, 1969) and pecans (Beuchat and Mann, 2010). The point at which breakage occurs will influence the potential source of microorganisms—from direct exposure to orchard soil, from float tank water, or from huller equipment.

Visible shell breakage dramatically increased as the walnuts moved from receipt at the huller to after drying (Fig 2.10), likely a result of pressure stresses on the shell as the nut moved over the various pieces of equipment. Greater than 90% of the walnuts sampled in this study had visibly intact shells upon arrival at the huller and through the hulling process, regardless of variety or shell thickness. After drying, the proportion and degree of breakage increased significantly, most substantially in ‘Chandler’ variety walnuts. This observation is consistent with Meyer and Vaughn (1969) who noted that dried samples of black walnuts had a higher proportion of shell breakage than wet (undried) samples. Dried shells are more brittle than undried shells, which potentially increases the opportunity for additional breakage during post-drying transport, especially for thin shell varieties. While there is some potential for mechanical damage as the drying bins are loaded, this is unlikely to explain the increase in breakage that was observed. A more likely explanation is that invisible shell fractures present before drying are exposed during drying as the shell contracts.

The thickness of the walnut shell differs among cultivars and also can be influenced by growing conditions or maturity at harvest. Various terms are used to describe the shell strength
of walnuts, but the general difference is whether a variety is primarily used for inshell (‘hard shell’ varieties), which generally have a stronger shell, or for kernels (‘thin shell’ varieties), which have a thinner shell that is easier to remove. Thin shell varieties, such as ‘Chandler’ and ‘Tulare’, have become increasingly popular with walnut growers and handlers due to the increased demand for walnut kernels. There was no significant difference between the microbial loads of walnut kernels from intact shells of ‘Chandler’ (thin shell variety) and ‘Hartley’ (hard shell variety) walnuts during harvest. However, microbial levels on ‘Chandler’ walnuts were significantly higher after hulling; it is possible that the higher level of shell breakage post-drying, lead to higher contamination rates. Food safety has not been a consideration in traditional breeding programs but varieties that are more resistant to shell breakage or the use of modifications to the harvest and hulling processes that would reduce breakage may offer opportunities to decrease microbial contamination of kernels.

**Microbial loads on huller equipment.** A guide for food safety and good manufacturing practices (GMPs) for walnut hullers and dryers (DFA, 2012) recommends regular replacement of float tank water to minimize organic loads that may be detrimental to product quality. The float tank is a large vessel of water ranging from 500 to 1500 liters through which all the walnuts are briefly (20 s) passed to remove rocks and other heavy debris which sink while the walnuts float.

The float tank used in this study was drained at the end of the day, rinsed with water and refilled the next morning. The high volume of walnuts passing through the float tank quickly lead to a darkening of the water due to a mixture of soil, hull material, tree debris and other organic matter. Although the float tank was drained and rinsed at the end of each day the volume of organic matter clinging to incoming walnuts quickly overwhelms the system. The float tank is an obvious place for significant cross contamination within and between loads of walnuts.
Maintaining the sanitary quality of the float tank water during operation is challenging due to the high organic load of the water. Chemical sanitizers would likely be rendered ineffective. Therefore, the application of an antimicrobial spray post float tank was evaluated as means to reduce the potential for widespread cross contamination.

After the float tank walnuts are typically rinsed with water. Some of this water clings to the nutshell and is carried through the huller system to the dryer bins. Water from the walnuts is transferred to various contact surfaces including conveyor belts and increases the potential for cross contamination of among walnuts. Without the application of antimicrobials, the microbial loads of conveyor belts increased rapidly (within 30 min of startup), and remained at 6 log CFU/100 cm² for the monitoring period.

**Effect of antimicrobials on walnut and conveyor microbial loads.** PAA sprays applied post float tank were evaluated as a means to reduce microbial loads on the walnuts and conveyor belts. Application of PAA did not significantly reduce natural microbial populations on walnut shells under laboratory conditions (data not shown) or under commercial conditions. In addition, the data were highly variable from sample to sample, with 2 log CFU/nut reductions and increases both before and after the treatment. Walnut shells have a rough surface which could reduce the effectiveness of PAA sprays. In addition, there were very short contact times (approximately 13 s) between the PAA and the walnuts. Extending contact time between the PAA and the walnuts could cause additional decreases in microbial populations. Initial microbial loads were inconsistent among samples, further complicating our ability to quantify small differences. PAA has been shown to significantly reduce *Salmonella* on various food products including tomatoes (Chang and Schneider, 2012) and pecans (Beuchat et al., 2012) under laboratory conditions. Beuchat et al. (2012) showed reductions of 2.4 log CFU/g for *Salmonella*
on inshell pecans when exposed to 40 ppm PAA for 1 min in a laboratory setting but they did not evaluate PAA under commercial conditions.

Not all walnut hullers are configured in the same manner and the potential benefits need to be weighed against the costs for each individual operation. During application of the PAA sprays, a fine mist of sanitizer was being created, which caused an irritation hazard for the employees working in close proximity to the spray bars. The irritation was observed even with the lowest tested concentration (100 ppm). Measures would need to be taken to ensure employee comfort and safety before the installation of this type of system in a walnut huller. The added cost of the equipment, water and sanitizers would also need to be taken into account when deciding to install an antimicrobial spray system.

The PAA sanitizers evaluated in the current study were mostly effective at reducing the microbial loads measured on the conveyor belts; significant reductions were observed for all but one treatment (BioSide 15%, 100 ppm) and in some instances the difference between water and the PAA was up to 2 log CFU/100 cm². The use of PAA to sanitize conveyer belts should be further explored. Consistent reduction in the microbial loads on conveyer belts may help prevent cross contamination. PAA has been found to be effective at reducing E. coli O157:H7 on a stainless steel meat grinder by 3 to 4 log CFU/cm² after processing artificially contaminated ground beef (Farrell et al., 1998). PAA has also been found to reduce E. coli on a stainless steel surface by 8 log CFU/ml in 3.1 min of contact time at 60 ppm (Kunigk and Almeida, 2001).

PAA-based sanitizers are an equilibrium mixture of PAA with hydrogen peroxide (H₂O₂) and acetic acid accompanied by various stabilizers designed to prevent the breakdown of the PAA. PAA breaks down into water and acetic acid, meaning no harmful byproducts are formed
and wastewater can be disposed of easily without negative environmental impact (Kitis, 2004). Because PAA is the primary bactericidal component in these formulations the focus was on PAA concentration rather than H₂O₂, which is more effective as a sporicide (Baldry, 1982).

**Microbial loads on inshell walnuts and kernels during storage.** Walnuts are stored for up to 1 year before sale and distribution. During the time period of the storage study the microbial populations declined significantly. The observed survival of natural populations of microbes in walnuts during commercial storage are similar to previously observed reductions in inoculated populations of bacteria (typically pathogens) on both inshell nuts (Beuchat and Heaton, 1975; Beuchat and Mann, 2010; Blessington, 2013; Kimber et al., 2012) and kernels (Beuchat and Mann, 2010; Blessington, 2012; Kimber et al., 2012; King et al., 1970; Kokal, 1939). Many of these studies have observed an initial period of reduction after which the microbial populations do not change for an extended period of time. The antimicrobial treatments did not have any additional significant effect on microbial loads during storage of the walnuts.

Currently, walnut huller-dehydrators are not considered to be food processors but rather the managers of raw agricultural commodities or RACs, similar to a fruit packinghouse. The huller-dehydrator serves a function between the grower and the handler or walnut processor. In a comprehensive food safety program the walnut grower would adhere to good agricultural practices (GAPs) to minimize the potential for the introduction of hazards into the harvested product. The huller-dehydrator would follow basic GMPs to prevent the introduction or amplification of hazards in their facility and the handler would have a hazard analysis and critical control points program in place.
As part of the Food Safety Modernization Act (FSMA), two major rules have been proposed: the Produce Safety Rule and the Preventative Controls Rule (FDA, 2013). As currently proposed, walnuts and other tree nuts will be covered under the Produce Safety Rule, which will require the application of science-based standards for the growing, harvesting, packing and holding of walnuts. Huller-dehydrators will be either covered by the Produce Safety Rule or the Preventative Controls Rule depending upon the source of the walnuts that are hulled. A risk analysis will need to be performed to identify sites of potential contamination for walnuts and appropriate preventative measures will need to be taken in order for walnut huller-dehydrators to be in compliance with the new legislation. Efforts to control cross contamination in a walnut huller would aid in ensuring that the potential risk posed by the hulling environment is being considered and a system is in place to minimize the risk.

