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Hot water treatment as a kill-step to inactivate *Escherichia coli* O157:H7, *Salmonella enterica*, *Listeria monocytogenes* and *Enterococcus faecium* on in-shell pecans

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ABSTRACT

In-shell pecans are susceptible to microbial contamination. This study was performed to investigate feasibility of using hot water treatment as a kill-step for food-borne pathogens during pecan shelling. In-shell pecans were subjected to hot water at 70, 80 or 90 °C for 1, 2, 3, 4 or 5 min. The time-temperature treatments to achieve a 5-log reduction of *Salmonella enterica*, *Escherichia coli* O157:H7, *Listeria monocytogenes,* and non-pathogenic *Enterococcus faecium* were determined. Thermal death values were determined for each tested condition. *L. monocytogenes* was most susceptible to heat treatment and were reduced by 4.6 ± 0.35 log CFU/g at 70 °C for 5 min, while 3–5 min at 80 and 90 °C treatments was required to achieve a similar reduction level for *S. enterica*, *E. coli* O157:H7, and *E. faecium*. *S. enterica* were most resistant and required 4 min treatment time to achieve a 5-log reduction at 80 and 90 °C. The D-values ranged from 1.15 to 1.72, 0.83 to 1.19, and 0.41–0.92 min at 70, 80 and 90 °C, respectively. *E. faecium* had the highest D-value (1.72 min at 70 °C), indicating a potential surrogate for process validation for pecan industries. Utilizing proper hot water treatment during pecan shelling could reduce food safety risk.

1. Introduction

Low-moisture foods such as tree-nuts with water activity lower than 0.7 are presumed to be low-risk food (Blessington, Theofel, & Harris, 2013; Harris, 2012). However, in the past few years tree nuts such as pecans, almonds, walnuts, pine nuts, pistachios, and mixed nuts have frequently been associated with various recalls and outbreaks due to contamination with foodborne pathogens such as *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* (Zhang et al., 2017). Even at low level of contamination (10–100 cells/gm), *S. enterica* have been reported for outbreaks associated with high fat and low moisture foods such as chocolate and peanut butter (Kapperud et al., 1990). Studies have shown that infectious dose was low possibly due to the high fat and low moisture in foods like nuts that protects organisms from the highly acidic condition of the stomach (Aviles, Klotz, Smith, Williams, & Ponder, 2013).

Pecans are one of the several most favored tree nuts consumed worldwide in different forms. The microbial food safety of pecans depends on the pre and post-harvest production and processing practices (Beuchat & Pegg, 2013). A quantitative risk assessment study by Farakos et al. (2017) shows that there is a possibility of risk of salmonellosis in U.S. population on consumption of *Salmonella* contaminated pecan. They reported that the shelling process of pecans during post-harvest treatments and acquiring illness at home by consuming uncooked pecans are well correlated. Post-harvest practice during pecan shelling includes conditioning of pecans to facilitate kernel separation, minimize kernel breakage and increase the shelling efficiency and can help to reduce the microbial levels from pecans (Beuchat & Pegg, 2013). Some of the conditioning methods currently used by industries are: (i) soaking in hot water at > 81 °C for 1–8 min or steam processing for 6–8 min; (ii) immersing in cold water (usually chlorinated) for 8 h and then draining for 16–24 h; or (iii) soaking in chlorinated water with a minimum free chlorine concentration of 200 ppm at 15–30 °C for 2 min (Beuchat & Mann, 2011; Farakos et al., 2017). However, as per our knowledge, none of the methods are scientifically validated as a "kill-step" which requires a 5 log reduction for a combination of potential pathogens such as *E. coli* O157:H7, *L. monocytogenes,* and *S. enterica*. Farakos et al. (2017) reported that hot conditioning, in comparison to

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cold, has a significant impact on reducing the potential risk of salmonellosis as it effectively reduces *Salmonella* by up to 4 log. Beuchat and Mann (2011) and Harris, Uesugi, Abd, and McCarthy (2012) demonstrated the efficacy of hot water treatment to reduce *S. enterica* by 5 log CFU/g from pecans and almonds, respectively. However, these studies do not evaluate the effect of hot water treatment on inactivation of pathogens like *E. coli* O157:H7 and *Listeria monocytogenes*.

