Food Microbiology 27 (2010) 332–338

Contents lists available at ScienceDirect

Food Microbiology

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

Inactivation of *Salmonella* and *Escherichia coli* O157:H7 on artificially contaminated alfalfa seeds using high hydrostatic pressure

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ARTICLE INFO

Article history:
Received 30 July 2009
Received in revised form
26 October 2009
Accepted 1 November 2009
Available online 13 November 2009

Keywords:
Salmonella
Escherichia coli O157:H7
High pressure
Alfalfa
Seeds

ABSTRACT

Alfalfa sprouts contaminated with *Salmonella* and *Escherichia coli* O157:H7 have been implicated in several outbreaks of foodborne illnesses in recent years. The seed used for sprouting appears to be the primary source of pathogens. Seed decontamination prior to sprouting presents a unique challenge for the sprouting industry since cells of the pathogenic survivors although undetectable after sanitizing treatments, can potentially multiply back to hazardous levels. The focus of this study was to therefore test the efficacy of high hydrostatic pressure to eliminate a ~5 log CFU/g load of *Salmonella* and *E. coli* O157:H7 on alfalfa seeds. Pressure treatment of 600 MPa for up to 25 min at 20 °C could not result in complete inactivation of *Salmonella*. High-pressure treatment was then carried out either at sub-ambient (4 °C) or elevated (40, 45 and 50 °C) temperatures to test the ability of high pressure to eliminate *Salmonella*. Pressure treatment at 4 and 20 °C did not deliver any satisfactory inactivation of *Salmonella* while high pressure at elevated temperatures achieved complete kill. Pre-soaking seeds prior to high-pressure treatment also enhanced pressure inactivation of *Salmonella* but at the expense of seed viability. High-pressure treatment of 500 MPa for 2 min at 45 °C was able to eliminate wild-type *Salmonella* and *E. coli* O157:H7 strains without bringing about any appreciable decrease in the seed viability.

1. Introduction

Sprouted seeds have a high nutritive value in addition to their anti-cholesterolemic and anti-carcinogenic properties (Meyerowitz, 1999). In fact, consumption of raw seed sprouts has become increasingly more popular among health conscious consumers. Unfortunately, this has led to a concomitant increase in the incidence of sprout-associated foodborne illnesses throughout the world (Fett and Cooke, 2003; Fett, 2006). *Salmonella* is the most frequently reported cause of foodborne outbreaks of bacterial gastroenteritis in the United States after *Campylobacter* and salmonellosis has been steadily increasing as a public health problem since reporting began in 1943 (Tauxe, 1991). In North America alone, there have been at least 29 reports of sprout-borne *Salmonella* outbreaks, involving more than 1500 cases since 1995 (Taormina and Beuchat, 1999; Chapman, 2009). *Salmonella* appears to be linked more frequently to alfalfa (*Medicago sativa*) sprout-related illnesses than other foodborne pathogens such as *Escherichia coli* O157:H7 and *Bacillus cereus* (Proctor et al., 2001; Hora et al., 2007). Early of 2009, a multi-state *Salmonella* outbreak linked to a variety of sprouts including alfalfa sprouts sickened approximately 166 people. The cases were of *Salmonella enterica* serovar Saintpaul strain while in 2008, *S. enterica* serovar *Typhimurium* was involved in another alfalfa sprouts outbreak (Chapman, 2009; MMWR, 2009).

On the basis of epidemiological evidence as well as direct isolation, the seeds used for sprouting have been found to be the most common source of these pathogens (Fett, 2006). As a result, the U.S. Food and Drug Administration and the California Department of Health Services currently recommends that sprout growers treat their seed with 20,000 ppm of free chlorine for 15 min or with an equivalent approved antimicrobial treatment in addition to testing spent irrigation water for the presence of *Salmonella* and *E. coli* O157:H7 (Waddell and Troxell, 2000; Fett and Cooke, 2003). Although seed decontamination has improved the safety of sprouted seeds, a number of sporadic outbreaks and product recalls incriminating sprouts continue to occur. The limited performance of calcium hypochlorite along with the fact that organic sprout growers are not allowed to treat seeds with such a high level of chlorinated sanitizer has spurred a search for alternative seed decontamination treatments. In addition, some treatments reduce sprouting efficacy, pose health hazards to workers or are regarded by consumers as “unnatural” or “unhealthy” (Kumar et al., 2006). The challenge encountered in disinfesting seeds is that pathogens can be located in deep cracks or crevices on seed coats or in damaged areas thereby protecting them from antimicrobial treatments. If pathogens are not completely eliminated, even low

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0740-0020/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2009.11.003

numbers of survivors can grow to potentially hazardous levels on growing sprouts as a result of the favorable environmental and nutritional conditions during the sprouting process (Holliday et al., 2001). Therefore, seed decontamination strategies applied must ensure complete inactivation of human pathogens while at the same time maintaining the viability and vigor of the seeds. This clearly indicates the need for new strategies that are effective to control diverse *Salmonella* spp. on seeds since it is known that multiple strains of *Salmonella* spp. have been implicated in sprout-related disease outbreaks (NACMCF, 1999).