Though low-moisture foods were once considered safe from a microbial perspective, recent outbreaks that have included tree nuts have shown these foods to be a potential source of foodborne pathogens. It is important for the walnut industry to take preventative measures to ensure they are doing everything possible to prevent contamination of walnuts. Understanding the routes of contamination and microbial survival during storage for walnuts will help in identifying where contamination risks exist and will allow for identification and evaluation of appropriate intervention methods. In the current study, antimicrobial sprays were not effective at reducing microbial populations on walnut shells, but the application of these sprays to control microbial loads on hulling equipment may aid in reducing the potential for cross contamination. All these factors should be considered by walnut handlers and used to supplement existing individualized food safety programs.

ACKNOWLEDGMENTS
Funds for this research were provided by the California Walnut Board. The cooperation of collaborating walnut growers and handlers is greatly appreciated. Special thanks to Dr. Anne-laure Moyne, Tyann Blessington, Vanessa Lieberman and Scott McCarthy for technical assistance during the field trials. Thanks also to all the companies who donated supplies for these trials: Spraying Systems Co., Enviro Tech Chemical Services, A&B Ingredients and BioSafe Systems. The contributions made by Sylvia Yada during the writing process are greatly appreciated.
REFERENCES


Figure 2.1. Diagram of huller-dehydrator used in trial 1 (2011). Walnut sampling points are indicated by black Xs. The peracetic acid (PAA) tank and spray line locations are shown in dark gray. The lauric arginate (LAE) tank and spray line locations are shown in light gray. The direction of flow through the system is indicated by black arrows.
Figure 2.2. Diagram of huller-dehydrator used in trial 2 (2012). Walnut sampling points are indicated by black Xs and the conveyor belt sampling points (A and B) are indicated by black squares. The peracetic acid (PAA) tank and spray line locations are shown in dark gray. The direction of flow through the system is indicated by black arrows.
Figure 2.3. In trial 1 two parallel PAA spray bars were placed across the conveyor immediately following the float tank (A) and two parallel LAE spray bars were placed immediately prior to the sort line conveyor, the first of which is pictured (B).
Figure 2.4. In trial 2, the PAA spray system consisted of two parallel spray bars running up the first conveyor belt after the squirrel cage (A) and a spray bar immediately following the shaker table prior to the sort line conveyor (B).
Table 2.1. Summary of antimicrobial products tested

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
<th>Concentration of active ingredient (%)</th>
<th>PAA(^a)</th>
<th>H(_2)O(_2)(^b)</th>
<th>LAE(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CytoGuard LA</td>
<td>A&amp;B Ingredients, Fairfield, NJ</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>10.0</td>
</tr>
<tr>
<td>StorOx 2.0</td>
<td>BioSafe Systems Co., East Hartford, CT</td>
<td></td>
<td>2.0</td>
<td>27.0</td>
<td>NA</td>
</tr>
<tr>
<td>SaniDate 5.0</td>
<td>BioSafe Systems Co.</td>
<td></td>
<td>5.3</td>
<td>23.0</td>
<td>NA</td>
</tr>
<tr>
<td>SaniDate 12.0</td>
<td>BioSafe Systems Co.</td>
<td></td>
<td>12.0</td>
<td>18.5</td>
<td>NA</td>
</tr>
<tr>
<td>BioSide HS 15%</td>
<td>EnviroTech, Modesto, CA</td>
<td></td>
<td>15.0</td>
<td>22.0</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(^a\) PAA, peracetic acid.

\(^b\) H\(_2\)O\(_2\), hydrogen peroxide.

\(^c\) LAE, lauric arginate ethyl ester.
Table 2.2. Concentration of peracetic acid (PAA) applied to walnuts in various antimicrobial treatments

<table>
<thead>
<tr>
<th>Trial</th>
<th>Antimicrobial treatment</th>
<th>Concentration of PAA (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BioSide HS 15%</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>BioSide HS 15% + 2% CytoGuard LA</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>StorOx 2.0</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>SaniDate 5.0</td>
<td>100, 200</td>
</tr>
<tr>
<td></td>
<td>SaniDate 12.0</td>
<td>100, 200</td>
</tr>
<tr>
<td></td>
<td>BioSide HS 15%</td>
<td>100, 200</td>
</tr>
</tbody>
</table>
Figure 2.5. Walnut breakage scale. Walnuts were sorted based on the description for each level of breakage. Images show examples of walnuts from each category.

0 – No visible breakage; suture appears tightly sealed (“intact”)

1 – Very minimal cracks in the shell, kernel not visible; very slight separation of suture

2 – More substantial cracks in the shell, no pieces of shell missing, kernel may be visible; suture open to a significant level but less than half of circumference along suture

3 – Small pieces of shell missing, kernel obviously visible; approximately half of suture open

4 – Up to one quarter of shell missing; substantially loosened suture (gentle tugging will remove one quarter to one half of the shell)

5 – More than one quarter of the shell missing; kernel very exposed; includes kernels without shells
Figure 2.6. Aerobic plate (A) and *E. coli* /coliform (B) counts on ‘Chandler’ inshell walnuts sampled at selected points from preharvest through hulling and drying. Dryer bin samples were collected after drying. Columns denoted with different letters are significantly different (*n* = 6, *P* < 0.05).
Figure 2.7. Aerobic plate (A) and *E. coli*/*coli*orm (B) counts on ‘Chandler’ walnut kernels sampled at selected points from harvest through hulling and drying. Kernels were extracted from walnuts with visibly intact shells (closed bars) or visibly broken shells (open bars). Counts denoted with different uppercase or lowercase letters are significantly different (*P* < 0.05); lowercase letters identify intact shells and uppercase letters identify broken shells. Counts for paired intact and broken shells marked with an asterisk (*, on the x-axis) are significantly different (*P* < 0.05). Limit of detection (LOD) = 1.7 log CFU/nut.
Figure 2.8. Aerobic plate (A) and *E. coli*/*coli*form (B) counts on inshell ‘Hartley’ (closed bars) and ‘Chandler’ (open bars) walnuts. Counts denoted with different uppercase or lowercase letters are significantly different (*P* < 0.05); lowercase letters identify ‘Hartley’ samples and uppercase letters identify ‘Chandler’ samples. Counts for paired ‘Hartley’ and ‘Chandler’ samples marked with an asterisk (*, on the x-axis) are significantly different (*P* < 0.05). Limit of detection (LOD) = 1.3 log CFU/nut.
Figure 2.9. Aerobic plate (A) and *E. coli*/*coliform* (B) counts on kernels extracted from visibly intact (breakage level 0) ‘Hartley’ (closed bars) and ‘Chandler’ (open bars) walnuts. Counts denoted with different uppercase or lowercase letters are significantly different (*P* < 0.05); lowercase letters identify ‘Hartley’ samples and uppercase letters identify ‘Chandler’ samples. Counts for paired ‘Hartley’ and ‘Chandler’ samples marked with an asterisk (*, on the x-axis) are significantly different (*P* < 0.05). Limit of detection (LOD) = 1.3 log CFU/nut.
Figure 2.10. Comparison of ‘Chandler’, ‘Tulare’ and ‘Vina’ variety walnuts for shell breakage (according to the scale in Fig. 2.5) distribution for walnuts collected at the receiving and after drying, with the degree of breakage from none visible (lightest gray) to over one quarter missing shell and exposed kernels (black).
Figure 2.11. Aerobic plate (A) and *E. coli*/coliform (B) counts from conveyor A (circles), conveyor B (squares) and float tank water (triangles) sampled from time of equipment startup. Water was applied to the walnuts during the 3-h sampling period.
Figure 2.12. Reductions in aerobic plate counts (A) and *E. coli*/*coli* counts (B) on walnut shells after treatment with water or antimicrobial product containing PAA at 100 or 200 ppm. Black circles indicate the mean of each treatment. Treatments marked with an asterisk (*) had significantly higher average reductions than water.
Figure 2.13. Aerobic plate (black) and *E. coli* /coliform (gray) counts on kernels extracted from intact ‘Chandler’ before (float tank) and after (sort table) being sprayed with water, 100 ppm PAA or 100 ppm PAA followed by 2% LAE. No significant difference was found for any point within either APC or ECC counts ($P > 0.05$).
Figure 2.14. Aerobic plate (black) and *E. coli/*coliform (gray) counts on two conveyor belts (belt A – closed bars, belt B – open bars) in a commercial huller when sprayed with water or antimicrobial product containing PAA at 100 or 200 ppm. All treatments produced significantly lower counts than water on both belts with the exception of BioSide 15% at 100 ppm.
Figure 2.15. Aerobic plate (A) and E. coli/coliiform (B) counts on inshell ‘Chandler’ walnuts treated with water (circles), PAA (squares), and PAA+LAE (triangles) through 9 months of commercial storage.
Figure 2.16. Aerobic plate (A, B, and C) and *E. coli*/*coli*form (D, E, and F) counts for kernels from intact (closed symbols) and broken (open symbols) Chandler walnuts treated with water (A and D), PAA (B and E), and PAA+LAE (C and F) through commercial storage. Intact/broken pairs marked with an asterisk (*) are significantly different ($P < 0.05$). Limit of detection (LOD) = 1 log CFU/nut.
Chapter 3: Prevalence and Survival of Foodborne Pathogens on Inshell California Walnuts

