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Elimination of *Escherichia coli* O157:H7 from Alfalfa Seeds through a Combination of High Hydrostatic Pressure and Mild Heat[▽]

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Escherichia coli **O157:H7 has been associated with contaminated seed sprout outbreaks. The majority of these outbreaks have been traced to sprout seeds contaminated with low levels of pathogens. Sanitizing sprout seeds presents a unique challenge in the arena of produce safety in that even a low residual pathogen population remaining on contaminated seed after treatments appears capable of growing to very high levels during sprouting. In this study, the effectiveness of high-pressure treatment in combination with low and elevated temperatures was assessed for its ability to eliminate** *E. coli* **O157:H7 on artificially contaminated alfalfa seeds. Inoculated seed samples were treated at 600 MPa for 2 min at 4, 20, 25, 30, 35, 40, 45, and 50°C. The pressure sensitivity of the pathogenic bacteria was strongly dependent on the treatment temperature. At 40°C, the process was adequate in eliminating a 5-log-unit population on the seeds with no adverse effect on seed viability. Three treatments carried out at reduced pressure levels and/or extended treatment time, 550 MPa for 2 min at 40°C, 300 MPa for 2 min at 50°C, and 400 MPa for 5 min at 45°C, were equally lethal to the pathogen. When all three treatments were compared in terms of their impact on seed viability, the process of 550 MPa for 2 min at 40°C was the most desirable, achieving final germination percentages and sprout sizes statistically similar to those of control untreated seeds (*P* > 0.05).**

In many countries worldwide, including the United States, the consumption of seed sprouts has increased in recent decades with the advent of neutraceuticals and phytochemicals and the shift of consumer preference toward health foods (18, 31). Alfalfa sprouts are one of the most common sprouts consumed in the United States due to availability and nutritional value (16, 24). However, the sprouting process makes the commodity susceptible to microbial contamination and growth, thereby compromising the safety and quality of the sprouts (17).

Recently, alfalfa and other types of sprouts that are often consumed raw have been designated as a special food safety problem by the National Advisory Committee on Microbiological Criteria for Foods because of the propensity of human pathogens, such as *Escherichia coli* O157:H7, to rapidly multiply on sprouts during sprouting and because of the lack of a postharvest lethal step in the processing of sprouts (28). Since 1995 in the United States, there have been two outbreaks caused by *E. coli* O157 (O157:H7 and O157:NM) due to the consumption of contaminated alfalfa and clover sprouts (6, 9, 15, 25). In most sprout-associated outbreaks, the initial source of contamination was thought to be the seeds themselves on the basis of direct isolation and/or epidemiological evidence (16).

Many studies have thus been performed to determine the effectiveness of a wide range of treatments for reducing the levels of pathogenic *E. coli* O157:H7 on seeds and sprouts (4). Decontamination by soaking the seeds in different chemical solutions has been thoroughly investigated. Chemicals used include chlorine, organic acids, hydrogen peroxide, and etha-

nol, and these chemicals have been reported to have various degrees of efficacy (3, 5, 20). Very often, the bacterial cells may reside in the seed crevices or between the seed coat and cotyledon (10), providing protection from chemical sanitation. Sprouting conditions may provide a suitable temperature and moisture conditions for the growth of *E. coli* O157:H7 and *Salmonella*. These conditions, together with nutrients released by sprouting seeds, help low levels of attached pathogens reach dangerous levels on the finished sprouts (35).

Conventional thermal processing has the potential to ensure food safety and lead to an extended shelf-life. However, it often leads to detrimental changes in the sensory and nutritional qualities of the product (27). With nonthermal processing technologies, such as high hydrostatic pressure (HHP) technology, more fresh product-like products can be obtained. HHP treatment is advantageous because of its ability to inactivate a number of enzymes and microorganisms contained in foods without significantly altering their sensory and nutritional properties (2).

The application of HHP alone to enhance the safety or quality of seeds or sprouts has been studied previously (1, 26, 29, 32, 39) with various degrees of efficacy reported. Research in our laboratory demonstrated that the application of pressure at a level of 650 MPa for 15 min at 20°C was adequate to eliminate *E. coli* O157:H7 from alfalfa seeds (29). The aim of this study was to develop a high-pressure process to achieve reduction of a 5-log-unit initial population of *E. coli* O157:H7 on alfalfa seeds to an undetectable level (cells were not detected using an enrichment method) and subsequently assess the effects of the process on the retention of viability of the seeds. To simplify sentence constructions, the term elimination was used in this paper interchangeably with "reduction of the bacterial population to an undetectable level."