To minimize the food safety risks, process validation should include use of various potential pathogens associated with the food or pathogens associated with known foodborne outbreaks (Swanson, 2011). Hence the main objectives of this study were to determine (i) hot water treatment conditions to achieve a 5 log reduction of *S. enterica, E. coli* O157:H7, *L. monocytogenes*, and *E. faecium* on in-shell pecans, and (ii) the rate of thermal lethality of tested organisms.

2. Materials and methods

2.1. Selection of pecans

Raw in-shell pecans (*Carya illinoinensis*) harvested from several Louisiana orchards during the October/November season of 2015–2016 were obtained from Louisiana State University Pecan Research and Extension Station at Bossier city, LA. These pecans were stored in woven polypropylene mesh bags at 4 °C, for approximately 3 months, until they were used in experiments.

2.2. Selection of bacteria

Several different outbreak strains of *S. enterica, E. coli* O157:H7, *L. monocytogenes* as well as non-pathogenic strain of *Enterococcus* spp., were used in this study. These pathogenic strains were provided by Dr. Michelle D. Danyluk at University of Florida and were similar to the ones used in their study on peanuts and pecan kernels (Brar, Proano, Friedrich, Harris, & Danyluk, 2015). *E. faecium* (ATCC 8459), a non-pathogenic organism was used as a surrogate organism for *S. enterica.* A mutant strain of *E. faecium* resistant to nalidixic acid was developed in our lab by following the method described by Parnell, Harris, and Suslow (2005).

2.3. Inoculum preparation

Frozen cultures of nalidixic acid resistant mutant of *S. enterica, E. coli* O157:H7, *L. monocytogenes,* and *E. faecium* were subcultured twice in tryptic soy broth (TSB) or TSBY (TSB with 0.6% yeast extract for *L. monocytogenes*) supplemented with nalidixic acid (TSBN) at 50 μg/ml with incubation at 37 °C for 24 h. Then, 1 ml of each overnight bacterial culture was plated on tryptic soy agar (TSA) supplemented with 50 μg/ml nalidixic acid (TSAN) and incubated at 37 °C for 24 h. Each strain was grown on TSAN plates to develop resistance towards subsequent stress conditions as suggested by Uesugi, Danyluk, and Harris (2006). The resultant lawn of bacteria on TSAN was scraped-off with a sterile glass rod using 7 ml of 0.1% sterile peptone water. In this manner, a total of 5 ml of inoculum was collected from each strain of pathogen/surrogate on TSAN plate, and separate cocktails of bacteria were prepared by mixing individual strains in a 400 ml stomacher® bag (Control Numero 5, Seward, UK). A total of 100 ml of inocula volume was maintained in 0.1% peptone water for each bacterial mixture.

2.4. Inoculation of pecans

Whole, undamaged in-shell pecans were selected and stored overnight inside the bio-safety cabinet at room temperature (21 °C). Pecans (n = 28) weighing 310 ± 10 g per batch were added to the stomacher bag containing 100 ml of a cocktail strain of each organism at 21 °C. Later the bags containing pecans and respective inoculums were hand massaged for a minute. The pecans in the bag were submerged in the inoculum for 1 h with frequent mixing and hand massaging. The inoculated pecans were then aseptically transferred to large petri dishes (150 × 15 mm) and air dried for 20 min inside the bio-safety cabinet. After that, pecans were placed in sterilized filter bags (T-Sac, tea filter bags, Model 1601; 2 pecans per bag) and sealed. Microbiological analysis of pecan samples at this point (as described in 2.6) before hot water treatment showed 7.88 ± 0.07 (*S. enterica*), 7.71 ± 0.07 (*E. coli* O157:H7), 7.58 ± 0.18 *(L. monocytogenes)* and 6.53 ± 0.23 (*E. faecium*) log CFU/g, respectively.