ABSTRACT

Over the past decade, outbreaks linked to low-moisture foods have resulted in increased interest in understanding the prevalence, levels, and survival of foodborne pathogens on tree nuts. The prevalence of *Salmonella* and *Escherichia coli* O157:H7 was determined for 2,839 raw inshell walnut samples collected in 2010, 2011, and 2012 from walnut handlers throughout California. *Salmonella* was not isolated from 100-g samples in 2010 but was isolated from 2 of 905 375-g samples in 2011 and 1 of 999 375-g samples in 2012 (average 0.11% prevalence). *E. coli* O157:H7 was not recovered from 1,904 375-g samples tested in 2011 and 2012.

Survival of five-strain cocktails of *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* inoculated onto inshell walnuts (at 8 log CFU/g) was measured under simulated commercial drying and storage conditions. All three pathogens declined by 2.5 to 3.0 log CFU/nut between inoculation and drying. During storage, *Salmonella* survived at higher levels for a longer time than either *E. coli* O157:H7 or *L. monocytogenes*. *Salmonella* populations remained at about 3 log CFU/nut through 7 months of storage. In contrast, *E. coli* and *L. monocytogenes* populations decreased rapidly to 3 and 2 log CFU/nut, respectively, within the first 8 and 27 days of storage; thereafter population densities declined slowly to about 2.2 and 1.2 log CFU/nut, respectively, by 2 and 5 months of storage, respectively. Although significant decreases in foodborne pathogens occurred during drying and initial storage of inshell walnuts, the potential exists for low-level long-term persistence during commercial storage.

INTRODUCTION
Although uncommonly linked to foodborne illness, several tree nuts (almonds, pine nuts, and inshell hazelnuts) have been associated with outbreaks of salmonellosis or *Escherichia coli* O157:H7 gastroenteritis (CDC, 2004, 2011a, 2011b). In 2011 there was an outbreak of *E. coli* O157:H7 gastroenteritis in Canada that was epidemiologically linked to walnuts (CFIA, 2011b; Health and Safety Watch, 2011; PHAC, 2011), although the pathogen was not detected in remaining samples of the implicated walnuts. Detection of *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes* in walnut samples has led to Class I recalls (CFIA, 2011a, 2011b, 2012; CDPH, 2010; Mojave Foods Corporation, 2010; Tropical Nut & Fruit of Charlotte, NC, 2010; FDA, 2009, 2010, 2011).

Surveys of agricultural commodities for the presence of foodborne pathogens are not common, but published data are available for the prevalence of *Salmonella* in California almonds and U.S. peanuts (Bansal et al., 2010, Calhoun et al., 2013; Danyluk et al., 2007, Lambertini et al., 2012). Some surveys on walnut contamination have been performed outside of the United States. *Salmonella* was not isolated from a total of 515 25-g walnut kernel samples collected in two surveys from retail stores in the UK (Little et al., 2009, 2010) or from walnuts (n = 80, 25-g samples) sampled at growers, manufacturers and retailers in Australia (NSW Food Authority, 2012). *Salmonella* was isolated from a single sample out of 50 10-g samples of walnut kernels collected in India (Riyaz-Ul-Hassan et al., 2003).

There are many points during harvest and hulling that can influence microbial loads of walnut shells and kernels. On the tree, walnut kernels are surrounded by a hard shell and fleshy hull that protect the kernel from contamination. Walnuts are harvested by mechanically shaking to the ground and then they are mechanically swept up for transport to a huller-dehydrator. At
the huller-dehydrator the walnut fruits are separated from rocks, sticks, leaves, and other debris. This process includes rapid passage through a water-filled “float” or “rock” tank to separate sinking rocks from the floating walnuts. Background microbial populations (aerobic and coliform counts) in the float tank water were shown to increase quickly to 6 log CFU/ml or greater during walnut processing (Blessington, 2012; Chapter 2). After passage through the float tank, walnuts are mechanically hulled under wet conditions to remove any flesh from the fruit which may remain after harvest, leaving the inshell nut. Aerobic plate counts and coliform counts for walnuts collected after the float tank were found to be 5 to 7 log CFU/nut (Chapter 2). Rinsing with water or an antimicrobial spray prior to drying did not consistently reduce levels of background aerobic or coliform bacteria (Chapter 2). Hulling is followed by drying inshell product for 4 to 48 h at low temperatures (up to 43°C) to preserve nut quality, thereby reducing the moisture content from a range of 10 to 30% to a target of 8% (Kader and Thompson, 2002). Reductions of 0.1 to 2.0 log CFU/nut in microbial loads have been observed at this step (Blessington, 2011, Chapter 2). Dried walnuts are then stored in the shell for up to 1 year in bins or silos at target temperatures of 10°C or less and a target relative humidity less than 65% (Lopez et al., 1995). During storage at cool ambient temperatures, aerobic plate counts and coliform counts decreased approximately 2 to 3 log over the first 4 months of storage; thereafter, further reductions were minimal (Chapter 2). As needed, walnuts are removed from storage and sorted and sold as inshell nuts or cracked and packaged for sale as kernel halves or pieces.

Blessington et al. (2013) evaluated the survival of Salmonella Enteritidis PT 30 inoculated onto dehydrated inshell walnuts. As the inoculum dried, levels of Salmonella declined by 0.7 to 2.4 log CFU/nut with generally higher reductions at lower inoculum levels. 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.

Blessington et al. (2013) also inoculated inshell walnuts with separate cocktails of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* and evaluated survival during storage. Commercially dehydrated walnuts were wet inoculated at relatively low target levels (4–5 log CFU/ml) with the inoculum suspended in 0.1% peptone and dried under ambient conditions for about 24 h before storage at 23°C. Populations of all three pathogens declined by 2 log CFU/nut during drying. Initial levels of the pathogens were near the limit of detection by plating at the initiation of storage, but most samples remained positive by sample enrichment over 3 months of storage.

To better understand the risk of *Salmonella* contamination in walnuts, a survey of inshell walnuts collected from California walnut handlers was performed from 2010 to 2012. In 2011 and 2012 the presence of *E. coli* O157:H7 also was assessed. These surveys provide data on the prevalence of these pathogens naturally found in walnuts. Also, inshell ‘Chandler’ walnuts were inoculated with *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* to better understand how these organisms survive in the walnut storage environment. This study expands upon the previous work of Blessington et al. (2013) by investigating pathogen survival with the following modifications to the study design: inoculating with previously unavailable strains of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* isolated from low-moisture foods or from produce; suspending the inoculum in water to reflect the organic loads found in the float tank; inoculating freshly hulled, undehydrated walnuts; drying walnuts in a manner that mimics commercial
dehydration; and storing walnuts under conditions that mimic commercial storage temperatures, humidity, and times.

MATERIALS AND METHODS

Walnuts samples for survey. Inshell walnuts were collected over a 3-year period (2010-2012) from 15 walnut handlers in the five walnut-growing regions of California (Sacramento Valley, Northern San Joaquin Valley, Southern San Joaquin Valley, North Coast, and Central Coast), representing approximately 50% of the total crop output in the state of California. The same handlers contributed samples each of the 3 years of sampling, with the exception of a single handler, which was replaced in 2012. A total of 935, 905 and 999 approximately 1 kg samples were collected in 2010, 2011 and 2012, respectively (2839 samples total).