Both Penas et al. (2008) and Ariefdjohan et al. (2004) investigated the application of high pressure on alfalfa seeds to improve the safety of the resultant sprouts. Penas et al. (2008) found that seeds pre-soaked in water and subsequently pressure-treated in the absence of water exhibited a significantly lower germination rate (<30%) than untreated alfalfa seeds. Ariefdjohan et al. (2004) found that pressurizing alfalfa seeds in the absence of water took longer to germinate, achieving final germination of up to 34%, while 95% of the control seed germinated. Recently we demonstrated that the presence of water during pressure treatment of alfalfa seeds was critical for pressure inactivation of nalidixic acid-resistant strains of *E. coli* O157:H7 and for seed viability retention (Neetoo et al., 2008). In later studies, we found that treatment temperature and soaking seeds prior to pressure treatment played an important role in the pressure inactivation of nalidixic acid-resistant strains of *E. coli* O157:H7 (Neetoo et al., 2009a,b). The results we obtained from those three studies were used in the current study to guide us to design experiments to obtain optimal pressure processing conditions to eliminate *Salmonella*, the etiological agent responsible for the great majority of sprout-related illnesses. The high-pressure processing parameters were first optimized using nalidixic acid-resistant *Salmonella* strains. The developed high-pressure processes were then validated for its ability to eliminate wild-type *Salmonella* and *E. coli* O157:H7 strains on alfalfa seeds. The effect of these pressure processes on the viability of the seeds was finally assessed.

2. Materials and methods

2.1. Determination of the pressure inactivation curve of Salmonella spp. on alfalfa seeds

2.1.1. Bacterial strains and preparation of inoculum

Five *Salmonella* strains were used in this study: three *Salmonella typhimurium* strains, T43, T45 and TDT 104, *S. enterica* serovar Enteritidis E44 and *S. enterica* serovar Montevideo Mo57 (Courtesy of Dr. Joerger, University of Delaware). The cells of *Salmonella* were adapted to grow in tryptic soy broth plus 0.6% yeast extract (Difco Laboratories, Sparks, MD) supplemented with nalidixic acid to a final concentration of 50 μg/ml (Fisher Scientific, Hampton, NH) (TSBYE-N). Individual cultures were grown in TSBYE-N overnight at 35 °C. Cultures were then transferred (one loopful) into 10 ml of fresh TSBYE-N and incubated at 35 °C for 24 h. Equal volumes of individual cultures were mixed to form a five-strain cocktail of *Salmonella* to a final cell density of 10^9 CFU/g.

2.1.2. Inoculation of seeds

Appropriate dilutions of the five-strain culture cocktail (5 ml) was mixed with 75 ml of sterile 0.1% peptone water (Fisher). Alfalfa seeds (*M. sativa*) (100 g), obtained from International Specialty Supply (Cookeville, TN), were added to the cell suspension and gently stirred for 5 min. The seeds were separated from the cell suspension by pouring the mixture over a double layer of cheesecloth supported by a wire screen and dried inside a biosafety hood at room temperature (21 ± 1 °C) for 24 h. Dried seeds with a water activity of 0.622 (AquaLab, Decagon Devices, Inc., WA) and an approximate inoculation level of 10^5 CFU of *Salmonella* per g were placed in sterile pouches and stored at 4 °C.

2.1.3. High-pressure treatment

Two grams of inoculated seeds and 3 ml of sterile deionized (DI) water were placed in a 3-mil-thick pouch (nylon/polyethylene, Koch Supplies, Kansas City, MO). Seeds were immersed in water during pressurization to enhance the baro-inactivation of pathogens and viability retention of pressure-treated seeds as demonstrated previously by Neetoo et al. (2008). To avoid leakage during pressure treatment, each sample pouch was placed in a larger pouch of an 8-mil-thick polyvinyl chloride plastic (McMaster-Carr, Elmhurst, IL) and heat sealed. Pressure treatment of samples was immediately carried out using a high-pressure unit with temperature control (model Avure PT-1; Avure Technologies, Kent, WA) and a maximum operating pressure of 650 MPa. The volume of the pressure chamber was approximately 50 ml. The experiments were conducted at 20 °C (initial seed sample temperature prior to pressure treatment) using water as a hydrostatic medium at a pressure level of 600 MPa. The pressure increase rate was approximately 22 MPa/s. The pressure release was almost immediate (<4 s). Pressurization time reported in this study did not include the pressure come up or release times.