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MATERIALS AND METHODS

Bacterial strains and preparation of inoculum. *E. coli* O157:H7 strains 1730, 250, 251, J58, and Cider were used. The sources of the strains were provided in the paper by Neetoo et al. (29). The cells of *E. coli* O157:H7 were adapted to grow in tryptic soy broth plus 0.6% yeast extract (Difco Laboratories, Sparks, MD) supplemented with 50 μg/ml of nalidixic acid (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 *E. coli* O157:H7.

Inoculation of seeds. The cocktail (10 ml) was mixed with 100 ml of sterile 0.1% peptone water (Fisher). Alfalfa seeds (*Medicago 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°C ± 1°C) for 24 h. Dried seeds (water activity of ~0.622) with an approximate inoculation level of 10^5 CFU of *E. coli* O157:H7 per g were placed in sterile pouches and stored at 4°C.

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). 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. HHP treatment of samples was carried out using a high-pressure unit with temperature control (model Avure PT-1; Avure Technologies, Kent, WA). The experiments were conducted at temperatures ranging from 4°C to 50°C (initial seed sample temperature prior to pressure treatment) using water as a hydrostatic medium at a pressure level of 600 MPa and a treatment time of 2 min. 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. 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 increase or release times.

Microbiological analysis. Pouches containing treated seeds were cut open aseptically. The sample 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 slurry 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 *E. coli* O157:H7 formed on the plates were enumerated. Occasionally, colonies were confirmed to be *E. coli* O157:H7 using either a BAX system PCR assay for screening *E. coli* O157:H7 (Qualicon-DuPont, Wilmington, DE) or rapid *E. coli* O157:H7 test methods (Strategic Diagnostics Inc., Newark, DE). Seed slurry samples were 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 sorbitol-containing MacConkey agar (Difco Laboratories, Sparks, MD) plates supplemented with 50 μg/ml of nalidixic acid. After 24 h of incubation, the presence of growth exhibiting morphological and biochemical characteristics typical of *E. coli* O157:H7 was determined by visually inspecting the plates.

Determination of percent germination of pressure-treated alfalfa seeds. To determine the effect of pressure treatment on the seed's germination potential, 2 g of uninoculated seeds were introduced into a pouch to which 3 ml of DI water was added. The samples were treated at 600 MPa for 2 min at 40°C. Pressure-treated and untreated seeds (control) were soaked in DI water for 3 h. 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 to 8 days after the germination system was set up, and the values were recorded as percent germination.

Application of mild heat and reduced pressure levels and/or extended treatment time on the inactivation of *E. coli* O157:H7. Two grams of inoculated seeds was mixed with 3 ml of sterile DI water, packaged, and treated at 250 to 550 MPa for 2 min at 40, 45, and 50°C or at 300 to 500 MPa for 5 min at 40 and 45°C. 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. In addition, inoculated samples were immersed for 10 min in the water bath at 50°C followed by a 3-min immersion at 61°C to determine the effect of temperature alone on the inactivation of *E. coli* O157:H7.

Assessment of germination rate and sprout length of HHP-treated seeds of different ages. Two grams of uninoculated seeds from either a freshly received batch or an old batch of alfalfa seeds (9 months storage at room temperature after receipt) was mixed with 3 ml of DI water, packaged, and treated at 300 MPa for 2 min at 50°C, 400 MPa for 5 min at 45°C, and 550 MPa for 2 min at 40°C. One hundred seeds were drawn from the pressure-treated and untreated (control) seeds and assayed for germinability as described above. Fifty seeds were also drawn from the treated and untreated samples and allowed to germinate for 8 days. The sprout lengths of germinated alfalfa seeds were determined with a digital vernier caliper.