Each sample consisted of a single cultivar of walnuts representing a number of major cultivars produced in California, in approximate proportion of their production volume. Samples were taken from incoming trucks arriving at the handler from various hullers. The number of samples collected from each handler was proportional to the amount of walnuts produced by the facility, with 20 samples taken from the smallest handler and 250 taken from the largest. Samples were taken either by DFA of CA (Fresno, CA) staff (60%) or handlers’ quality control (QC) staff (40%) using typical QC sampling techniques. Samples were shipped to the lab for analysis, coded to blind the origin, and stored at 4°C before testing to minimize decreases in Salmonella populations; samples were tested within 2 months of collection. Subsamples (100 g in 2010, 375 g in 2011 and 2012) of walnuts were analyzed for Salmonella (2010-2012) and E. coli O157:H7 (2011 and 2012) by DFA of CA using AOAC Official Methods 2001.09 (AOAC, 200a) and AOAC Research Institute (AOAC-RI) 060903, respectively.
Enrichment for *Salmonella* from walnuts. In accordance with AOAC Official Method 2001.09, survey walnuts were mixed, and 100 g or 375 g subsamples were combined with 900 ml or 3,375 ml of buffered peptone water (BD Diagnostic Systems, Sparks, Md.), respectively in sterile 946-ml or 4,000-ml plastic jars (Bel-Art Products, Pequannock, N.J.) and shaken for 120 s. Following mixing, the samples were loosely capped and incubated at 35 ± 2°C for 18 to 24 h. The overnight preenrichment culture was then subjected to immunoconcentration by the automated mini-VIDAS system (bioMérieux, Hazelwood, Mo.). Pre-enrichment broth (800 μl) was processed on an immunoconcentration *Salmonella* (ICS) test strip (bioMérieux), and the resulting concentrate was used to inoculate a 2-ml vial of ICS broth (bioMérieux); vials were incubated at 41°C for 5 h. After incubation, 1 ml of the ICS broth culture was boiled for 15 min and then cooled to room temperature. To screen for *Salmonella*, 500 μl of the boiled ICS culture was added to an SLM (*Salmonella*) test strip (bioMérieux) and tested for *Salmonella* by the mini-VIDAS system. The VIDAS screening system is based on an enzyme-linked fluorescent assay; a relative fluorescence value greater than 0.23 was considered a positive result.

If a sample was positive for *Salmonella* by the VIDAS system, the remaining (unboiled) portion of the ICS broth culture from the vial was streaked onto three selective agars: bismuth sulfite, Hektoen Enteric, and xylose lysine desoxycholate. Plates were examined for typical *Salmonella* colonies after incubation at 35°C for 24 h (Hektoen Enteric and xylose lysine desoxycholate agars) or 48 h (bismuth sulfite agar). Suspect colonies were checked for purity by restreaking them onto plates of MacConkey agar (BD Diagnostic Systems). MacConkey agar plates were incubated at 35°C for 24 h and examined for typical *Salmonella* colonies. The identity of *Salmonella*-suspect colonies was confirmed with API 20E test strips according to the manufacturer’s instructions.
**Enrichment for E. coli O157:H7 from walnuts.** In accordance with AOAC-RI Official Method 060903, 100 g or 375 g subsamples were combined with 900 ml or 1,125 ml of buffered peptone water (BD) with vancomycin additive, respectively, in sterile 946-ml or 4,000-ml plastic jars (Bel-Art Products, Pequannock, N.J.) and shaken for 120 s. Following mixing, the samples were loosely capped and incubated at 42 ± 1°C for 10 to 24 h. Pre-enrichment broth (500 µl) was added to an ECPT strip and heated for 5 ± 1 min prior to performing the VIDAS assay. If a positive result was obtained, a second sample (500 µl) was taken from the unboiled enrichment broth and subjected to a VIDAS ICE assay. The immunoconcentrated solution was then collected with a calibrated swab and streaked onto CT SMAC plates and incubated at 37 ± 1°C for 18 to 24 h.

After incubation, plates were examined for colonies typical of *E. coli* O157:H7 (colorless or neutral/gray with a smoky center). Two or more colonies were picked and tested using a latex agglutination test for the O157 and H7 antigens (Remel, Lenexa, KS). Up to 10 additional colonies were streaked onto TSAye (TSA + yeast extract) and incubated at 37 ± 1°C for 18 to 24 h. The identity of *E. coli* O157:H7-suspect colonies was confirmed with API 20E test strips according to the manufacturer’s instructions.

**Identification of Salmonella isolates.** Confirmed *Salmonella* isolates from survey walnuts were stored at –80°C in tryptic soy broth (Difco, Becton Dickinson) containing 15% glycerol. All cultures were submitted to the California Animal Health and Food Safety Laboratory System (Davis, Calif.) for serotyping. When an isolate was serotyped as *Salmonella* Enteritidis it was submitted to the National Veterinary Services Laboratory (Ames, Iowa) for phage typing.
Microbiological analysis of survey walnuts. For each survey year, every tenth sample and two of the three *Salmonella*-positive samples were screened for aerobic plate count (APC), *E. coli*, yeasts, and molds. For these tests, 90 g of walnuts was combined with 90 ml of sterile Butterfield’s phosphate buffer (3M, St. Paul, MN.) to obtain a 100 dilution and then shaken 50 times through a 30-cm arc. The mixture was allowed to stand for 3 to 5 min and then shaken five times through a 30-cm arc to resuspend the sample before serial dilution in Butterfield’s phosphate buffer and inoculation of media. The APCs were carried out by AOAC Official Method 966.23 (AOAC, 2000b). *E. coli* was quantified via MPN by a modified version of AOAC Official Method 966.24 (AOAC, 2000c); the modification was to use API 20E test strips to confirm positive *E. coli* colonies. Yeast and mold counts were determined by following the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (FDA-BAM) method (Tournas et al., 2001), except that tempered agar pour plates rather than spread plates of phytone yeast extract agar (Difco, Becton Dickinson) were used. This modified method is commonly used by the dry fruit and nut industry. When evaluated previously, counts from pour and spread plates were not significantly different (data not shown).

Walnut samples for inoculation and preparation of walnut rinse water. Freshly hulled, undehydrated, inshell Chandler variety walnuts were obtained from a commercial huller in Stockton, CA, and were collected from the sort table after the nuts had been hulled and passed through three separate water rinses; walnuts were stored at 4°C for up to 3 days prior to inoculation and drying. Walnuts with no remaining hull and no visible shell breakage were selected for inoculation. For the preparation of walnut rinse water, walnuts were collected from the same commercial huller after hulling and a single water rinse. The suspending liquid (walnut rinse water) was prepared by placing 100 inshell walnuts immediately after collection into 4
liters of the well water used in the huller. The mixture was agitated by hand for 2 min, and then the walnuts were removed; the resulting rinse water was placed into sterile bottles and frozen at −20°C until use.

**Culture and growth conditions.** The strains used in this study were as follows: *Salmonella enterica* Enteritidis phage type 30 (ATCC BAA-1045), isolated from raw almonds associated with an outbreak (Isaacs et al., 2005); *S. enterica* Tennessee, a clinical isolate from a peanut butter–associated outbreak (CDC, 2007); *S. enterica* Montevideo, isolated from pistachios (provided by FDA); *S. enterica* Saintpaul and *S. enterica* Muenchen, isolated from walnuts (this study); *Escherichia coli* O157:H7 (Odwalla strain 223), isolated from a juice-associated outbreak; *E. coli* O157:H7 (CDC 658), a clinical isolate from a cantaloupe-associated outbreak; *E. coli* O157:H7 (EC4042), a clinical isolate from a spinach-associated outbreak (Kotewicz et al. 2008); *E. coli* O157:H7 (EC1738), isolated from cookie dough (suspected source contaminated flour) (Neil et al., 2012; provided by Mark Mammel, FDA); *E. coli* O157:H7 phage type 4 (Health Canada NML# 11-1865), a clinical isolate from a walnut-associated outbreak (provided by Alex Gill, Health Canada); *Listeria monocytogenes* (4b) LJH552, isolated from tomatoes; *L. monocytogenes* (4b) (LCDC81-861), isolated from raw cabbage associated with an outbreak; *L. monocytogenes* (4b) (Scott A), a clinical isolate from a milk-associated outbreak; *L. monocytogenes* (1/2a) (V7), isolated from milk associated with an outbreak; and *L. monocytogenes* (PTVS 335), isolated from cantaloupe (provided by Trevor Suslow, UC Davis).

A stepwise procedure (Ruiz et al., 2008) was used to isolate rifampicin (rif)resistant mutants of each parental strain to facilitate enumeration of each organism separate from the background microbiota Unless otherwise specified, culture media were Difco brand obtained
from BD (Franklin Lakes, NJ) and were supplemented with rif at 50 mg/liter (Sigma, St. Louis, MO) when resistant cultures were used. The isolates were stored at −80°C in tryptic soy broth (TSB) supplemented with 15% glycerol (Fisher Scientific, Fair Lawn, NJ).