2.1.4. Microbiological analysis

Pouches containing treated seeds were cut open aseptically. The sample consisting of the seed-water mixture was transferred into a stomacher bag to which 8 ml of sterile 0.1% peptone water was added and subsequently stomached for 2 min at 260 rpm (Seward 400 Stomacher; Seward Medical Co., London, United Kingdom). The seed mixture was serially diluted in sterile 0.1% peptone and surface-plated in duplicate on tryptic soy agar with 0.6% yeast extract (Difco Laboratories, Sparks, MD) supplemented with nalidixic acid to a final concentration of 50 μg/ml (TSAYE-N). TSAYE-N plates were incubated for 3 days at 35 °C. Presumptive colonies of *Salmonella* formed on the plates were enumerated. The seed mixture was also directly enriched in 90 ml of TSBYE-N and incubated for 48 h at 35 °C to allow resuscitation of sublethally injured cells. Samples were streaked onto Xylose Lysine Deoxycholate (XLD) agar (Difco Laboratories, Sparks, MD) plates supplemented with 50 mg/ml of nalidixic acid. After 24 h of incubation, the presence of black or black-centered colonies typical of *Salmonella* was recorded as positive.

2.2. Application of low or elevated temperatures and/or extended treatment time on the inactivation of Salmonella spp.

Two grams of inoculated seeds was mixed with 3 ml of sterile DI water, packaged, and treated at 300–600 MPa for 2 min at 4, 20, 40, 45, and 50 °C or at 300–600 MPa for 5 min at 40, 45 and 50 °C. Samples were submerged in the water bath surrounding the pressure cell for 10 min for samples to equilibrate to the water bath temperature before pressurization. Immediately after pressure treatment, the samples were cooled in an ice–water mixture. Samples were then microbiologically assayed as described previously and enriched for the detection of survivors.

2.3. Effect of pre-soaking alfalfa seeds in water for varying times prior to pressure treatment on inactivation of Salmonella spp.

Two grams of inoculated seeds was soaked in 20 ml sterile DI water for 0 (without soaking), 15, 30 45 and 60 min at 20 °C. At the end of the soaking period, the excess water was subsequently decanted and seeds were placed into a pouch in the presence of 3 ml of fresh sterile DI water and pressure-treated at 300–550 MPa

for 2 min or 300–450 MPa for 5 min at 40 °C as described above. The handling of samples (3–5 min), temperature equilibration step (10 min) and the actual pressure treatment (2 or 5 min) represented an additional seed-soaking step lasting a total of 15–20 min. Samples were then immediately cooled and microbiologically assayed post-treatment as described previously.

2.4. Germination test on selected treatments to determine optimal conditions for pressure processing of seeds

To determine the effect of pressure treatment on the seed's germination potential, two grams of un-inoculated alfalfa seeds was mixed with 3 ml of DI water, packaged, and treated at 600 MPa for 2 min at 40 °C, 500 MPa for 2 min at 45 °C, 500 MPa for 5 min at 40 °C, 400 MPa for 5 min at 45 °C and 350 MPa for 5 min at 50 °C as described previously. In addition, 2 g of un-inoculated seeds were soaked for 45 min and pressure-treated at 500 MPa for 2 min at 40 °C or soaked for 60 min, the excess water decanted and pressure-treated at 400 MPa for 5 min at 40 °C as described above. Untreated (control) and pressure-treated seeds were then soaked in DI water after pressure treatment for an overall soaking time of 3 h prior to setting them to germinate. One hundred seeds were drawn from the soaked seeds and spread evenly on pieces of wet paper towels on a plastic rack, which in turn was placed in a water-filled bucket to provide a moist environment for the seeds. The water level was maintained below the level of seeds. The bucket was kept at room temperature for 8 days (suggested by the seed provider) and misted daily. The bucket was covered loosely with a piece of plastic film to allow exchange of air between the inside and outside of the bucket. Sprouted seeds characterized by the emergence of the root tip (radicle) were enumerated 3–8 days after the germination system was set up, and the values were recorded as percent germination. The incubation duration was extended to up to 8 days to give sufficient time for seeds with a delayed sprouting onset to germinate.

2.5. Assessment of the robustness of selected treatments with respect to complete elimination of wild-type Salmonella and E. coli O157:H7

Two grams of alfalfa seeds inoculated with wild-type *Salmonella* was mixed with 3 ml of DI water, packaged, and treated at 600 MPa for 2 min at 40 °C, 500 MPa for 2 min at 45 °C or 500 MPa for 5 min at 40 °C. Samples were then pre-enriched in Lactose broth (90 ml) for 24 h at 35 °C. After the pre-enrichment step, 0.1 ml of the mixture was transferred to 10 ml Rappaport-Vassiliadis (RV) medium and incubated for another 24 h. After the secondary enrichment step, the sample was then streaked onto XLD plates (U.S. FDA, 2007). Wild-type *E. coli* O157:H7 strains, Strains 250, 251 and 1730, Cider strain and J58, were also used (Neetoo et al., 2008). Two grams of alfalfa seeds inoculated with a cocktail of the five wild-type *E. coli* O157:H7 strains were mixed with 3 ml of DI water, packaged, and treated at 500 MPa for 2 min at 45 °C or 500 MPa for 5 min at 40 °C as described previously. Samples were then enriched in 90 ml of EHEC Enrichment Broth (EEB) for 24 h at 35 °C. After the enrichment step, the sample was then streaked onto CT-SMAC plates and incubated for 24 h (U.S. FDA, 2002).