Validation of the selected optimum HHP process. Two grams of inoculated seeds from a freshly received batch or an old batch of alfalfa seeds (12 months storage at room temperature after receipt) was mixed with 3 ml of sterile DI water, packaged, and treated at 550 MPa for 2 min at 40°C. Immediately after pressure treatment, samples were cooled in an ice-water mixture. Samples were then microbiologically assayed as described above and enriched for the detection of survivors. In addition, the same procedure was repeated for uninoculated seeds from the same batch of fresh and old seeds. One hundred seeds were drawn from the pressure-treated (aged and fresh) seeds and their untreated counterparts and assayed for germinability as described above. To evaluate the reliability of the process, a total of 10 trials were carried out for both pathogen inactivation and germination tests.

Statistical analysis. All experiments were replicated three times with the exception of the validation experiment, which was replicated 10 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 *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 ($P < 0.05$).

RESULTS

Effect of temperature on pressure inactivation of *E. coli* O157:H7 on alfalfa seeds and resulting seed viability. Results for the pressure inactivation of *E. coli* O157:H7 on alfalfa seeds at different initial sample temperatures are shown in Fig. 1. *E. coli* O157:H7 responded quite similarly to a pressure level of 600 MPa at initial sample temperatures of 4 and 25°C, achieving 2.4- to 2.8-log-unit CFU/g reduction. When the initial sample temperature was increased to ≥30°C, the pressure treatments resulted in ≥5-log-unit reduction of the pathogen, although survivors were still detected postenrichment for treatments at 30 and 35°C. Survivors were not detected using the enrichment method when the temperature was raised to ≥40°C.

Since a treatment of 600 MPa for 2 min at 40°C was adequate in eliminating a >5-log-unit initial population of *E. coli* O157:H7 on the seeds, the seeds pressure treated under this condition were assayed for germination. Results are shown in Fig. 2. Overall, the pressure treatment did not bring about any statistically significant difference between the control and pressure-treated seeds ($P > 0.05$) after 8 days of sprouting.

Effects of the combined application of mild heat and reduced pressure level and/or extended treatment time on the inactivation of *E. coli* O157:H7. Since a combination of high pressure of 600 MPa and elevated temperature of 40°C was able to eliminate the pathogen on the seeds, further experiments were conducted to investigate the application of lower pressure levels in combination with mild heat to achieve an equivalent kill. Results for this experiment are summarized in
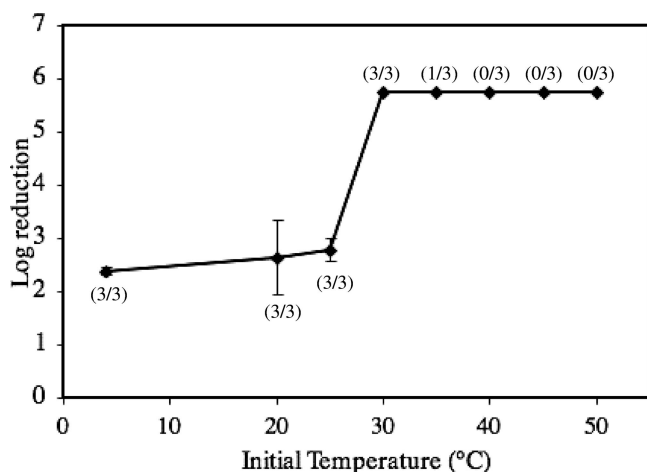
FIG. 1. Effect of temperature on pressure inactivation of *E. coli* O157:H7 on alfalfa seeds. Initial populations of *E. coli* O157:H7 on inoculated and dried seeds were 5.7 log CFU/g. Inoculated seed samples were treated at 600 MPa for 2 min. Log reduction = log counts of untreated samples − log counts of treated samples. Data are the means of three replicates. Numbers in parentheses represent the number of samples testing positive after enrichment/total number of trials. Error bars represent ±1 standard deviation. Error bars may not be visible for most data points, as they are smaller than the size of the data marker.

Table 1. At 40°C and 45°C, the lowest pressure that could achieve consistent reduction of the pathogens to undetectable level during a 2-min treatment was found to be 550 MPa. By increasing the initial sample temperature to 50°C, a pressure treatment at a magnitude as low as 300 MPa successfully eliminated a ~5-log-unit population of *E. coli* O157:H7 on the seeds.