**Inoculum preparation.** Separate cocktails of *Salmonella*, *E. coli* O157:H7 or *L. monocytogenes* containing a mixture of five strains of each organism were prepared by streaking frozen stocks of each strain onto tryptic soy agar (TSA: TSB plus 1.5% granulated agar) and incubating at 37°C for 24 h. A loop (1 μl) of this culture was transferred two successive times into 10 ml of TSB and incubated at 37°C for 24 h. The second overnight culture (1 ml) was spread over large TSA plates (150 × 15 mm) and incubated at 37°C for 24 h to produce a bacterial lawn. After incubation, 9 ml of 0.1% peptone was added to each plate and the bacterial lawn was suspended in the buffer by scraping the agar with a sterile spreader (L-shaped cell spreader, Fisherbrand, Fair Lawn, NJ). For each organism, the resulting suspension was decanted and equal volumes of each strain were mixed to make a five-strain cocktail of each pathogen. Each cocktail was diluted with walnut rinse water to give a concentration of 8.5 log CFU/ml.

**Inoculation.** Undehydrated, inshell Chandler walnuts were inoculated as described by Blessington et al. (2013). To achieve an average of 8 log CFU/nut, inshell walnuts (400 g) were weighed into a sterile bag, 25 ml of the liquid inoculum was added, then the bag was sealed, and each bag was shaken and rubbed by hand for 2 min.

**Simulated dehydration and storage.** Inoculated walnuts were laid in a single layer on a mesh wire rack placed in a metal tray. The trays were then placed into a 0.13 m³ (4.5 ft³) environmental chamber (Model 9145-1110) equipped with a MG90 dryer (Parameter Generation & Control Inc., Black Mountain, NC). Walnuts were dried at 43°C (110°F) and 35% relative
humidity with hourly sampling until a kernel moisture content of between 6 and 8% was achieved. Walnuts were then stored in the same environmental chamber used for dehydration, but the chamber was set to 10°C and 65% relative humidity to mimic the commercial storage conditions measured previously (Chapter 2). Uninoculated walnuts were also stored under the same conditions.

**Moisture determination.** Moisture content of uninoculated walnuts was determined during drying as well as at 1, 6, 9 and 12 months during storage using the method described by Blessington et al. (2012). Walnuts were cracked and approximately 10 g of walnut kernels were extracted and homogenized for 20 s in a food processor (Waring 2.4 liter, Pro Food Processor, Torrington, CT). Samples were sieved through a standard #12 testing sieve (1.7 mm, Fisher Scientific, Pittsburgh, PA) to eliminate large particles. Four grams of the homogenized sample was weighed into an aluminum dish and spread into an even layer. Moisture was measured using an HG63 moisture analyzer (Mettler Toledo, Columbus, OH).

**Enumeration.** Individual inshell walnuts (approximately 10 to 12 g) were added to 10 ml of 0.1% peptone in sterile 530-ml (18-oz) Whirl-Pak bags (Nasco, Modesto, CA) and manually shaken and rubbed for 2 min. Appropriate dilutions were plated onto TSA + rif (TSAR) and incubated at 37°C for either 24 h (for *Salmonella* and *E. coli* O157:H7) or 48 h (for *L. monocytogenes*). In order to increase the limit of detection as microbial populations decreased, four 250-µl aliquots of the lowest dilution were spread plated onto each media and a further 2.5 ml was filtered using a 0.45 µm analytical test filter funnel (Fisher). Filters were removed from the funnel and incubated on TSA + rif for 6 h to allow injured cells to recover prior to transferring filters to appropriate selective media for each organism. *Salmonella*-inoculated samples also were plated onto bismuth sulfite agar + rif (BSAR) and incubated at 37°C for 48 h.
E. coli O157:H7-inoculated samples also were plated onto sorbitol MacConkey agar + rif(SMACR) and incubated for 24 h. L. monocytogenes-inoculated samples also were plated onto modified Oxford medium + rif (MOXR) and incubated at 37°C for 48 h. When the counts on the selective agar fell to below the limit of detection on TSAR (10 CFU/nut or 1 log CFU/nut), when possible, 10 randomly-selected colonies from TSAR plates (or all colonies when less than 10 were present) were streaked onto appropriate selective media for each organism and incubated as described above. Salmonella-inoculated samples were streaked onto BSAR and considered positive if shiny black colonies with a black halo were observed. E. coli O157:H7-inoculated samples were streaked onto either SMACR or CHROMagar O157 (CHROM O157) + rif and considered positive if colorless to white colonies or mauve colonies, respectively, were observed. L. monocytogenes-inoculated samples were streaked onto MOXR and considered positive if black colonies with a black halo were observed. Uninoculated walnuts (n = 3) were also analyzed at each sampling point by the same methods for enumerating inoculated walnuts and plated on each medium to determine the contribution of background populations that were able to grow in the presence of 50 mg/liter rif to the counts of inoculated samples, if any.

Enrichment. When populations dropped below the limit of detection by plating (0.6 log CFU/nut), samples were enriched. After plating as described above, 50 ml of TSB supplemented with 50 mg/liter rif was added to the remaining sample. Bags were shaken at a 30° angle for 1 min and then incubated at 37°C for 24 h. One microliter from each sample bag was streaked using a sterile loop onto either BSAR for Salmonella, SMACR for E. coli O157:H7, or MOXR for L. monocytogenes. After streaking, the enrichment was incubated for an additional 24 h before being streaked again on the respective media for each organism.
**Statistical analysis.** For each organism, two separate inoculations were performed using separately prepared inoculum. At each sampling point, three samples were taken from each of the two inoculations \((n = 6)\). When counts were below the limit of detection but a sample was positive by enrichment it was assigned a value equal to the limit of detection \((0.6 \log \text{CFU/nut})\). All statistical analyses were performed using the JMP 10 software package (SAS Institute, Cary, NC) including analysis of variance and \(t\)-tests. Differences between means were considered significant at \(P < 0.05\).

**RESULTS AND DISCUSSION**

**Survey of California walnuts for *Salmonella* and *E. coli* O157:H7.** A total of 2,839 inshell walnut samples were analyzed from the 2010, 2011, and 2012 harvests. In 2010, 935 100-g samples were enriched for the presence of *Salmonella*. None of the samples were positive (Table 3.1). Thus the sample size was increased to 375 g for 2011 and 2012. In early 2011, an outbreak of *E. coli* O157:H7 was linked to consumption of inshell hazelnuts (CDC, 2011b) and a separate outbreak epidemiologically linked to consumption of inshell walnuts (CFIA, 2011a, 2011b). Therefore, in 2011 and 2012, 325-g samples of inshell walnuts were analyzed for both *Salmonella* and *E. coli* O157:H7. In 2011 and 2012, two of 905 and one of 999 samples were *Salmonella*-positive, respectively (prevalence = 0.22% and 0.10%, respectively); none of the samples were positive for *E. coli* O157:H7. The average prevalence of *Salmonella* in 375-g samples of inshell walnuts was 0.16%.

The California almond industry analyzed more than 13,000 almond samples over 8 years, which indicated an average 1% prevalence of *Salmonella* in 100-g samples of raw almond kernels (range 0.6 to 1.6%) (Bansal et al., 2010, Danyluk et al., 2007, Lambertini et al., 2012);
the prevalence in raw inshell almonds was 1.5% of 100-g samples (Bansal et al., 2010). A similar prevalence of *Salmonella* has been observed for 100-g samples of inshell pecans (Danyluk, personal communication) and inshell pistachios (Lieberman and Harris, unpublished), and a 2.3% prevalence of *Salmonella* was reported in 375-g samples of peanut kernels (Calhoun et al., 2013).

When *Salmonella* is detected in nuts, levels of the organism are often near or less than one cell per gram even in outbreak-associated product. Levels of 1.2 MPN/g were predicted for raw almonds associated with an outbreak (Lambertini et al., 2012). Levels of *Salmonella* in positive survey samples ranged from 0.0079 to 0.16 MPN/g in almonds (Bansal et al., 2010, Danyluk et al., 2007, Lambertini et al., 2012), and <0.3 to 2.4 MPN/g in peanuts (Calhoun et al., 2013). Two retail samples of Brazil nuts had concentrations of 0.23 and 0.09 MPN/g of *Salmonella* (Little et al., 2010). Concentrations of *Salmonella* were not determined for positive walnut samples in the current study but would likely be low based on data for other tree nuts.