2.6. Testing of sprouted seed samples and waste irrigation water for surviving pathogens

Two grams of seeds inoculated with a ~5 log burden of wild-type *Salmonella* or *E. coli* O157:H7 were pressure-treated at 500 MPa at 45 °C for 2 min as described previously. Treated seeds were then soaked for 3 h at ambient temperature in sterile DI water. After soaking, the water was then decanted and the seeds were placed onto a pre-wet Whatman filter paper in sterile plastic boxes for germination at room temperature. Sterile DI water was sprayed onto the sprouting seeds daily. After 48 h of germination, sprouted and non-sprouted seeds were scooped and transferred into sterile stomacher bags, enriched and analyzed for *Salmonella* or *E. coli* O157:H7 as described above. In addition, 10 ml of the spent water was collected at the bottom of the plastic boxes and similarly tested. The presence of growth on CT-SMAC and XLD plates was further confirmed for identification using the RapidChek® tests for *E. coli* O157 and *Salmonella*, respectively (Strategic Diagnostics Inc., Newark, DE, USA).

2.7. Statistical analysis

All experiments were replicated three times. Where appropriate, statistical analyses were conducted using Minitab release 15 (Minitab Inc., University Park, PA). One-way analysis of variance and Tukey's one-way multiple comparisons were used to determine differences in the populations of *Salmonella and E. coli* O157:H7 recovered on treated alfalfa seeds and differences in the germination rates of seeds. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

3. Results and discussion

3.1. Determination of the pressure inactivation curve of Salmonella spp. on alfalfa seeds

Survival curve of a five-strain *Salmonella* cocktail on seeds pressurized at 20 °C is shown in Fig. 1. Pressure treatment for 5–25 min significantly ($P < 0.05$) reduced the population and an increase in treatment time was accompanied by a progressively higher degree of inactivation, achieving a maximum reduction of 4.5 log CFU/g of *Salmonella*. The inactivation curve exhibited a nearly linear decline in surviving cells with time, marked by a steady drop in bacterial counts, in contrast to the more frequently observed pressure inactivation curve characterized by a rapid initial drop followed by tailing caused by a diminishing inactivation rate.

Pressure inactivation of *Salmonella* has been widely studied at ambient temperature. Patterson (2005) showed that a pressure treatment of 350 MPa and 15 min at 20 °C reduced *S. typhimurium* by 5 log cycles. Chen et al. (2006) demonstrated a 6.5-log reduction of *S. enteritidis* at 600 MPa for 10 min at 21.5 °C in UHT whole milk. Tholozan et al. (2000) exposed *S. typhimurium* Mutton (ATCC 13311) in sodium citrate (pH 5.6) and sodium phosphate (pH 7.0)

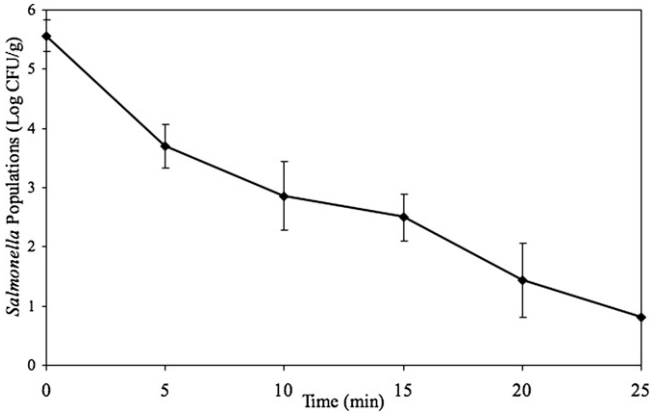

Fig. 1. Pressure inactivation curve of nalidixic acid-resistant strains of *Salmonella* spp. on alfalfa seeds treated at 600 MPa and 20 °C. Error bars represent ± one standard deviation.

buffers to 400 MPa for 10 min and 20 °C for complete inactivation (>8 log reduction). Guan et al. (2005) showed that pressure treatment of 450 MPa for at least 30 min or 20 min at 500 and 550 MPa at ambient temperature brought about a 5.0-log reduction of *S. typhimurium* DT 104 in UHT milk. In general, inactivation curves did not follow first-order kinetics but rather tended to be exponential, with an initial rapid decrease in numbers followed by a "tail" indicating that a small fraction of the population was highly baro-resistant (Chen, 2007). In addition, as several authors already mentioned, the composition and water activity of the substrate in which the microorganisms are suspended are also likely factors that affect their high-pressure tolerance (Oxen and Knorr, 1993; Palou et al., 1997; Patterson and Linton, 2008).

