Efficacy of High Hydrostatic Pressure Treatment in Reducing *Escherichia coli* O157 and *Listeria monocytogenes* in Alfalfa Seeds


ABSTRACT: The application of high hydrostatic pressure (HHP) technology as a seed decontamination technique was evaluated. Alfalfa seeds inoculated with *Escherichia coli* O157 and *Listeria monocytogenes* were air-dried and subjected to independent HHP treatments of 275 to 575 MPa for 2 min or at 475 MPa for 2 to 8 min (40 °C). There were 1.4-log and 2.0-log reductions in *E. coli* O157 populations at 575 MPa (2 min) and 475 MPa (8 min), respectively. However, these treatments caused only 0.8-log and 1.1-log reductions in *L. monocytogenes* counts. Treated seeds took longer to germinate, achieving germination rate of up to 34%, whereas 95% of the control germinated. Results suggest that *L. monocytogenes* is more resistant to the bactericidal effects of HHP than *E. coli* O157. Although HHP treatments achieved a greater reduction in *E. coli* O157, it was at the expense of seed germination. Overall, our results indicate that although HHP treatments reduced the populations of *E. coli* O157 and *L. monocytogenes* in alfalfa seeds, they did not completely eliminate these microorganisms.

Keywords: high hydrostatic pressure, alfalfa seed, seed germination, *E. coli*, *L. monocytogenes*

Introduction

With the recent shift in consumer lifestyle toward healthy living and healthier foods, the consumption of raw sprouts in salads and sandwiches has increased in popularity (Feng 1997; Kurtzweil 1999). According to the International Sprout Growers Association (ISGA), some 475 sprout growers in the United States produce 300000 tons of sprouts annually contributing to a $250 million market and as many as 10% of Americans eat sprouts regularly (Kurtzweil 1999). However, the popularity of sprouts dropped dramatically in 1995, when the consumption of sprouts was implicated in salmonellosis and *Escherichia coli* O157 outbreaks worldwide. Most of these outbreaks were caused by the consumption of sprouts grown using unsanitary practices and/or from contaminat ed seeds (Feng 1997). In 1998, the U.S. Food and Drug Administration (USFDA) declared alfalfa sprouts as a high-risk food (the 3rd food item after raw oysters and unpasteurized juices to carry such classification), making it unsuitable to be consumed by immunocompromised individuals, the elderly, pregnant women, and children (Burros 1998).

The USFDA recommends treating seeds with sanitizers before sprouting. This involves the use of highly concentrated sanitizers. Milder alternative processing methods need to be developed to produce microbiologically safe high-quality sprouts. Various seed decontamination methods studied in the past decade have not shown promising results in terms of bacterial inactivation capability. Techniques ranging from surface sterilization using gas to immersion in chemicals caused sub-lethal cell injury instead of completely eliminating pathogens from seeds (Delaquis and others 1999; Weissinger and Beuchat 2000). Irradiation, which was considerably successful in inactivating pathogens in spices, satisfactorily eliminated pathogens from seeds but inadvertently lowered the yield ratio of seeds (Rajkowski and Thayer 2001). Sub-lethal injury is an important aspect when evaluating the efficacy of any food preservation method because the presence of injured bacterial cells could pose as much danger as fully viable ones. Under favorable conditions during storage, injured cells could undergo cellular repair and proliferate once recovery had been completed (Everis 2000). Studies have shown that sub-lethally injured pathogens present in cracks and crevices of seeds could proliferate to microbiologically hazardous levels during sprouting process where water and nutrients are plentiful (Feng 1997). Therefore, the development of a decontamination technique that could completely eliminate pathogenic contaminants in seeds is needed.

Past studies have demonstrated the efficacy of high hydrostatic pressure treatment (HHP) in inactivating a wide spectrum of Gram-negative and Gram-positive bacteria in suspensions, as well as in various solid food items (Patterson and others 1995; Arroyo and others 1997). Multidimensional HHP treatment incorporating a combination of time, pressure, heat, and antimicrobial compounds or performed consecutively with other decontamination methods create “hurdle” effects that could significantly eliminate various pathogens (Earnshaw and others 1995). Generally, bacterial inactivation by HHP is caused by damages on the cell membrane leading to alteration in membrane permeability, inactivation of intracellular enzymes, and even rupture of the cell wall (Hoover and others 1998; Vachon and others 2002). On the basis of these studies, HHP technology could present itself as an alternative technique for decontaminating seeds.