The three *Salmonella* isolates from walnuts were serotyped to Saintpaul, Muenchen, and Enteritidis phage type RDNC (routine dilution, none confirmatory) (Table 3.1). With only three isolates, it is difficult to make an assessment on the source of the organism. For surveys where greater numbers of isolates were available, a wide range of serotypes was observed. Over an 8-year survey *Salmonella* was isolated from 151 almond samples; 49 different serovars were identified among these isolates (Bansal et al., 2010; Danyluk et al., 2007; Bansal and Harris, unpublished); 13 serovars were identified from 22 *Salmonella*-positive peanut samples over 3 years (Calhoun et al., 2013). The population diversity observed suggests that the *Salmonella* present in these products came from a variety of environmental sources.
Background microbial populations on dried walnuts from walnut surveys. A total of 284 samples were analyzed for background populations during the 3 years of this survey (Fig. 3.1). Aerobic plate counts (APC) ranged from <1.0 to >5.4 log CFU/nut, with an average of 3.4 log CFU/g. *E. coli* was detected in 10 samples (3.5%) and, when detected, ranged in concentration from 0.4 to 110 MPN/g (−0.4 to 2.0 log MPN/g) and averaged 26.4 MPN/g (1.4 log MPN/g). Populations of mold were also enumerated and ranged from <1 to 4.7 log CFU/g with an average of 2.5 log CFU/g. Yeast was detected in 15 samples (5.3%) and populations ranged from 1.0 to 5.2 log CFU/g, with an average of 2.3 log CFU/g.

Background populations were determined for two of the three *Salmonella*-positive walnut samples. The APC were 2.4 and 3.8 log CFU/g, mold counts were 2.4 and 2.6 log CFU/g, and yeasts were below the limit of detection (<1 log CFU/g). MPN analysis did not detect *E. coli* in the one sample tested. These levels of background populations are near average for walnuts in this survey. Similarly, the presence of *E. coli* or high background counts were not predictive of the presence of *Salmonella* in almonds (Danyluk et al., 2006) or in seeds (Willis et al., 2010).

Survival of pathogens on inoculated walnuts. Freshly hulled, inshell, undried walnuts collected from a commercial processor were inoculated with five-strain cocktails of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*. As commonly recommended for these types of studies (NACMCF, 2010), cocktails of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* were used in this study. Where possible, the strains selected were associated with tree nuts or low moisture foods or were clinical or food isolates from produce-associated outbreaks. These strains include two *Salmonella* strains isolated from walnuts in the survey described above, an *E. coli* O157:H7 clinical strain isolated from an outbreak epidemiologically linked to walnuts (CFIA 2011a, 2011b), an *E. coli* O157:H7 strain from a cookie dough–associated outbreak in which
contaminated flour was thought to be the source of the pathogen (Neil et al., 2012), and a *L. monocytogenes* strain from a large 2011 cantaloupe outbreak. These strains, which have been isolated from dried products, or in the case of cantaloupe, from a dry surface, may have increased desiccation tolerance and, therefore, provide a more accurate representation of survival of each pathogen type in a dried food matrix. In the absence of data that would justify selection of a single strain, including a diversity of strains in an inoculum cocktail increases the potential that isolates more adapted to survival under the experimental conditions are included. However, using a cocktail does not allow for identification of those more desiccation tolerant individual strains.

While equal volumes of each strain were used in preparation of the cocktails, the initial amount of each strain was not determined either by OD or plate count; previous studies have shown that this method produces approximately equal concentrations of each strain in the final cocktail (Kimber et al., 2012). Kimber et al. (2012) used a combination of antibiotic-resistance, PCR, and pulse field gel electrophoresis profiles to determine the survival of individual *Salmonella* strains from cocktails of organisms applied to almonds and pistachios. Distribution of four of the five isolates was remarkably similar at over 1 year of storage.

Walnuts were inoculated at 8.0 log CFU/nut wet and dried to a target of 8% moisture, which resulted in final concentrations of 5 to 6 log CFU/nut or 4 to 5 log CFU/g after drying, which was the desired starting concentration. These levels are orders of magnitude higher than the natural contamination levels reported in almonds and peanuts (Bansal et al., 2010; Danyluk et al., 2007; Lambertini et al., 2012; Calhoun et al., 2013), and over the levels estimated in an almond outbreak (Lambertini et al., 2012). Evaluating the survival of pathogens in products where the anticipated natural contamination levels are below the limit of detection by plating is a
challenge. Inoculation at levels above the limit of detection allows for a longer time for quantification of survival, as more accurate counts can be obtained. *Salmonella* declined at a faster rate on inshell walnuts and kernels when initial inoculum levels were lower (Blessington et al., 2012, 2013). Based on this observation, the rates of decline measured in this study would be conservative since they are expected to be higher at the natural concentrations typically observed in walnuts and similar products.

Freshly hulled walnuts (pre-dehydration) were used for inoculation. Float tank contamination was emulated by inoculating walnuts with microorganisms that were suspended in water exposed to freshly hulled walnuts. Inoculated walnuts were then dried for 10 to 11 h to a target moisture of less than 8% in an environmental chamber at temperatures typical of a commercial dehydrator. The walnut industry generally uses a non-destructive method (in-bin steel plates that measure electrical resistance) to determine moisture content for the whole nut (shell and kernel) and to monitor loads of dehydrating walnuts. When a walnut is dried, the shell typically has a higher moisture content than the kernel, effectively raising the moisture measurement of the whole nut (Thompson et al., 1998). The commercial whole-nut measurement methods were not appropriate in this study for the small batches of walnuts handled in the laboratory, and walnut shells are not easily blended for laboratory moisture measurements. Therefore, kernel moisture was measured instead and is reported here.

Measured final moisture content of walnut kernels after drying ranged from 5.5 to 7.9%, with an average moisture content across all trials of 6.3% (data not shown). Walnuts were stored at consistent conditions of 10°C and 65% relative humidity, which were chosen to align with those measured in a commercial walnut storage facility (Chapter 2). Due to the limited space in the environmental chamber, samples were evaluated for moisture at selected points during
storage. The moisture content of walnuts stored for 1 and 6 months (4.6% ± 0.15% and 4.4% ± 0.36%, respectively), was not significantly different. The consistent temperature and humidity in the environmental chamber allowed the walnuts to remain at approximately the same moisture through 6 months of storage.

Initial populations for all three pathogen cocktails prior to drying (t = 0 days), as determined on TSA, were approximately 8 log CFU/nut (8.0 to 8.3 log CFU/nut). Populations on selective media for each organism were not significantly different but were 0.1 to 0.2 log CFU/nut lower than the TSA counts, ranging from 7.9 to 8.2 log CFU/nut. Uninoculated control samples were also plated at each sampling point to determine if microorganisms capable of growth on media supplemented with rifampicin were present; colonies were not detected on either TSA or selective media at any time point.

As storage progressed and the populations approached the limit of detection the difference between TSA and the selective media increased, especially for E. coli O157:H7 and L. monocytogenes (Table 3.2). In some cases the differences were as much as 1 log CFU/nut higher on TSAR compared with the selective medium for those pathogens. In almost every case, when colonies selected from TSA were streaked onto selective agar they produced colonies typical of the pathogen on that agar. When colonies did not produce a positive result on selective media, the counts for TSA were modified appropriately. The latter situation occurred very infrequently (less than once per sampling point) and only in instances where the populations were at or near the limit of detection. Because colonies were not detected on any of the media for uninoculated control walnuts, it was expected that colonies on TSA were representative of the inoculated pathogens. The discrepancy between selective and nonselective media indicates that some cells are viable, but unable to produce colonies on selective media.
After drying \((t = 0.5\) days), populations decreased by 2.5, 3.0, and 2.8 log CFU/nut to 5.8, 5.0, and 5.2 log CFU/nut for *Salmonella*, *E. coli O157:H7*, and *L. monocytogenes*, respectively. These reductions are on the high side of those observed in the background microbiota during commercial drying (0.9 to 2.3 and 0.2 to 4.0 log CFU/nut, for aerobic and coliform counts, respectively (Chapter 2)). The wide range of reductions observed in a commercial walnut dehydrator is not unexpected given the relatively uncontrolled nature of this process. In this study, inoculated walnuts were placed in a single layer within a chamber with a strictly controlled atmosphere. This is much more precise than would be expected in a commercial walnut dehydrator where walnuts may be stacked in layers that span several feet with warm air flowing in a single direction. Thus the reductions observed in the laboratory during drying are not likely representative of what may be observed in a commercial process. Commercial dehydration as currently practiced is unlikely to be considered an appropriate reduction step for *Salmonella* given the lack of process control and the potential for post dehydration recontamination.