Patterson and Linton (2008) noted that significant variation in pressure resistance has been reported among different strains of the same genus which may be attributed to differences in susceptibility to membrane damage, although the exact nature of the lethal effect and the role of the membrane structure still have to be clarified. Chen et al. (2009) observed that when thirty strains of *Listeria monocytogenes* were exposed to a treatment of 400 MPa for 2 min at 21 °C, the strains exhibited reductions ranging from 1.9 to 7.1 log CFU/ml. In recognition of the natural variability and uniqueness of strains, it is preferable to use a cocktail of strains in inactivation and validation studies conducted on food (Balasubramaniam et al., 2004). Hence, in our challenge studies, a composite of five strains of *Salmonella* were used to that effect. *S. typhimurium* DT 104 was included as part of the five-strain cocktail since this strain has been the object of rising alarm, spreading at a fast pace through Britain and the United States over the past 10–15 years (Keene, 1999).

3.2. Application of low or elevated temperatures and/or extended treatment time on the inactivation of Salmonella

To achieve more than 5 log cycles reduction of *Salmonella* on seeds, higher pressure (>600 MPa) and/or extended treatment time (>25 min) could be used. However, the upper pressure limit for commercial high-pressure applications is 600 MPa and long pressurization time is not economically feasible. The effect of treatment temperature is therefore of great practical interest, because combined pressure-temperature processing may deliver greater microbial inactivation ratios while operating at lower pressure levels and/or for shorter periods of time. The economical advantage of combining pressure with temperature would result in less costly equipment at a higher throughput, with a considerable decrease in the production cost (per mass unit of food) (Van den Berg et al., 2001).

Hence, our next step was to investigate the ability of high pressure to completely eliminate *Salmonella* spp. on alfalfa seeds subjected to various pressure/time/temperature combinations. Results for the pressure inactivation of *Salmonella* on alfalfa seeds at different initial sample temperatures are shown in Table 1. The enhancement of temperature on pressure inactivation of *Salmonella* is clearly evident marked by the reduction to below detectable limits of the initial inoculum load under various ternary combinations of pressure/time/temperature. The most effective treatment was observed at 50 °C, followed by 45 and 40 °C. The degree of inactivation increased with increasing pressure with lower survival rates at 600 MPa than at 300 MPa for all temperatures tested.

When the initial sample temperatures were set to 4 or 20 °C, *Salmonella* spp. responded quite similarly to pressures ranging from 300 to 600 MPa with no statistically significant enhancement ($P > 0.05$) at a sub-ambient temperature, achieving a maximum reduction of 1.5 and 2.0 log CFU/g at 600 MPa at 4 and 20 °C, respectively. Previous studies have shown that the combination of pressure and low or subzero treatment temperatures can be more effective at killing microorganisms than treatment at ambient temperature (20 °C). Lopez-Caballero et al. (2002) found greater microbial inactivation when the pressure treatment was carried out at refrigeration temperatures rather than at ambient temperature. They reported 1- to 2-log greater inactivation of *Pseudomonas fluorescens* at 5 °C than with pressure treatment at 20 or 35 °C. When *L. innocua* was pressure-treated at 300 MPa for 15 min in liquid whole egg, a 1.5–2 log inactivation was achieved at −15 or 2 °C, compared with virtually no inactivation at 20 °C (Ponce et al., 1998). Kural and Chen (2008) also found that cold temperature enhanced pressure inactivation of *Vibrio vulnificus* when compared with treatment at ambient temperature. However, we did not observe any enhancement in pressure inactivation of *Salmonella* at low temperatures compared to ambient temperatures, demonstrating that the effect of sub-ambient temperature on pressure inactivation of microorganisms are strain-dependent.

When the initial sample temperature was 40 °C and higher, inactivation was significantly higher ($P < 0.05$) and more pressure-dependent, achieving >5 log reductions at ≥300 MPa (50 °C), ≥400 MPa (45 °C) and ≥450 MPa (40 °C) although survivors were still detected post-enrichment. However, survivors were not detected using the enrichment method when the pressure was increased to 600 MPa (40 °C) and 500 MPa (≥45 °C). Increasing the treatment temperature from 40 through 50 °C greatly enhanced the pressure inactivation of *Salmonella* reducing the pressure required to completely eliminate the pathogen. Bacterial cells are known to be relatively less barosensitive at 20–35 °C, but become more sensitive to pressurization above 35 °C, due to phase transition of membrane lipids (Kalchayanand et al., 1998a,b; Ludwig, 2002).