2.5. Hot water treatment of inoculated in-shell pecans

Inoculated in-shell pecans were subjected to hot water treatment in a 500 ml wide-mouthed glass bottles using a water bath (VWR, Model 10128–126, Radnor, PA, U.S.A.). Briefly, the glass bottles were first filled with sterile distilled water up to the neck (~450 ml) and then brought to a temperature of 1.5 °C higher than the set temperatures of either 70, 80, or 90 °C, respectively. This ensured that the water inside the bottles was maintained at 70, 80 and 90 °C as measured with a calibrated thermometer. Individual groups of four inoculated pecan samples (i.e., two tea filter bags) were dipped in hot water and treated for 1, 2, 3, 4 or 5 min at 70, 80 or 90 °C. Pecan processors mostly use hot water > 81 °C for 1–8 min to condition the pecans (Beuchat & Mann, 2011; Farakos et al., 2017). Thus, test temperatures were selected close to what pecan processors have in place already. In addition, preliminary trials were conducted at 70, 80 and 90 °C for 3–12 min (data not shown) which helped us to select tested time –temperature combinations.

2.6. Enumeration

Enumeration of surviving bacterial cells was performed by either crushing or using whole pecans. For organisms other than *L. monocytogenes*, four hot water treated pecans were taken in a puncture resistant stomacher® bag (Control Numero 5, Seward, UK) and crushed into pieces using a sterile pestle. After crushing, 100 mL of 0.1% peptone water was added to each bag and placed in an ice bath for 10 min to lower the temperature. Pecan samples were not subjected to crushing for the enumeration of *L. monocytogenes*.

This modification of protocol was done based on the results of our preliminary studies (data not shown) where recovery of *L. monocytogenes* cells from crushed pecans was lower than other bacteria used in this study. Few studies reported higher susceptibility of *Listeria* to bioactive compounds in pecan shells compared to other pathogens (Babu, Crandall, Johnson, O'Bryan, & Ricke, 2014; Caxambu et al., 2016; Prado et al., 2014). This might be one probable cause for the discrepancy in our preliminary study. However, understanding this mechanism is beyond the scope of the current study.

Later the pecan samples in the bag were hand massaged and shook for 1 min to dislodge the organisms. Appropriate serial dilutions of the samples were prepared, and survived organisms were enumerated by plating on Xylose Lysine Deoxycholate agar containing nalidixic acid at 50 μg/ml (XLDN) for *S. enterica*, Cefixime-Tellurite Sorbitol MacConkey Agar containing nalidixic acid at 50 μg/ml (CT-SMACN) for *E. coli* O157:H7, Oxford Listeria Agar base containing nalidixic acid at 50 μg/ml for *L. monocytogenes* and non-selective media TSAN for *E. faecium* and incubation at 37 °C for 24–48 h.

2.7. Determination of D -values

Log reduction of each organism was plotted at different temperatures on y-axis against treatment time on x-axis. D-values were calculated at each test temperature for each organism by taking the inverse of the slope of linear regression line from the log reduction graph and expressed in minutes. The D values calculated were plotted and the negative inverse slope of this curve was calculated as Z value