In this study, we sought to evaluate the feasibility of using HHP as a seed decontamination technique. The specific objectives of...
this research were to determine and to compare the effects of HHP on the survival of Gram-negative (E. coli O157:NM) and Gram-positive (Listeria monocytogenes) microorganisms inoculated on alfalfa seeds and to assess the effects of HHP on the germination capability of seeds.

Materials and Methods

Bacterial culture and inoculation of alfalfa seeds
Nonpathogenic variants of E. coli O157:NM (MFT7123A) (Deng and Fratamico 1996) and L. monocytogenes (ATCC9113) (Pine and others 1991; Lathrop and others 2003) from our collection were streaked on trypticase soy agar slants (TSA) (Difco Laboratories, Detroit, Mich., U.S.A.) and incubated at 37 °C. Cells of E. coli O157:NM and L. monocytogenes obtained from 3 successive 24-h transfer cultures were collected by centrifugation (4000 × g, 20 min), resuspended in 50 mL 0.1% peptone, and pooled to 500 mL in separate sterile stomacher bags (Fisher Scientific, Pittsburgh, Penn., U.S.A.). Alfalfa seeds (500 g) purchased from the Sprout House (Forest Hills, New York, U.S.A.) were added into the stomacher bag and mixed with respective inoculum (500 mL) for approximately 5 min with a consistent gentle shaking to thoroughly mix the content. After decanting the suspension, seeds were air-dried at room temperature under a laminar flow hood (Biohazard Class II) on sterile cheesecloth for at least 5 h. Once dried, inoculated seeds were stored in the refrigerator (4 °C) for no more than 24 h and packed into plastic pouches for pressure treatments. From preliminary experiments, it was observed that inoculated seeds consistently retained approximately 10⁵ colony-forming units (CFU)/g of E. coli O157:NM and 10⁶ CFU/g of L. monocytogenes.

Sample packing and HHP treatment
HHP treatments were conducted using a Quintus Model QFP-6 High Pressure Food Processor (ABB Autoclave Systems, Inc., Columbus, Ohio, U.S.A.) with 50% glycol solution used as a pressure-transmitting medium. The initial temperature was adjusted so that the final process temperature of 40 °C was achieved for various pressure-holding time combinations (that is, final process temperature = initial temperature + compression heating factor × target pressure) (Balasubramanian and Balasubramaniam 2003). Come-up time ranged from 85 to 145 s for pressures ranging from 275 to 827 Mpa, with depressurization completed in less than 2 s. In all cases, the duration of treatment does not include the pressure come-up or depressurization time. Seed treatments were performed as independent sequences consisting of (1) increasing pressure (275, 375, 475, and 575 MPa) at constant treatment duration of 2 min and (2) increasing treatment duration (2, 4, 6, and 8 min) at constant pressure of 475 MPa. Temperature during all treatments was continuously monitored using a thermocouple attached to top closure of the HHP chamber. For the pressure treatment, 50 g of dried seeds were pressurized in a heat-sealed sterile polyethylene pouch (8 x 5 cm; Fisher Scientific). To avoid leakage during pressure treatment, each sample pouch was placed inside a larger pouch of a thicker material (FoodSaver® Rolls, Tilia®, San Francisco, Calif., U.S.A.) and then vacuum-packed (FoodSaver Vac350, Tilia).

Determination of bacterial inactivation and enumeration of injured cells
Pouches containing treated seeds were rinsed with 70% ethanol and then opened aseptically. Contents were stirred using a sterile glass rod to obtain a homogeneous sample. Seeds (10 g) were added to 40 mL of sterile 0.1% peptone, stomached for 1 min at medium speed (Seward 400 Stomacher, Seward Medical Co., London, U.K.), serially diluted in sterile 0.1% peptone, and surface-plated (100 μL) in duplicate on Sorbitol MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, U.K.) supplemented with Cefixime (Dyna1, Inc., Lake Success, N.Y., U.S.A.) (C-SMAC) or modified Oxford agar (MOA) (Difco Laboratories) containing antimicrobial supplement (Difco Laboratories). C-SMAC plates were incubated at 37 °C for 24 h whereas MOA plates at 37 °C for 48 h. Presumptive E. coli O157:NM colonies were confirmed using the E. coli O157 latex agglutination assay (Oxoid Inc., Ogdenburg, N.Y., U.S.A.) and presumptive L. monocytogenes colonies using the API Listeria assay (BioMerieux Vitec, Hazelwood, Miss., U.S.A.).