Since the findings showed that a 2-min application of high pressure at a level of 300–450 MPa at 40 and 45 °C resulted in

Table 1
Effect of high pressure (treatment time of 2 min) in combination with low or elevated temperatures on the inactivation of nalidixic acid-resistant strains of *Salmonella*. The initial population of *Salmonella* spp. on alfalfa seeds was 5.3 log CFU/g.

Pressure (MPa)	Temperature (°C)				
	4	20	40	45	50
300	4.5 ± 0.5	4.6 ± 0.0	2.4 ± 1.0	1.4 ± 0.2	<0.8 (3/3)
350	4.6 ± 0.3	4.4 ± 0.1	1.4 ± 0.6	0.8 ± 0.0	<0.8 (2/3)
400	4.3 ± 0.3	4.3 ± 0.1	1.2 ± 0.8	<0.8 (3/3)	<0.8 (1/3)
450	4.2 ± 0.2	4.2 ± 0.2	<0.8 (2/3)	<0.8 (1/3)	<0.8 (1/3)
500	4.4 ± 0.1	3.9 ± 0.2	<0.8 (1/3)	<0.8 (0/3)	<0.8 (0/3)
550	4.0 ± 0.1	3.6 ± 0.2	<0.8 (1/3)	<0.8 (0/3)	<0.8 (0/3)
600	3.8 ± 0.5	3.3 ± 0.2	<0.8 (0/3)	<0.8 (0/3)	<0.8 (0/3)

Data representing mean log survivors (CFU/g) ± standard deviation. Numbers in parentheses represent number of samples testing positive after enrichment out of a total of 3 trials.

Table 2
Effect of high pressure (treatment time of 5 min) in combination with mild heat on inactivation of nalidixic acid-resistant strains of *Salmonella*. The initial population of *Salmonella* spp. on alfalfa seeds was 5.2 log CFU/g.

Pressure (MPa)	Temperature (°C)		
	40	45	50
300	1.0 ± 0.3	<0.8 (3/3)	<0.8 (1/3)
350	<0.8 (2/3)	<0.8 (2/3)	<0.8 (0/3)
400	<0.8 (3/3)	<0.8 (0/3)	<0.8 (0/3)
450	<0.8 (1/3)	<0.8 (0/3)	<0.8 (0/3)
500	<0.8 (0/3)	<0.8 (0/3)	<0.8 (0/3)
550	<0.8 (0/3)	<0.8 (0/3)	<0.8 (0/3)
600	<0.8 (0/3)	<0.8 (0/3)	<0.8 (0/3)

Data representing mean log survivors (CFU/g) ± standard deviation. Numbers in parentheses represent number of samples testing positive after enrichment out of a total of 3 trials.

Table 3
Effect of pre-soaking on pressure (300–550 MPa for 2 min at 40 °C) inactivation of nalidixic acid-resistant strains of *Salmonella*. The initial population of *Salmonella* spp. on alfalfa seeds was 5.9 log CFU/g.

Pressure (MPa)	Soaking time (min)				
	0	15	30	45	60
300	3.1 ± 0.1	2.7 ± 0.1	1.7 ± 0.7	<0.8 (3/3)	<0.8 (3/3)
350	1.6 ± 0.4	1.3 ± 0.2	0.8 ± 0.0	<0.8 (3/3)	<0.8 (2/3)
400	1.2 ± 0.3	1.4 ± 0.6	<0.8 (2/3)	<0.8 (2/3)	<0.8 (2/3)
450	<0.8 (3/3)	<0.8 (3/3)	<0.8 (3/3)	<0.8 (1/3)	<0.8 (1/3)
500	<0.8 (3/3)	<0.8 (3/3)	<0.8 (2/3)	<0.8 (0/3)	<0.8 (0/3)
550	<0.8 (3/3)	<0.8 (3/3)	<0.8 (1/3)	<0.8 (0/3)	<0.8 (0/3)

Data representing mean log survivors (CFU/g) ± standard deviation. Numbers in parentheses represent number of samples testing positive after enrichment out of a total of 3 trials.

>5 log inactivation, the pressure treatment time was thus extended to 5 min to investigate whether a lower pressure level could result in an equivalent kill in an attempt to reduce the treatment pressure level. When the pressure exposure time was extended to 5 min, as anticipated, a lower pressure bracket (300–500 Ma) brought about an equivalent kill. Specifically, exposure to 500 MPa at 40 °C or ≥400 MPa at 45 °C or ≥350 MPa at 50 °C for 5 min completely eliminated a 5 log load of *Salmonella* (Table 2).