Since the findings showed that a 2-min application of high pressure at a level of 350 to 500 MPa at 40 and 45°C resulted in >5-log-unit inactivation, the pressure treatment time was extended to 5 min to investigate whether a lower pressure level could result in complete kill. A treatment time of 5 min was chosen to make the process economically feasible. Results for

FIG. 2. Comparison of the germination rates of pressure-treated alfalfa seeds relative to control (untreated) seeds. Error bars represent ±1 standard deviation.

TABLE 1. Effect of pressure treatments for 2 or 5 min in combination with mild heat on inactivation of *E. coli* O157:H7 on alfalfa seeds[a]

Pressure level (MPa)	Log survivors (CFU/g) (mean ± SD) after treatment at the following temp and pressure treatment time[b]:					
	40°C		45°C		50°C	
	2 min	5 min	2 min	5 min	2 min	5 min
250	3.5 ± 0.3	ND	ND	ND	1 ± 0.35	ND
300	2.9 ± 0.5	ND	<0.8 (3)	<0.8 (2)	<0.8 (0)	ND
350	<0.8 (3)	<0.8 (2)	<0.8 (1)	<0.8 (2)	<0.8 (0)	ND
400	<0.8 (2)	<0.8 (1)	<0.8 (1)	<0.8 (0)	<0.8 (0)	ND
450	<0.8 (1)	<0.8 (1)	<0.8 (0)	<0.8 (0)	<0.8 (0)	ND
500	<0.8 (1)	<0.8 (1)	<0.8 (1)	<0.8 (0)	<0.8 (0)	ND
550	<0.8 (0)	ND	<0.8 (0)	ND	<0.8 (0)	ND

[a] The initial populations of *E. coli* O157:H7 on inoculated and dried seeds were 5.7 log CFU/g, as determined by plating appropriate dilutions on TSAYE-N.

[b] The number of survivors (log survivors [CFU/g]) after treatment at three temperatures for 2 or 5 min at different pressures. Data representing log survivors (CFU/g) are the means of three replicates. The numbers in parentheses are the number of samples testing positive after enrichment out of a total of three trials. ND, not done.

the 5-min experiment also displayed in Table 1 show that the treatments of 300 to 500 MPa at 40°C resulted in >5-log-unit reductions, but survivors were still detected at a low frequency. However, reduction of the pathogens to undetectable levels was consistently achieved (across three replicates) when a pressure magnitude ranging from 400 MPa to 500 MPa was applied at 45°C for 5 min. To determine the effect of heat alone in the absence of HHP, inoculated seed samples were immersed for 10 min in the water bath at 50°C and 3 min at 61°C (to represent a worst case scenario of adiabatic heat during pressure application of 300 MPa for 2 min at 50°C). It was found that *E. coli* O157:H7 underwent >5-log-unit inactivation, although survivors could still be detected postenrichment (results not shown).