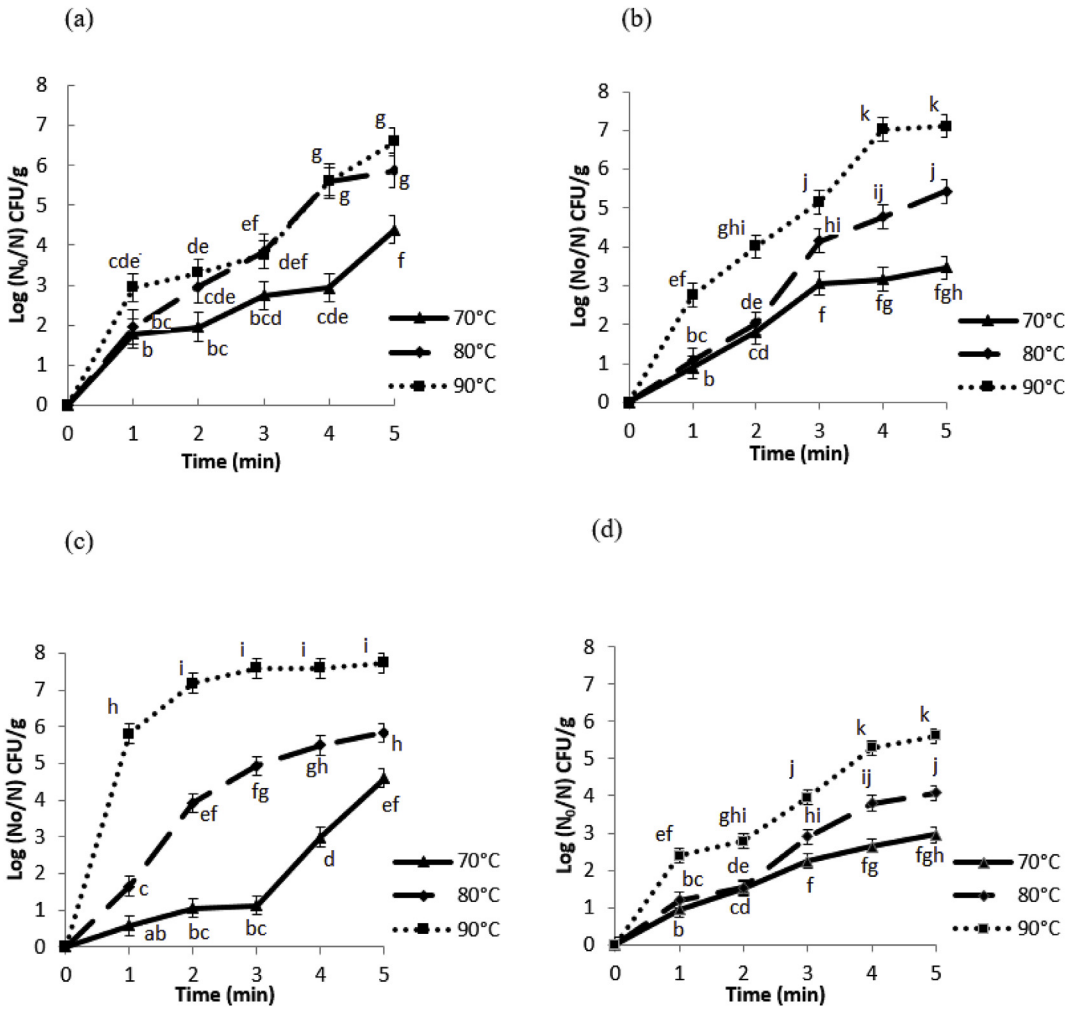

Fig. 1. Reduction (log CFU/g) of (a) *S. enterica,* (b) *E. coli* O157:H7, (c) *L. monocytogenes,* and (d) *E. faecium* observed in in-shell pecans when treated with hot water at 70, 80 and 90 °C for 5 min. Mean values with different letters in each figure represent significant difference ($P < 0.05$).

(temperature change needed for a log change in D value).

2.8. Statistical analysis

All the experiments were replicated three times and the data were analyzed by ANOVA using SAS software (Version 9.1, SAS Institute Inc., Cary, NC). The Fisher's least significant difference test was used to determine the significant differences in mean values with significance considered at $P < 0.05$.