3.3. Effect of pre-soaking alfalfa seeds in water for varying times prior to pressure treatment on inactivation of Salmonella

To further reduce the pressure level needed for complete elimination of *Salmonella* on seeds, the effect of pre-soaking of seeds on pressure inactivation of *Salmonella* was determined. Tables 3 and 4 show that treatments at 300–600 MPa without prior soaking brought about 2.8–5.8 log CFU/g reduction at 40 °C. When seeds were soaked for >30 min, a significant enhancement ($P < 0.05$) in pressure inactivation of *Salmonella* relative to un-soaked seeds was observed. In addition, a direct relationship between the degree of inactivation and the soaking time as evidenced by larger population reductions was observed across the entire pressure spectrum (300–550 MPa) investigated. Seeds soaked for >45 min followed by pressure treatment at >500 MPa at 40 °C for 2 min produced no detectable counts and tested negative after enrichment (Table 3). When the pressure holding time was extended to 5 min, a pressure level of >400 MPa was adequate to completely decontaminate seeds with a minimum soaking time of 60 min (Table 4). Again, the same trend was observed i.e. the longer the soaking time, the greater the degree of pressure inactivation. It should be noted that the additional seed-soaking step during the handling of samples, temperature equilibration prior to pressure treatment and the actual pressurization for a total of 15–20 min might have also enhanced the pressure inactivation of the inoculated pathogen.

Table 4
Effect of pre-soaking on pressure (300–450 MPa for 5 min at 40 °C) inactivation of nalidixic acid-resistant strains of *Salmonella*. The initial population of *Salmonella* spp. on alfalfa seeds was 5.9 log CFU/g.

Pressure (MPa)	Soaking time (min)				
	0	15	30	45	60
300	1.6 ± 0.2	1.6 ± 0.2	<0.8 (3/3)	<0.8 (3/3)	<0.8 (3/3)
350	1.1 ± 0.4	<0.8 (3/3)	<0.8 (3/3)	<0.8 (3/3)	<0.8 (2/3)
400	<0.8 (3/3)	<0.8 (3/3)	<0.8 (3/3)	<0.8 (2/3)	<0.8 (0/3)
450	<0.8 (3/3)	<0.8 (3/3)	<0.8 (2/3)	<0.8 (1/3)	<0.8 (0/3)

Data representing mean log survivors (CFU/g) ± standard deviation. Numbers in parentheses represent number of samples testing positive after enrichment out of a total of 3 trials.

Previously, Delaquis et al. (1999) performed a microscopic examination of alfalfa seeds and reported that although the seed surface is relatively smooth, the stem scar is relatively porous with areas capable of harboring pathogens, thus affording protection to hidden bacterial cells. In addition to their topographic complexity, the surface of alfalfa seeds is covered with a waxy cuticle (cutin) thus lowering their water wettability. Charkowski et al. (2001) however mentioned that by pre-soaking of seeds in water for a certain period of time, water is believed to permeate the seed coat causing bacteria trapped in cracks, crevices or other discontinuities to be released. It is also thought that the pre-soaking step may be important in raising the water activity of the seeds which is known to have a bearing on the pressure inactivation of bacterial cells. It is well documented that high water activity enhances pressure inactivation of microorganisms (Oxen and Knorr, 1993; Palou et al., 1997; Goodridge et al., 2006; Kingsley and Chen, 2008). The critical role played by water in pressure inactivation of *E. coli* O157:H7 on seeds was also demonstrated by Neetoo et al. (2008, 2009b). Hence it can be concluded that there is an intimate interplay between the soaking time, pressure magnitude and treatment temperature which are critical factors in ensuring the efficacy of HHP to decontaminate alfalfa seeds.

3.4. Germination test on selected treatments to determine optimal conditions for pressure processing of seeds

Results from Sections 3.2 and 3.3 have shown that complete inactivation of *Salmonella* spp. is possible when seeds are pressure-treated under selected conditions of pressure exposure time, temperature, pressure level and soaking time. The germination percentages as determined 3–8 days from the onset of germination showed that seeds pressure-treated under the different conditions were affected to variable extents depending on the conditions of the treatment (Table 5). The treatments at 600 MPa at 40 °C for 2 min, 500 MPa at 40 °C for 5 min, 500 MPa at 45 °C for 2 min and

Table 5
Effect of selected high-pressure treatment conditions on seed germination rate (%).