Effects of selected high-pressure treatments on the germination rate and sprout length of fresh and aged seeds. Figure 3 compares the germination rates of pressure-treated seeds relative to untreated seeds for fresh and aged seeds, respectively. Untreated fresh seeds had a higher final germination percentage than their aged counterparts did. When fresh seeds were pressure treated under the three different conditions, their germination rates underwent a maximum reduction of 5% relative to the control, although the difference was not statistically significant ($P > 0.05$). The average sprout lengths for fresh and aged seeds subjected to the various pressure treatments are shown in Fig. 4. As far as sprout length is concerned, the treatment of 550 MPa for 2 min at 40°C was the most promising, as it had the least impact on sprout growth ($P > 0.05$). The pressure treatment conducted at 50°C, on the other hand, was the least desirable, bringing about the most noticeable decrease in sprout length, although the difference was not significant statistically ($P > 0.05$). Similar to fresh seeds, the final germination rates of pressure-treated aged seeds determined after 8 days was found to be reduced by a maximum of 5%, although the difference was not significant statistically ($P > 0.05$). Aged seeds also exhibited a decrease in sprout length when pressurized under the different conditions,
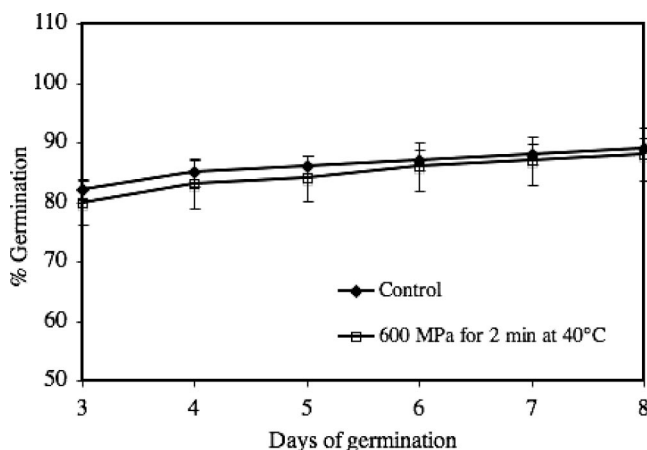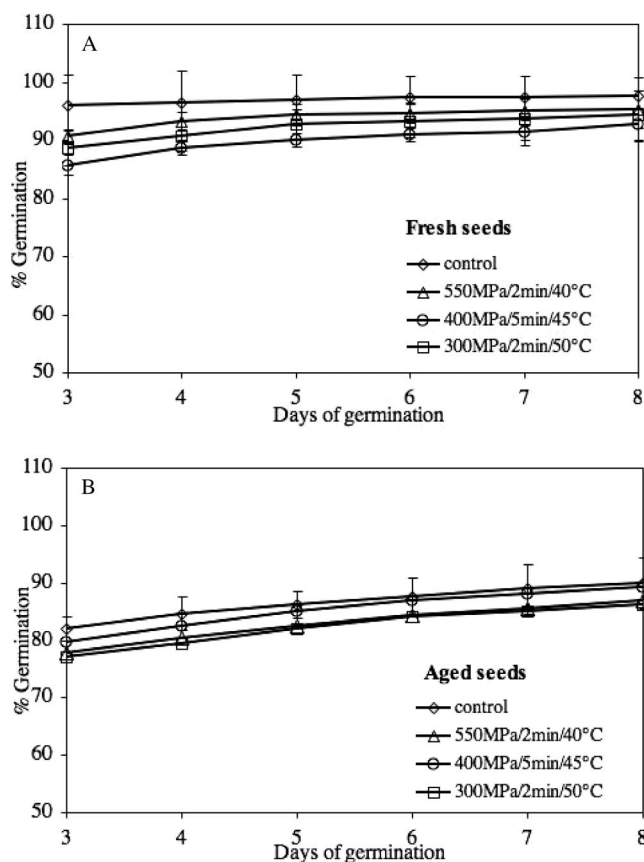
FIG. 3. Comparison of the germination rates of fresh (A) and aged (B) alfalfa seeds pressure treated under various conditions relative to control (untreated) seeds. Error bars represent ±1 standard deviation. The y-axis scale was adjusted to a maximum of 110% to fit the error bars into the chart area.

and the treatment of 550 MPa for 2 min at 40°C was again the most preferable.

Validation of the selected optimum HHP process. It was found that in either fresh or aged seeds inoculated with 5 log units of *E. coli* O157:H7, the treatment of 550 MPa for 2 min at 40°C was able to consistently reduce the populations to undetectable levels in all 10 trials conducted. With respect to seed viability, 10 samples each of uninoculated fresh and aged

seeds were pressure treated and assayed for germination. It was found that the average percent sprouted seeds determined after 3 days of germination were ~80 and ~92 for pressure-treated old and fresh seeds, respectively, while their untreated counterparts achieved an average germination of ~87 and ~97%, respectively. After 8 days of germination, the final percent germination reached >86 and >97% for treated old and fresh seeds, respectively, while their untreated counterparts reached an average of 91 and 100%, respectively.