3. Results and discussion

3.1. S. enterica

Fig. 1(a) shows the effect of hot water treatment of pecans on *S. enterica*. Temperature of hot water and the treatment time were found to have significant effect on the log reduction. Pecans when subjected to hot water treatment for 1 min showed a reduction of 1.79, 1.95, and 2.95 log CFU/g at 70, 80 and 90 °C, respectively; however, no significant difference ($P > 0.05$) in the reduction was observed among the three temperatures. Increasing the treatment time for up to 4 min at 70 °C and 3 min at 80 and 90 °C showed no significant difference ($P > 0.05$). Further increasing the treatment time to 4 min at 80 or 90 °C showed a significant increase (5.60 log CFU/g) in the reduction. Moreover, a maximum reduction of 4.39 ± 0.38, 5.87 ± 1.43, 6.59 ± 0.95 log CFU/g were achieved after 5 min treatment at 70, 80, and 90 °C, respectively. The results of our study show that a 5 min treatment of in-shell pecans with hot water at 70 °C is not sufficient to achieve a 5-log reduction of *S. enterica*. Increasing the treatment temperature to 80 °C achieved a 5-log reduction within 4 min. Further increasing treatment temperature to 90 °C at 4 min showed no significant difference. A similar reduction of *S. enterica* (> 5 log at 85 °C for 4 min) on in-shell pecans was reported by Beuchat and Mann (2011). As per their study *S. enterica* cells that have survived drying and refrigerated storage condition of in-shell pecans are found to be more resistant (> 5 log reduction at 80 or 90 °C for 5 min) to heat treatment than the cells that were treated after drying overnight (> 5 log reduction at 85 °C for 4 min or 90 °C for 1.33 min). Beuchat and Mann (2011) study used inoculated pecans that were forced air dried at 30 °C for 18 h and then stored for weeks prior to hot water treatment while the current study used inoculated in-shell pecans that were air-dried for only 20 min (until the surface is visibly dry) and subjected to hot water treatment. *S. enterica* has showed comparable reductions to hot water treatment in both the scenarios. This implies that hot water treatment of in-shell pecans (either freshly contaminated or long time stored after contamination) at optimum time-temperature conditions is equally efficient in reducing the levels of *S. enterica*.

3.2. E. coli O157:H7

The reduction of *E. coli* O157:H7 when treating pecans with hot water is shown in Fig. 1 (b). Hot water treatment of pecans for 1 min

showed a reduction of 0.9, 1.08 and 2.76 log CFU/g at 70, 80 and 90 °C, respectively. Increasing the temperature from 70 to 80 °C and treatment time from 1 to 2 min had no significant effect on reduction ($P > 0.05$). Increasing the treatment time to 3 min showed a significant increase ($P < 0.05$) in the reduction 3.05, 4.15, and 5.16 log CFU/g at 70, 80 and 90 °C, respectively. Further increasing the treatment time to 5 min showed a reduction of 5.43 and 7.02 log CFU/g at 80 and 90 °C, respectively. Like *S. enterica*, hot water treatment of pecans at 70 °C for 5 min was not sufficient to achieve target 5-log reduction of *E. coli* O157:H7. A minimum of 3 min hot water treatment at 90 °C or 5 min treatment at 80 °C is required to achieve a 5-log reduction of *E. coli* O157:H7.

Several studies reported that the factors such as type of heat treatment, time-temperature conditions, and the type of food matrix has significant effect on the microbial inactivation (Chang, Han, Reyes-De-Corcuera, Powers, & Kang, 2010; Komitopoulou & Aloza, 2009; Phebus et al., 1997). For example, hot water treatment of mung beans seeds (at 90 °C for 90 s) showed a 6.08 log reduction of *E. coli* O157:H7 (Bari, Inatsu, Isobe, & Kawamoto, 2008) while steam treatment of almonds, pistachios, watermelons, cantaloupes (at 200 °C for 10–30 s) and whole flax seeds and sunflower kernels (at 75 °C for upto 5 min) showed more than 5 log reduction (Ban & Kang, 2016; Kwon, Song, & Kang, 2018; Shah, Asa, Sherwood, & Graber, 2017). Orchard contamination of pecans with *E. coli* (Marcus & Amling, 1973) and infiltration of in-shell pecans with microorganisms (Beuchat & Mann, 2010; Blanchard & Hanlin, 1973) have been reported. Based on the findings of this study, hot water treatment of pecans has potential to mitigate the risk of *E. coli* O157:H7 in pecans.