Injured cells of E. coli O157:NM were enumerated using the membrane transfer method (McCarthy and others 1998; Singh and others 2003). Appropriate diluents of seed slurries (100 μL) were surface-plated in duplicate on sterile polycarbonate filter membranes (0.4-μm pore size, 90-mm dia) (Osmonics Co., Westboro, Mass., U.S.A.) that had been placed on plate count agar plates (PCA) (Difco Laboratories). Following incubation at 37 °C for 4 h to resuscitate cells, membranes were aseptically transferred onto C-SMAC plates and further incubated at 37 °C for 20 h. Injured cells of L. monocytogenes were enumerated by the overlay method (Kang and Fung 1999). PCA plates inoculated with appropriate dilutions of L. monocytogenes were incubated at 37 °C for 4 h and then layered with liquefied MOA (tempered 38 °C). Once the MOA layer solidified, plates were incubated at 37 °C for 44 h. Presumptive colonies of E. coli O157:NM and L. monocytogenes were confirmed using the assays stated earlier.

Determination of seed germination
One hundred seeds were spread evenly between 2 pieces of water-saturated filter paper (nr 4; Whatman Intl. Ltd., Kent, U.K.) in a petri dish (90-mm dia; Fisher Scientific). Five plates were prepared for respective seed samples. Seeds were germinated at room temperature, and filter papers were moistened daily with sterile deionized water to maintain a high-moisture environment. Seeds were visually examined daily and considered germinated when 2 mm of radical protruded from seed coat. Germination percentage was calculated by averaging total number of seeds germinated in 5 plates at the end of day 7.

Statistical analysis
All experiments were replicated 3 times, and results were recorded as means ± standard deviations. One-way analysis of variance (ANOVA) and Duncan’s multiple range tests were used to determine differences in the populations of E. coli O157:NM and L. monocytogenes recovered on treated alfalfa seeds, as well as to assess differences in germination percentage of seeds as a consequence of various pressure treatments.

Results and Discussion
Reduction of E. coli O157:NM and L. monocytogenes as a function of pressure and time
The inactivation of microorganisms by HHP is essentially a 3-dimensional process consisting of a combination of pressure, treat-
Efficacy of high hydrostatic pressure . . .

ment time, and temperature (Ting and others 2002). This design was established as the basis of our study. To compare the effects of HHP treatments on Gram-positive and Gram-negative microorganisms, E. coli O157:NM and L. monocytogenes were used in our study. Although L. monocytogenes has not been linked to illnesses associated with sprout consumption, this ubiquitous pathogenic is a potential contaminant of seeds (NACMF 1999). In addition, being a psychrotroph, L. monocytogenes poses an additional safety hazard to the consumption of sprouted seeds because alfalfa sprouts are typically marketed under refrigeration.

The reduction of E. coli O157:NM and L. monocytogenes populations in alfalfa seeds after HHP treatments is summarized in Table 1 and 2. We observed that the highest reduction for both E. coli O157:NM and L. monocytogenes was obtained at the highest pressure of 575 MPa (2 min) and at the longest exposure to pressure (475 MPa, 8 min). Negative control did not contain any E. coli O157:NM and L. monocytogenes. Although HHP treatments had been reported to reduce the populations of E. coli O157:H7 and L. monocytogenes by 5 to 6 logs (at treatments up to 345 MPa) and 5 logs (300 MPa), respectively (Alpas and others 1999; Vachon and others 2002), our results did not corroborate with their findings. Overall, treatments at 575 MPa (2 min) reduced the populations of E. coli O157:NM by 1.4 logs and L. monocytogenes by 0.8 logs, whereas treatment for 8 min (475 MPa) produced 2.0-log and 1.1-log reduction of E. coli O157:NM and L. monocytogenes, respectively. We infer that this is most likely because of differences in test substrates used in our study and others. A higher bacterial reduction is usually observed when HHP treatment is subjected to culture-in-suspension than when bacteria is incorporated into food matrix because certain food components such as protein, carbohydrates, and lipids may confer baro-protective effects to bacterial cells (Paterson and others 1995). We also deduced that because seeds were inoculated with E. coli O157:NM and L. monocytogenes by soaking, these microorganisms may have been imbibed into the seeds and subsequently made them inaccessible to the bactericidal actions of HHP. Furthermore, cracks and crevices in the seed coat may have provided additional sites for bacterial attachment, as well as created a shield protecting these bacteria from the effects of HHP. This mode of contamination and bacterial attachment may or may not be true for naturally contaminated alfalfa seeds.