DISCUSSION

Effect of temperature on pressure inactivation of *E. coli* O157:H7 on alfalfa seeds and resulting seed viability. The results of Fig. 1 show that the pressure sensitivity of *E. coli* O157:H7 had a strong dependence on the treatment temperature, exhibiting the highest pressure resistance at temperatures ranging from 4 to 25°C and becoming more sensitive the higher the temperature of the treatment. It is well documented that the temperature of food during pressurization plays a significant role in the inactivation of bacteria and viruses. In particular, elevated temperatures above 30°C have been shown to enhance the pressure inactivation of bacteria as reported for *Listeria monocytogenes* (14), *Staphylococcus aureus* (12), *Escherichia coli* (33), *Salmonella enterica* serovar Enteritidis (34), *Vibrio vulnificus* (22), and *Vibrio parahaemolyticus* (23). Studies conducted by Chen et al. (11) and Kingsley et al. (21) demonstrated that temperatures of >30°C also increased the pressure inactivation of feline calicivirus and hepatitis A virus, respectively. However, unlike other authors (8, 13, 14, 22, 23), we did not observe any enhancement in pressure inactivation at low temperatures (i.e., <20°C). In fact, *E. coli* O157:H7 exhibited the highest baroresistance at the lowest temperature investigated, i.e., at 4°C. Similarly, Ponce et al. (34) and Kingsley et al. (21) showed that the respective inactivation of *Salmonella* Enteritidis and hepatitis A virus was lower at lower temperatures.

Uninoculated seed samples treated at 600 MPa for 2 min at 40°C did not exhibit a significantly different germination rate relative to that of untreated seeds after 8 days of sprouting (P > 0.05). When the adiabatic compression heating component of the pressure treatment was taken into consideration, the temperature of the samples increased to a maximum of
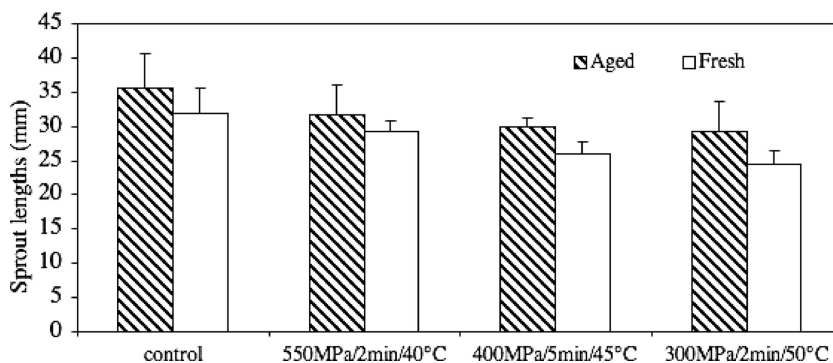

FIG. 4. Effects of pressure treatments carried out under various conditions on the average sprout lengths of fresh and aged alfalfa seeds. Error bars represent ±1 standard deviation.

59.2°C during pressure treatment. Bari et al. (4) showed that the use of very high temperature for short times (hot water treatment at 90°C for 90 s followed by dipping in chilled water for 30 s) resulted in the elimination of *Salmonella* and *E. coli* O157:H7 and the process did not affect the germination yield of mung bean seeds to any significant extent. Weiss and Hammes (37) indicated that the treatment of alfalfa and radish seeds with hot water could be an effective seed sanitizing step, achieving >95% germination. They demonstrated that treating seed inoculated with *Salmonella* sp. or *E. coli* O157:H7 for 0.5 to 8 min at 53 to 64°C reduced all pathogens by more than 5 log CFU/g, although no evidence of elimination was provided. Hence, studies conducted by other authors have pointed to the possible application of brief high-temperature treatment to inactivate seed-borne pathogenic microorganisms with minimal impact on the seed viability. However, the drawback associated with high-temperature treatments is the reported lack of consistency of the effect of the treatment on seed viability (20) and on the safety of sprouts. Under commercial practice, the ability of hot water treatments to ensure consistent elimination of bacterial human pathogens from alfalfa seed was put into question by a recent multistate outbreak of salmonellosis due to contaminated alfalfa sprouts grown from seed treated with hot water followed by a soak in low levels (2,000 ppm) of chlorine (38).