Reductions in populations of inoculated pathogens during drying were less than 1 log CFU/nut higher than those reported previously for inshell walnuts (2.1, 2.2, and 1.9 log CFU/nut reductions for *Salmonella*, *E. coli O157:H7* and *L. monocytogenes*, respectively) (Blessington et al., 2013) but 2 log CFU/nut higher than reported for inshell pistachios (1.2, 1.2, and 1.4 CFU/g for *Salmonella*, *E. coli O157:H7*, and *L. monocytogenes*, respectively) (Kimber et al., 2012). Inoculated inshell walnuts and pistachios were dried at ambient conditions for 24 h or 3 days, respectively while walnuts in the current study were dried at a higher temperature for a shorter time. The higher drying temperatures along with different strains that were used in these studies may, in part, account for these differences.
During storage at 10°C and 65% RH, *Salmonella* counts steadily decreased over the first 85 days from 5.2 log CFU/nut to 3.0 CFU/nut. From 85 to 210 days (7 months) the population of *Salmonella* was relatively stable ranging from 3.0 to 3.6 log CFU/nut. Similarly, *Salmonella* inoculated onto inshell pecans at a similar level as applied here (~6 log CFU/g) and stored at 21°C declined steadily to about 3 log CFU/g after 1.5 years (Beuchat and Mann, 2010). In contrast, *E. coli* O157:H7 populations declined from 5.0 to 3.0 CFU/nut within the first 8 days of storage, (a total of 5 log CFU/nut within the first week after inoculation). From 8 to 120 days of storage the counts declined slowly to 1.8 log CFU/nut and then fluctuated from 1.7 to 2.7 log CFU/nut. However, by 183 days (7 months), two of six samples were below the limit of detection by plating but were positive by enrichment of a single 10- to 12-g nut. Similarly, *L. monocytogenes* counts dropped rapidly in the first week of storage to 3.7 CFU/nut (a total reduction of 4.7 log CFU/nut), followed by a steady decline through 143 days of storage after which counts remained near the limit of detection. Some samples were below the limit of detection by plating at day 143 (but not days 183 and 211), but all samples were positive by enrichment of a single 10- to 12-g nut.

The overall trends in pathogen survival observed in the current study are consistent with previous studies on almonds, pistachios and walnuts (Kimber et al., 2012; Blessington et al., 2012, 2013). In general, the decline of *Salmonella* during storage at room temperature (21°C or higher in most studies) was slower than for either *E. coli* O157:H7 or *L. monocytogenes*. Regardless of initial inoculum level, the rates of decline during desiccation were relatively consistent among the three pathogens but declines differed during the initial week to 6 months of storage. When populations approach the limit of detection by plating, a low-level persistence is
observed for extended periods of time, sometimes in excess of 1 year (Beuchat and Mann, 2010; Blessington et al., 2012, 2013; Kimber et al., 2012).

A number of factors including temperature, relative humidity, kernel size (Beuchat and Mann, 2010) and, in some cases, inoculation level, influence survival of microorganisms on nuts during storage (Blessington et al., 2012, 2013). Lower temperatures have been shown to increase survival of foodborne pathogens in a number of dried products including almonds, pecans, pistachios and walnuts (Abd et al., 2012; Kimber et al., 2012; Beuchat and Heaton, 1975; Beuchat and Mann, 2010; Blessington et al., 2012, 2013). When stored at refrigeration or freezing temperatures (4 or -20°C, respectively) there is virtually no decline in pathogen concentrations over extended storage periods. Higher relative humidity in storage of dried products may be associated with greater rates of decline (Crumrine and Foltz, 1969). Inshell walnuts in the current study were stored at 10°C and 65% RH, conditions that mimic commercial walnut storage. This cooler temperature and moderate humidity reduces the rate of rancidity in walnuts but also favors survival of pathogens to a greater extent than would be expected during storage at higher temperatures and RH.

Linear declines in *Salmonella* populations of 0.20 and 0.15 log CFU/g/month at 23°C were previously observed on inoculated almond kernels and inshell pistachios, respectively (Kimber et al., 2012). The decline of *E. coli* O157:H7 on inshell pistachios was also linear (Kimber et al., 2012). However, in almond kernels, a rapid die off early on in storage was followed by long-term survival at or below the limit of detection (Kimber et al., 2012) similar to the current observations on inshell walnuts. On almond kernels, a similar trend was seen for *L. monocytogenes*; populations declined to about 1.5 log CFU/g within 120 days and persisted at a similar level for over a year. On inshell pistachios the decline was slower; the limit of detection
(0.3 log CFU/g) was reached after 210 days and populations persisted at this level for the next 150 days (Kimber et al., 2012).

Blessington et al. (2013) was not able to determine rates of decline for *Salmonella, E. coli* O157:H7 and *L. monocytogenes* on inshell walnuts because the population levels after the inoculum dried were near the limit of detection by plating. All three pathogens persisted at low levels for the 3-month study. The study design was significantly modified in the current study, by increasing the initial inoculum level and attempting to more closely mimic potential commercial contamination, temperatures and RH typical of commercial storage, and typical commercial storage times. Despite these changes, the overall results between the two studies were very similar.

The walnut shell is more exposed to the environment than the kernel and this is reflected in microbial loads on the shell and kernels extracted from intact inshell nuts (Chapter 2). While the shell is not consumed, there is a risk of illness for nuts sold in-the-shell, as confirmed with an outbreak of *E. coli* O157:H7 linked to consumption of inshell hazelnuts (Miller et al., 2012). When inshell walnuts are sold in North America they are often subjected to a lightening or “brightening” process by exposing the nuts for a short time to 3 to 4% bleach (sodium hypochlorite) to lighten the shell for better visual appearance. This process has been shown to reduce populations of *Salmonella* by more than 2 log CFU/nut (Blessington et al., 2013). Although brightening may reduce pathogens on nuts sold inshell and cracked by the consumer, it is not used for nuts cracked commercially and so would not be a control step for product sold as kernels.
Although the walnut shell does provide some protection to the kernel, as evidenced by higher microbial levels on kernels extracted from broken shells, it is not an impenetrable barrier. Significant microbial populations also can be enumerated from kernels with visibly intact shells (Chapter 2). The commercial process of cracking or removing the shell from the nut to extract kernels introduces the potential for transfer of bacteria from the shell to the kernel (Blessington, 2011; Frelka and Harris, unpublished data). Foodborne pathogens have been shown to persist for extended periods at low levels on walnut kernels (Blessington et al., 2012), so potential for transfer of microorganisms from shell to kernel should be considered when assessing food safety risks.

Although the primary food safety concern for walnuts is *Salmonella*, the risk from other pathogens should not be overlooked. The observed prevalence of *Salmonella* and *E. coli* O157:H7 is low on inshell walnuts, which is consistent with previous observations for other nuts and nut products. This low prevalence still can present a food safety risk if the pathogens are present and environmental factors (i.e. temperature or moisture) allow growth or persistence in walnuts. In cases where pathogens are present, having an understanding of their survival can aid in establishing adequate food safety programs.

The observed survival of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* provide data that can be used for risk assessment modeling. Each pathogen displayed a unique survival curve with *Salmonella* surviving at the highest level for the longest period of time (approximately 3 log CFU/nut for 7 months) and *E. coli* O157:H7 and *L. monocytogenes* displaying more rapid die off followed by persistence at lower levels (1 to 2 log CFU/nut). The use of these survival curves in the creation of models requires making the assumption that the microorganisms will decline during storage, but making this assumption also requires an
understanding of the state of the bacteria at the time of a contamination event. If the routes of walnut contamination were better understood, more accurate models could be created taking into account physiological state of the cells and data could be generated to ensure survival is accurately accounted for.

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Table 3.1. Summary of walnut surveys from 2010 to 2012

<table>
<thead>
<tr>
<th>Survey year</th>
<th>Total no. of samples</th>
<th>Sample size (g)</th>
<th>No. of samples positive for <em>E. coli</em> O157</th>
<th><em>E. coli</em> prevalence (%)</th>
<th>No. of samples positive for <em>Salmonella</em></th>
<th><em>Salmonella</em> prevalence (%)</th>
<th><em>Salmonella</em> serovars isolated</th>
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<td>375</td>
<td>0</td>
<td>0.0</td>
<td>1</td>
<td>0.10</td>
<td>Enteritidis (phage type RDNC&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

<sup>a</sup>RDNC, routine dilution no conformity.
Figure 3.1. Distribution of populations of APC (A), *E. coli* (B), molds (C) and yeasts (D) on walnuts from 2010 to 2012 survey sampling (*n* = 284).
Figure 3.2. Survival of five-strain cocktails of *Salmonella* (circles), *E. coli* O157:H7 (squares), and *L. monocytogenes* (triangles) on inshell walnuts stored at 10°C and 65% RH. Time at 0 days represents wet inoculum and time at 0.5 days represents dried inoculum recovered from the walnuts. Colonies enumerated on TSA + rif plates. Dashed line indicates the limit of detection (0.6 log CFU/nut).
Table 3.2. Populations of inoculated pathogens over time on selective and non-selective media.