Comparative assessments on the number of E. coli O157:NM and L. monocytogenes recovered after HHP treatments (Table 1 and 2) suggest that lower populations of L. monocytogenes were eliminated than E. coli O157:NM for the same level of pressure or duration of exposure. These results indicate that L. monocytogenes may be more resistant to the effects of HHP than E. coli O157:NM, which reiterated previous findings involving pure culture-in-suspension and other food systems (Simpson and Gilmour 1997b; Alpas and others 1999). Thicker cell wall and complex cellular membrane composition have been speculated to confer Gram-positive bacteria a higher resistance to the bactericidal effects of HHP than other less resistant cells and were able to undergo cellular repair under a suitable condition.

The effectiveness of HHP treatments was evaluated by comparing the number of E. coli O157:NM and L. monocytogenes recovered without repair step (surface-plating) to number enumerated with normal feature associated with the mechanism of bacterial inactivation by HHP that is attributed to a small portion of the bacterial populations, which is relatively resistant to a certain pressure applied (Metrick and others 1989). These resistant cells pose potential microbial hazards because their recovery indicate that they were either totally not affected by HHP or suffered less damage than other less resistant cells and were able to undergo cellular repair under a suitable condition.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Pressure (MPa)</th>
<th>With repair step</th>
<th>Without repair step</th>
<th>Total log reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli O157:NM</td>
<td>275</td>
<td>5.1 ± 0.6</td>
<td>4.4 ± 0.8</td>
<td>3.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>375</td>
<td>5.0 ± 0.4</td>
<td>4.0 ± 0.8</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>475</td>
<td>5.1 ± 0.3</td>
<td>4.1 ± 0.4</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>575</td>
<td>5.1 ± 0.5</td>
<td>3.7 ± 1.1</td>
<td>2.9 ± 1.2</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>275</td>
<td>7.2 ± 0.5</td>
<td>6.7 ± 0.8</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>375</td>
<td>7.3 ± 0.5</td>
<td>6.6 ± 0.7</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>475</td>
<td>7.2 ± 0.5</td>
<td>6.6 ± 0.7</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>575</td>
<td>7.4 ± 0.4</td>
<td>6.5 ± 0.7</td>
<td>6.3 ± 0.6</td>
</tr>
</tbody>
</table>

Table 1—Survival of Escherichia coli O157:NM* and Listeria monocytogenes† in alfalfa seeds after high hydrostatic pressure (HHP) treatment at 275, 375, 475, and 575 MPa for 2 min at 40 ± 2°C

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Time (min)</th>
<th>Control</th>
<th>With repair step</th>
<th>Without repair step</th>
<th>Total log reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli O157:NM</td>
<td>2</td>
<td>5.2 ± 0.3</td>
<td>4.0 ± 0.5</td>
<td>3.4 ± 0.4</td>
<td>1.1 ± 0.3b</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.6 ± 0.7</td>
<td>4.1 ± 0.3</td>
<td>3.3 ± 0.5</td>
<td>0.8 ± 0.4a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.1 ± 0.3</td>
<td>3.7 ± 0.6</td>
<td>3.3 ± 0.9</td>
<td>0.8 ± 0.4a</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4.6 ± 0.1</td>
<td>3.7 ± 0.7</td>
<td>3.4 ± 0.9</td>
<td>0.8 ± 0.4a</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>2</td>
<td>7.2 ± 0.5</td>
<td>6.7 ± 0.6</td>
<td>6.5 ± 0.7</td>
<td>0.5 ± 0.2c</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.1 ± 0.2</td>
<td>6.2 ± 0.1</td>
<td>6.2 ± 0.2</td>
<td>0.9 ± 0.1b</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>7.3 ± 0.1</td>
<td>6.4 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>0.9 ± 0.1b</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>7.2 ± 0.2</td>
<td>6.0 ± 0.2</td>
<td>5.7 ± 0.4</td>
<td>1.1 ± 0.2a</td>
</tr>
</tbody>
</table>