Neetoo et al. (29) recently demonstrated that pressure treatment at 600 MPa for 20 min at 20°C was inadequate in completely eliminating *E. coli* O157:H7 from seeds. In the current study, the combined application of pressure and mild temperatures to enhance the safety of alfalfa seeds was investigated. By increasing the initial sample temperature to 40°C, the HHP process at 600 MPa proved to be an effective intervention technology to enhance the safety of alfalfa seeds. Both Penas et al. (32) and Ariefdjohan et al. (1) also investigated the application of high pressure on alfalfa seeds to improve the safety of the resultant sprouts. Penas et al. (32) found that seeds presoaked in water and subsequently pressure treated at 400 MPa and 40°C in the absence of water exhibited a significantly lower germination rate (<30%) than untreated alfalfa seeds. On the other hand, Ariefdjohan et al. (1) found that dry (not presoaked) alfalfa seeds pressure treated at 40°C (275 to 575 MPa for 2 min or 475 MPa for 2 to 8 min) took longer to germinate, achieving final germination of up to 34%, while 95% of the control seed germinated. Unlike findings reported by those authors, our data show that alfalfa seeds pressure treated in the presence of water at the same temperature of 40°C and at an even higher pressure magnitude of 600 MPa still achieved a final germination percentage comparable to that of the control ($P > 0.05$). It is most likely because the other authors pressure treated presoaked (32) or dry (1) seeds in the absence of water, while in our study, seeds were pressure treated while immersed in water. In order to demonstrate the adverse effect of pressure treatment of presoaked seeds in the absence of water, we immersed alfalfa seeds in DI water for 3 h and then pressure treated them unimmersed in water at 600 MPa for 2 min at 40°C. The average final germination percentage of pressure-treated seeds after 8 days of germination was ~19. On the other hand, seeds pressure treated using the process we developed consisting of HHP treatment of seeds immersed in water without prior soaking resulted in a significantly higher final

germination percentage of 96. It is possible that the presence of immersion water surrounding the seeds acts as a cushion, protecting the seeds against any physical and structural damage during pressure treatment.

Effect of the combined application of mild heat and reduced pressure level and/or extended treatment time on the inactivation of *E. coli* O157:H7. The experimental data of Table 1 display a slight degree of interexperimental variability as evidenced by the positive enrichment results for the two treatments of 350 MPa and 45°C, 1/3 for the 2-min treatment and 2/3 for the 5-min treatment. It would be expected that increasing the treatment time would increase the lethal effect on microorganisms. However, it has been widely reported that pressure inactivation of microorganisms is not always first order and the tailing effect of survival curves has been found in many species, including *E. coli* O157:H7 (13). The occurrence of tails is usually attributed to the presence of a more resistant subpopulation of cells. Therefore, a small increase in treatment time may not necessarily be able to enhance the inactivation effect on these resistant cells. The chance of bacterial elimination might also depend on the samples being treated, as the samples might have slight variations with respect to cell age or genetic constitution of the inoculum.

The results shown in Table 1 demonstrate that the pressure level of the treatment could be substantially reduced by increasing the sample temperature to achieve an equivalent lethality. Since the capital costs of the high-pressure equipment increase exponentially with operating pressures and process costs are related to operating pressure (14), it is economically beneficial to use lower levels of pressure in combination with other hurdles, such as elevated temperatures to inactivate *E. coli* O157:H7 on alfalfa seeds. Munoz et al. (27) showed that the inactivation of microorganisms in orange juice and vegetable soup increased when the temperature was increased from 20°C to 60°C, demonstrating that a treatment at 60°C brought about the greatest reductions during the application of high pressure. Hence, it can be concluded that elevated temperature can reduce the pressure magnitude needed to achieve a target level of pathogen inactivation. The manipulation of the process parameters can thus greatly influence the outcome and economics of a high-pressure process.

In addition, Table 1 shows that the combinations that were effective in eliminating an initial population of ~5 log units of *E. coli* O157:H7 included treatments at a pressure of ≥550 MPa for 2 min at 40°C (the maximum temperature reached due to adiabatic heating was 57.6°C at 550 MPa), treatments at a pressure of ≥400 MPa for 5 min at 45°C (the maximum temperature reached due to adiabatic heating was 58.2°C at 400 MPa), and treatments at a pressure of ≥300 MPa for 2 min at 50°C (the maximum temperature reached due to adiabatic heating was 60.2°C at 300 MPa). Temperature changes are known to occur during compression and pressure release as a result of adiabatic heating and cooling (36). This adiabatic temperature change is predictable; all compressible substances will change temperature during physical compression as a result of this unavoidable thermodynamic effect (36). The magnitude of this change depends mainly on the compressibility of the substance, the specific heat of the substance, and the initial sample temperature. Since the early work of Bridgman (7), the adiabatic compression behavior of water has been well char-