3.3. L. monocytogenes

Among all three pathogens tested in this study, *L. monocytogenes* showed the most heat susceptibility (Fig. 1(c)). A reduction 4.6 log CFU/g was observed when pecans were subjected to hot water treatment at 70 °C for 5 min. Upon increasing the treatment temperature to 80 °C a reduction of 4.93–5.49 log CFU/g was achieved within 3–4 min. Whereas, a reduction of > 5 log CFU/g was achieved within 1 min of treatment at 90 °C. Further increasing the treatment time (i.e. ≥ 2 min at 90 °C) had no significant effect ($P > 0.05$) on the log reduction.

L. monocytogenes has shown heat susceptibility on various food products such as beef (Ikeda, Samelis, Kendall, Smith, & Sofos, 2003; Ozdemir et al., 2006), cantaloupe, watermelon surfaces (Kwon et al., 2018) and RTE turkey breast (Murphy, Duncan, Driscoll, Marcy, & Beard, 2003). A study (Muriana, Quimby, Davidson, & Grooms, 2002) on RTE deli-style meats found that by increasing the hot water temperature from 85-88 °C to 90.6–96.1 °C significantly increased the reduction (≥2 log) of *L. monocytogenes* within 2–4 min. Contamination of nuts/nut products with *L. monocytogenes* has often led to various recalls (FDA, 2017a, 2017b); however, to our knowledge there are no literature regarding heat inactivation of *L. monocytogenes* on nuts. Thus, the result of this study provides evidence that hot water treatment can adequately inactivate *L. monocytogenes* on in-shell pecans.

3.4. E. faecium

Of all the tested organisms in the study, *E. faecium* showed the highest resistance to hot water treatment (Fig. 1(d)). When pecans were subjected to hot water treatment *E. faecium* levels were reduced by 0.95–2.24, 1.20–2.91 and 2.39–3.96 log CFU/g within 1–3 min at 70, 80 and 90 °C, respectively. Further increasing the treatment time to 4–5 min has no effect at 70 °C while a significant increase ($P < 0.05$) in the reduction was observed at 80 and 90 °C, respectively. As per the results of this study a minimum of 4 min hot water treatment at 90 °C is required to achieve a 5 log reduction of *E. faecium*.

E. faecium NRRL B-2354 (ATCC 8459) has been found to be just as resistant as *Salmonella* PT 30 (Shah, Asa, Sherwood, Graber, & T, 2017) and it has been considered effective to be used as a surrogate organism in thermal process validation in the food manufacturing areas (Kopit, Kim, Siezen, Harris, & Marcoa, 2014). The Almond Board of California recommends using *E. faecium* as a surrogate organism for validation of processing equipment used for almond processing. However, it is recommended to validate if the organism can be used as a surrogate for products other than almonds (ABC, 2014). There have been many studies determining the heat resistance of *E. faecium* in other foods. For example, vacuum steam pasteurization of flaxseed, quinoa, and sunflower kernels showed that *E. faecium* was the most heat resistant among tested organisms and it could be used as an effective surrogate for *Salmonella* PT 30 and *E. coli* O157:H7 (Shah et al., 2017). Similar results were reported by Bianchini et al. (2014) when balanced carbohydrate-protein meal was subjected to heat treatment. They observed a 5 log reduction of *E. faecium* and *S. enterica* at 73.7 and 60.6 °C, respectively, when the extruder was operated for 5 min after reaching desired temperature. These findings and the results from our study indicate that *E. faecium* is more resistant to heat treatment as compared to bacterial pathogens such as *S. enterica, E. coli* O157:H7 *and L. monocytogenes.*