Table 2—Survival of Escherichia coli O157:NM* and Listeria monocytogenes† in alfalfa seeds after high hydrostatic pressure (HHP) treatment at 475 MPa for 2, 4, 6, and 8 min at 40 ± 2°C

*a All numbers are in log_{10} colony-forming units (CFU)/g.
*b Populations of E. coli O157:NM and L. monocytogenes recovered from positive control.
*c E. coli O157:NM were repaired using the membrane transfer method and L. monocytogenes using the overlay method.
*d E. coli O157:NM were surface-plated on sorbitol MacConkey agar with Cefixime and L. monocytogenes on modified Oxford agar containing antimicrobial supplement.
*e Difference in log_{10}CFU/g of bacteria in control and number recovered with repair step.
*f Means ± S.D. with the same letter are not significant to each other (n = 3; P > 0.05); comparisons were made between rows (log_{10}CFU/g at 275 to 375 to 475 to 575 MPa) and independent comparison was made for each microorganism.

URLs and E-mail addresses are active links at www.ift.org
repair procedure (membrane-transfer method or overlay method). For each treatment, lower numbers were recovered by surface plating than when repair step was incorporated (Table 1 and 2). This difference was more prominent in *E. coli* O157:NM (up to 0.8 log) than *L. monocytogenes* (up to 0.3 log). These observations suggest that surface plating on C-SMAC and MOA did not allow for resuscitation of injured cells. Consequently, the efficacy of a decontamination method may be overestimated when repair procedure is not incorporated into the enumeration protocol. This finding is consistent with the observations of other investigators (Simpson and Gilmour 1997a, 1997b; Kalchayanand and others 1998). These results also indicate that cells subjected to pressurization did not experience an equal degree of cellular damage even though HHP treatments are considered to be isostatic. In turn, this implied that the parameters of HHP treatments used in this study caused sub-lethal injury to bacterial cells instead of completely eliminating them.

### Seed viability

Seed viability (that is the ability of seeds to germinate) was observed to be severely affected by HHP treatments. Reduction in germination was significantly correlated to increasing pressure level or exposure to HHP. Seeds treated at 275, 375, 475, and 575 MPa for 2 min achieved a germination rate of 34%, 34%, 27%, and 23%, respectively, whereas seeds treated for 2, 4, 6, and 8 min (at 475 MPa) attained a germination rate of 28%, 22%, 14%, and 9%, respectively. Untreated seeds, however, achieved a germination rate of 95%. In addition, untreated seeds -2 began to germinate after 48 h, whereas control seeds germinated within 36 h. When examined under a light microscope, the seed coats of treated seeds (475 MPa for 8 min; 575 MPa for 2 min) were damaged, showing cracks or completely broken seeds. On the basis of these observations, we concluded that the structure of dry alfalfa seeds may not be able to withstand pressures exerted by HHP. Similar to previous studies on seed decontamination techniques, this study found that alfalfa seeds were sensitive toward treatments (Delaquis and others 1999; Weissinger and Beuchat 2000; Rajkowski and Thayer 2001). Generally, longer exposure or higher concentration of chemicals used during treatment will lead to lower germination. Softening the seed coat with water or water-containing antimicrobials before HHP treatment may alleviate seed damage and thus improve seed germination. Wuytack and others (2003) reported a higher germination rate when they included sterile, deionized water in seed pouches during HPP treatments.

### Conclusions

Results of this study suggest that Gram-positive bacteria (*L. monocytogenes*) are more resistant to the effects of HHP than Gram-negative bacteria (*E. coli* O157:NM). In addition, HHP treatment of alfalfa seeds at pressure levels of 575 MPa (2 min) or 475 MPa (8 min) did not produce the recommended 5-log reduction (NACMF 1999) in both *E. coli* O157:NM and *L. monocytogenes*. Instead of complete elimination, HHP sub-lethally injured the bacterial cells at the expense of seed germination. Pretreatment procedures to soften the seed coat and/or incorporation of liquid-based antimicrobials in seed pouches may alleviate damage to seeds and improve germination capabilities after pressure treatments. Given these overall findings, HHP treatment by itself had limited success eliminating bacterial contaminants in alfalfa seeds.

### Acknowledgments

The authors wish to thank the USDA-ARS (1935–42000–035) for funding the project and the Nail Center for Food Safety and Technology, Summit Argo, Ill., U.S.A. for the use of their high hydrostatic pressure unit and their technical assistance.

### References