<table>
<thead>
<tr>
<th>Day</th>
<th>Salmonella</th>
<th>E. coli O157:H7</th>
<th>Listeria monocytogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSA + rif</td>
<td>BSA + rif</td>
<td>MOX + Rif</td>
</tr>
<tr>
<td></td>
<td>(log CFU/nut)</td>
<td>(log CFU/nut)</td>
<td>(log CFU/nut)</td>
</tr>
<tr>
<td>0</td>
<td>8.3 ± 0.24</td>
<td>8.2 ± 0.12</td>
<td>6</td>
</tr>
<tr>
<td>0.5</td>
<td>5.8 ± 0.37</td>
<td>5.6 ± 0.57</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>5.5 ± 0.79</td>
<td>5.2 ± 0.77</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>5.0 ± 0.34</td>
<td>4.8 ± 0.42</td>
<td>6</td>
</tr>
<tr>
<td>27</td>
<td>4.9 ± 0.62</td>
<td>4.7 ± 0.64</td>
<td>6</td>
</tr>
<tr>
<td>55</td>
<td>4.4 ± 0.40</td>
<td>4.0 ± 0.45</td>
<td>6</td>
</tr>
<tr>
<td>85</td>
<td>3.4 ± 0.44</td>
<td>2.3 ± 0.62</td>
<td>6</td>
</tr>
<tr>
<td>120</td>
<td>3.5 ± 0.34</td>
<td>3.2 ± 0.34</td>
<td>6</td>
</tr>
<tr>
<td>148</td>
<td>3.9 ± 0.69</td>
<td>3.6 ± 0.72</td>
<td>6</td>
</tr>
<tr>
<td>183</td>
<td>3.7 ± 0.48</td>
<td>3.3 ± 0.74</td>
<td>6</td>
</tr>
<tr>
<td>211</td>
<td>3.1 ± 0.60</td>
<td>2.6 ± 0.57</td>
<td>6</td>
</tr>
</tbody>
</table>

\(a\) E represents the number of replicates that were above the limit of detection (4 CFU/nut); \(b\) 1° - 24 h represents the number of replicates positive after primary enrichment for 24 h; \(c\) 1° - 48 h represents the number of replicates positive after primary enrichment for 48 h.
FUTURE RESEARCH

This research presented in this thesis has answered many questions about microorganisms on walnuts and has also raised additional questions. Future research should further explore a number of aspects of the walnut microbiota and how various harvest, hulling, drying and cracking processes influence these populations.

The current research increased the understanding of potential routes of contamination for walnut kernels, but it is still unclear how microorganisms penetrate walnut shells. Future research should further explore mechanisms of microbial infiltration of walnut shells to determine what factors have the greatest influence on contamination events. One such method may involve imaging. The infiltration of Salmonella through almond hulls and shells has been visualized using Salmonella labeled with green fluorescent protein (gfp) and confocal laser scanning microscopy (Danyluk et al., 2008). A similar study could be performed on walnuts. In addition to illustrating the migration of the cells through the shell, it would be possible to pinpoint areas of suspected weakness in the shell and determine if the bacteria are localized to any specific point thus demonstrating the weakness of the shell. This should be performed during the walnut harvest season on undried walnuts as water uptake and potentially microbial infiltration are likely influenced by the moisture content of the walnuts and with scenarios that mimic commercial handling.

The outer shell of a walnut is more likely to be contaminated than the kernel. Thus understanding the potential for transfer of microorganisms from the shell to the kernel during cracking would be valuable. Preliminary experiments demonstrated that increases in microbial loads on walnut kernels do increase during commercial cracking. Trials could be performed to
determine the rate of transfer of microorganisms from the shell to the kernel during commercial cracking processes. Determining the rate of transfer from the shell to the kernel would aid in modeling the risk due to cracking in walnuts.

In order to understand what is happening through the hulling and dehydration process, an examination of the microbial ecology of walnuts at various points during harvest, hulling and drying may shed light on how the microbes are behaving in these various processes. Examination of the different species associated with walnuts could be done by performing culture-independent techniques, such as DNA sequencing. By utilizing high-throughput sequencing, such as Illumina or pyrosequencing, the diversity of the microbes present on walnuts can be assessed; changes in microbial diversity may be assessed after various steps in the harvest and postharvest handling of walnuts to determine if particular groups of microorganisms are influenced by different practices., for instance, it is possible that drying is causing decreases in specific subpopulations on the walnuts and selecting for microbes which are more heat and/or desiccation tolerant.

The survival study was ongoing at the time this thesis was completed. Future studies could include an analysis of the surviving populations of the three pathogens to determine if there are strains that are more prevalent on the stored walnuts. Although levels of each strain were not determined initially populations have declined by over five orders of magnitude. Presence of any of the strains should indicate a reasonable ability to tolerate desiccation and storage of walnuts. A variety of techniques (antibiotic susceptibility, PCR, and PFGE profiles) could be used to identify individual isolates as outlined by Kimber et al. (2012).

Some of the data collected in this research will be used to develop a quantitative microbial risk assessment (QMRA) model for walnuts. This model will allow the walnut industry
to run various scenarios to evaluate how changes in production volume, handler practices or other parameters of walnut production will affect the risk of pathogens in the supply. QMRA has proven successful for the almond industry and has been used to determine an appropriate process control, a 4-log reduction, to reduce risk of *Salmonella* to an acceptable level (Danyluk et al., 2006; Lambertini et al., 2012) and would prove a useful tool for the walnut industry as well.
REFERENCES


### APPENDIX

Table A.1. Summary of walnut shell breakage scores (0 to 5) for inshell walnuts according to variety and sampling location

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Sampling location</th>
<th>Number of samples</th>
<th>Number (and %) of samples in each breakage score category</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Chandler’</td>
<td>Orchard floor</td>
<td>252</td>
<td>243 (96) 4 (1.6) 5 (2.0) 0 (0.0) 0 (0.0) 0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>Receiving pit</td>
<td>239</td>
<td>232 (97) 0 (0.0) 1 (0.40) 1 (0.40) 2 (0.80) 3 (1.3)</td>
</tr>
<tr>
<td></td>
<td>Sort table</td>
<td>326</td>
<td>324 (99) 0 (0.0) 1 (0.30) 0 (0.0) 1 (0.30) 0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>Dryer bin</td>
<td>376</td>
<td>86 (23) 178 (47) 47 (12) 20 (5.3) 27 (7.2) 18 (4.8)</td>
</tr>
<tr>
<td>‘Hartley’</td>
<td>Dryer bin</td>
<td>122</td>
<td>30 (25) 11 (9.0) 23 (19) 22 (18) 17 (14) 19 (16)</td>
</tr>
<tr>
<td>‘Tulare’</td>
<td>Receiving pit</td>
<td>260</td>
<td>253 (97) 2 (0.80) 0 (0.0) 4 (1.5) 0 (0.0) 1 (0.40)</td>
</tr>
<tr>
<td></td>
<td>Dryer bin</td>
<td>151</td>
<td>102 (68) 19 (13) 22 (15) 8 (5.3) 0 (0.0) 0 (0.0)</td>
</tr>
<tr>
<td>‘Vina’</td>
<td>Receiving pit</td>
<td>464</td>
<td>459 (99) 0 (0.0) 0 (0.0) 4 (0.90) 0 (0.0) 1 (0.20)</td>
</tr>
<tr>
<td></td>
<td>Sort table</td>
<td>824</td>
<td>774 (94) 1 (0.10) 9 (1.1) 19 (2.3) 9 (1.1) 12 (1.5)</td>
</tr>
<tr>
<td></td>
<td>Dryer bin</td>
<td>702</td>
<td>495 (70) 108 (15) 46 (6.6) 33 (4.7) 10 (1.4) 10 (1.4)</td>
</tr>
</tbody>
</table>
Figure A.1. Percentage of walnuts within each breakage category (0 to 5) at the receiving pit (A), sort table (B), and after drying (C). Charts represent a composite of all ‘Chandler’, ‘Hartley’, ‘Vina’, and ‘Tulare’ walnuts sampled.