3.5. Heat resistance of organisms

D-values of tested organisms are shown in Table 1. The D-values of organisms ranged from 1.15 to 1.72, 0.83 to 1.19, and 0.41–0.92 min at 70, 80, and 90 °C, respectively. The corresponding z-values are 75.86 (*E. faecium*), 76.22 (*S. enterica*), 61.10 (*E. coli* O157: H7), and 49.57 °C (*L. monocytogenes*), respectively. Among the tested organisms, *L. monocytogenes* was found to be least heat resistant with the lowest D-value of 0.41 min at 90 °C while *E. faecium* has the highest D-value of 1.72 min at 70 °C. Further increasing the treatment temperature to 80 and 90 °C significantly ($P < 0.05$) reduced the decimal reduction time of *E. faecium* to 1.19 and 0.92 min, respectively. *S. enterica*, and *E. coli* O157: H7 showed similar thermal death times whose D-values at 70 and 80 °C were significantly lower ($P < 0.05$) than that of *E. faecium.* However, no significant difference was observed at 90 °C. These results indicate that *E. faecium* can be used as a surrogate for heat inactivation studies involving in-shell pecans in place of pathogenic strains of *S. enterica* or *E. coli* O157:H7 or *L. monocytogenes*. Similar observations were also reported when almonds were heat treated with moist-air (Jeong, Marks, & Ryser, 2011) and hot water (Harris et al., 2012). In both of these studies, *Enterococcus* showed equal or higher resistance than *Salmonella* spp. Thus, using the D-values for the most heat resistant organism i.e. *E. faecium* from Table 1, a minimum of 8.6, 6.0, and 4.6 min treatment will be required at 70, 80, and 90 °C, respectively, to achieve a 5-log reduction of tested pathogens on in-shell pecans.

Table 1
Calculation of D-values of each pathogen at each hot water treatment temperature.

Organisms	D-values (min)			z-values (°C)
	70 °C	80 °C	90 °C	
Enterococcus faecium	1.72 ± 0.16a	1.19 ± 0.12 b	0.92 ± 0.02 cd	75.86
Salmonella enterica	1.36 ± 0.19^{b}	0.88 ± 0.25^{d}	0.85 ± 0.09^{d}	76.22
E. coli O157:H7	1.38 ± 0.09^{b}	0.87 ± 0.07^{d}	0.73 ± 0.18^{d}	61.10
Listeria monocytogenes	1.15 ± 0.09 ^{bc}	0.83 ± 0.02^{d}	0.41 ± 0.01^{e}	49.57

Experiments were run in triplicates and analyzed using ANOVA with $P < 0.05$. The different superscripts represent the significant difference between organisms at each temperature and between different temperatures.

4. Conclusions

This study investigated the feasibility of using hot water treatment as a kill-step to mitigate the risk of foodborne pathogens on in-shell pecans. Treatment temperature and the time has significant effect on the log reduction. Among the tested pathogens, *S. enterica* was found to be the most resistant while *L. monocytogenes* was the least resistant to hot water treatment. Our data suggested that 5 log reductions of all the tested pathogens can be achieved when in-shell pecans were hot water treated for 8.6, 6.0, and 4.6 min at 70, 80 and 90 °C, respectively. Also, non-pathogenic *E. faecium* showed similar resistance to hot water as *S. enterica* and *E. coli* O157:H7, indicating a potential surrogate for process validation in pecan industries. Thus, the hot water treatment showed promise in being incorporated as a kill-step to mitigate the risk of foodborne pathogens during post-harvest processing of in-shell pecans. Further studies need to be conducted to understand the effect of hot water treatment of pecans on their physico-chemical properties, sensory characteristics and consumer acceptance.

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