Treatment conditions				Days of germination					
P[a] (MPa)	T[b] (°C)	t[c] (min)	St[d] (min)	3	4	5	6	7	8
600	40	2	0	91 ± 1	92 ± 1	94 ± 1	95 ± 2	96 ± 2	96 ± 2
500	45	2	0	91 ± 2	93 ± 0	96 ± 2	96 ± 2	97 ± 3	97 ± 3
500	40	5	0	90 ± 1	93 ± 1	94 ± 2	96 ± 1	96 ± 1	96 ± 1
400	45	5	0	87 ± 3	90 ± 2	94 ± 1	94 ± 1	95 ± 1	95 ± 1
350	50	5	0	70 ± 4	79 ± 5	83 ± 5	85 ± 5	85 ± 5	85 ± 5
500	40	2	45	46 ± 1	54 ± 1	58 ± 2	58 ± 2	58 ± 2	58 ± 2
400	40	5	60	51 ± 8	58 ± 7	59 ± 7	60 ± 7	60 ± 7	60 ± 7
Control				98 ± 1	99 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0

[a] P = pressure.
[b] T = temperature.
[c] t = pressure holding time.
[d] St = soaking time before pressure treatment.

400 MPa at 45 °C for 5 min were the most promising as they had no significant adverse effects ($P > 0.05$) on the seeds' viability achieving a germination percentage of ≥95% after 8 days of germination. Pressure treatments on un-soaked alfalfa seeds at 40 and 45 °C brought about greater viability retention than when the temperature was set to 50 °C.

As far as soaked seeds are concerned, it is not clear what mechanisms are responsible for the lower germination yields but it is possible that denaturation of proteinaceous molecules such as enzymes might have occurred during pressurization following their activation during prolonged water imbibition. It is also likely that during soaking, seeds uptake water and as a result of the increased moisture content, the seeds advance into an active state whereby physiological changes associated with the initial stages of germination start to take place (Neetoo et al., 2009b).

3.5. Assessment of the robustness of selected treatments with respect to complete elimination of wild-type Salmonella and E. coli O157:H7

The three optimum treatments determined in Section 3.4 were able to completely inactivate *Salmonella* nalidixic acid-resistant mutants; however only two treatments, 500 MPa for 5 min at 40 °C and 500 MPa for 2 min at 45 °C were able to consistently eliminate wild-type *Salmonella* strains on alfalfa seeds. Further testing on wild-type *E. coli* O157:H7 strains showed that pressure treatment at 500 MPa for 2 min at 45 °C was the only treatment that could achieve a complete inactivation. Since seeds pressure-treated under 500 MPa at 45 °C for 2 min achieved >95% germination rate (Table 5), this study thus indicates that high pressure applied in conjunction with mild heat can be an effective method for inactivating *Salmonella* and *E. coli* O157:H7 on alfalfa seeds, hence pointing to the plausibility of pressure application for effective control of enteric pathogens on alfalfa seeds (Table 6).

3.6. Testing of sprouted seed samples and waste irrigation water for surviving pathogens

Overall, sprouted seeds originally inoculated with *E. coli* O157:H7 and pressure-treated at 500 MPa and 45 °C for 2 min along with their spent irrigation water samples produced bright pink growth on CT-SMAC plates in out of three cases. Seeds initially inoculated with *Salmonella* and their corresponding collected water samples produced bright yellow growth on XLD plates in two out of three trials. Since colony morphology and color were atypical of *E. coli* O157:H7 and *Salmonella* respectively, we confirmed the absence of both pathogens in all cases using immunoassay-based tests.

Table 6
Assessment of the robustness of selected treatments with respect to complete elimination of wild-type *Salmonella* and *E. coli* O157:H7. The initial populations of *Salmonella* spp. and *E. coli* O157:H7 on alfalfa seeds were 5.8 and 5.2 log CFU/g, respectively.

Treatment conditions			Frequency of survivors	
P[a] (MPa)	T[b] (°C)	t[c] (min)	*Salmonella*	*E. coli* O157:H7
600	40	2	(1/3)	ND
500	45	2	(0/3)	(0/3)
500	40	5	(0/3)	(1/3)

Numbers in parentheses represent number of samples testing positive after enrichment out of a total of 3 trials.
ND = Not done.
[a] : P = pressure.
[b] : T = temperature.
[c] : t = pressure holding time.

4. Conclusions

In conclusion, this study demonstrated that the pressure inactivation of *Salmonella* including *S. typhimurium* DT 104 on alfalfa seeds followed a fairly linear rate of inactivation. Results clearly indicate that destruction of *Salmonella* spp. on alfalfa seeds depended on pressure and the temperature at which it was applied. Also, inactivation was less pronounced at low and room temperatures while higher temperatures gave better results. High pressure (500 MPa) combined with mild heating (45 °C) could be an effective means to decontaminate seeds whilst retaining seed viability. The inactivation data garnered in this study should be invaluable for seed processing for safety, especially as exemplified using *S. typhimurium* DT 104. With the ever expanding applications of HHP for the preservation and processing of food, reliable determination of optimum processing parameters, with regard to temperature, time and pressure magnitude, will become critical process factors towards ensuring the safety of pressure-treated foods.

Acknowledgement

This study was supported by a start-up fund from the Department of Animal and Food Sciences at the University of Delaware.

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