acterized. At the pressures typically encountered during HHP (400 to 1,000 MPa), under adiabatic conditions near room temperature, water (the hydrostatic medium used) typically changes by about 3°C for every 100-MPa pressure change. Since the seeds were immersed in water during pressure treatment, the adiabatic heating compression factors of pressure-treated seeds were very similar to those of water. Temperature increases during pressure treatment of the samples were 2.8, 2.9, 3.2, and 3.4°C/100 MPa at 20, 30, 40, and 50°C, respectively. For samples pressure treated at the highest starting temperature of 50°C (the harshest treatment) to a final increase in pressure of 300 MPa, the sample temperature rose to a maximum of 60.2°C. Inoculated seed samples subjected to a control thermal treatment at 61°C still harbored *E. coli* O157:H7 survivors. This finding therefore indicates that the thermal effect during high-pressure processing was inadequate on its own in eliminating *E. coli* O157:H7, pointing to the critical contribution of pressure treatment in achieving a complete kill.

Effects of selected high-pressure treatments on the germination rate and sprout length of fresh and aged seeds. Since the germinability of alfalfa seeds is known to decrease with the age of the seeds (19, 30), it was deemed worthwhile to determine the effect of the pressure treatments on the germination characteristics of treated seeds of different ages. The germination percentages and the average sprout lengths of fresh and aged seeds pressure treated at 550 MPa for 2 min at 40°C, 400 MPa for 5 min at 45°C, or 300 MPa for 2 min at 50°C were affected to a variable extent depending on the treatment conditions (Fig. 3 and 4). The treatment at 550 MPa at 40°C was the most promising, as it had no significant adverse effect on the viability of the seeds ($P > 0.05$). When the seeds were observed under the light microscope, no differences could be discerned between untreated seeds and seeds pressurized at 550 MPa at 40°C (results not shown). This contrasts with observations made by Ariefdjohan et al. (1) who pressure treated seeds at 275 to 575 MPa for 2 min at 40°C and observed structural damage to the seeds with a concomitant decrease in viability. We attribute this difference to the fact that our seeds were pressurized while submerged in water, thus alleviating the seed damage and retaining the germination capability. In the light of these results, we recommend a pressure treatment of 550 MPa for 2 min at 40°C on alfalfa seeds, since this condition has the ability to eliminate *E. coli* O157:H7 on seeds while having the least impact on the germination rate and sprout length of seeds regardless of the age of the seeds.

Validation of the selected optimum HHP process. The pressure treatment developed in this study carried out at 550 MPa for 2 min at 40°C was able to consistently reduce a 5-log-unit pathogen load on aged and fresh seeds to an undetectable level throughout all trials with minimal impact on seed viability. It should be pointed out that small samples of seeds (2 g) were used in this study due to the limitation of our pressure unit. A scale-up study using a much larger quantity of seeds would be needed before commercialization of this process. Although it is anticipated that a large-scale pressure unit would differ with respect to pressure increase time and temperature profile of seeds during pressurization, these differences are not expected to affect the inactivation efficacy and seed viability retention significantly. In addition, we recommend testing the robustness

of the developed pressure process by conducting studies on seeds from different sources.

Conclusion. The application of pressure treatment at optimum temperatures on alfalfa seeds is highly desirable, since results in this study demonstrated that temperature plays a significant role in pressure inactivation of *E. coli* O157:H7. Although high-pressure processing is more expensive than the currently available methods for decontamination of seeds, HHP appears to be highly effective against *E. coli* O157:H7 with minimal adverse impact on seed viability. Since alfalfa seeds are very small (~2 mm long), it is anticipated that a large number of seeds could be processed at one time, thus helping to achieve a reasonably high throughput. In addition, through a combination of high-pressure processing with additional hurdles, such as mild heat and optimization of the various processing parameters (pressure, time, and temperature), the pressure levels needed to achieve a desired target level of bacterial inactivation can be substantially reduced. Therefore, HHP could be feasible for use to enhance the safety of sprouts as these niche products are becoming increasingly popular natural foods.

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