Research Note

Thermal Inactivation of *Salmonella* and *Escherichia coli* O157:H7 on Alfalfa Seeds

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ABSTRACT

Alfalfa seeds inoculated with five strains of *Salmonella* or *Escherichia coli* O157:H7 were subjected to dry heat at 55°C for up to 8 days. Five-log reductions in *Salmonella* or *E. coli* O157:H7 on seeds were observed. No pathogens were detected on the sprouted seeds, which were initially inoculated with ca. 2 log CFU/g of *Salmonella* or more than 8 log CFU/g of *E. coli* O157:H7. The percentages of germination of the alfalfa seeds did not significantly decrease after 6 days of heating at 55°C. These results showed that heat treatment of alfalfa seeds at 55°C for up to 6 days was effective in enhancing the safety of alfalfa sprouts without affecting germination significantly.

Since the first large international outbreak of salmonellosis in the United States and Finland in 1995, the safety of eating fresh raw sprouts, especially alfalfa sprouts, has been a major concern for consumers (21). The pathogens found on seeds are thought to originate from environmental sources during seed production at the crop level. The contamination levels of seeds are usually low in numbers (21). However, during sprouting, temperatures of approximately 30°C and high humidity create favorable conditions for the rapid growth of pathogens that initially may be below detectable levels on seeds but increase to more than 10⁷ CFU/g within 24 h of sprouting, which poses a health risk to consumers.

A number of physical and chemical methods have been evaluated for their effectiveness in inhibiting or eliminating bacterial foodborne pathogens on seeds and sprouts. Soaking alfalfa seeds inoculated with 2.4 to 3.23 log CFU/g of *Escherichia coli* O157:H7 in 20,000 ppm of active chlorine, in combination with heating at 55°C, was found to be effective in reducing pathogens to below detectable levels. However, pathogens were still detected by enrichment (20). In response to this finding, the U.S. Food and Drug Administration recommended that soaking alfalfa seeds in 20,000 ppm of calcium hypochlorite for 15 min be used as a seed decontamination treatment prior to sprouting. However, in 1999, still another multistate outbreak of salmonellosis due to alfalfa sprouts occurred, despite the sprout manufacturer following U.S. Food and Drug Administration–recommended chlorine treatment prior to sprouting. This suggested that the hypochlorite treatment of the seeds insufficiently reduced the levels of contaminating pathogens associated with the seeds used for sprouting (17). Although it was reported that 20,000 ppm of free chlorine soak for 10 min was still effective in eliminating *Salmonella* Mbandaka from naturally contaminated seeds (19), other studies showed that the efficacy of 20,000 ppm of chlorine was questionable in eliminating *Salmonella*, which grew to more than 7 log CFU/g on sprouts, even with spray applications of 100 ppm of chlorine during germination (10). Regrowth of *E. coli* O157:H7 to 7 log CFU/g on sprouts after a treatment of 20,000 ppm of hypochlorite for 15 min has also been observed (14). Many other studies have examined the effectiveness of various chemicals, including chlorine at various concentrations (3–5, 11, 13, 15, 16, 18, 24, 25), indicating that none of the sanitizers were able to eradicate *Salmonella* or *E. coli* O157:H7 without affecting germination, although significant reductions of the pathogens were observed. Physical treatments, including gamma radiation (22) and heating in hot water (23) as a means to decontaminate *Salmonella* and *E. coli*, were confirmed to be capable of reducing the population on seeds and sprouts significantly, but complete elimination was not observed. High hydrostatic pressure can reduce *E. coli* O157:H7 populations by 2 log, but it results in extremely poor germination of the seeds (1). Biocontrol of *Salmonella* by plant-associated pseudomonads was studied and found to be effective in inhibiting the growth of *Salmonella*, but it was unable to reduce the populations on sprouts (9).

It is the physiology of alfalfa seeds that complicates the disinfection of pathogenic bacteria. Researchers have postulated that removing pathogens from alfalfa seeds is more difficult than from other seeds because alfalfa seeds have wrinkles (7) and crevices on the surface, allowing pathogens inside these protective structures and making them inaccessible to chemical sanitizers. The biofilms formed by pathogens on sprouts may provide protection.

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against antimicrobial compounds used for decontamination (8).

Heat treatment, by soaking seeds in hot water, has been tried by many researchers, resulting in limited reductions of the pathogens and usually decreases in seed germination rates (18, 19, 23). Dry heat has been shown to be an effective decontamination treatment for mung bean without affecting the seed viability (12). However, dry heat has not been extensively studied for alfalfa seeds. Dry heat could be a promising treatment, because heat transfer will not be hindered by seed physiological structures, such as crevices or wrinkles that provide protection to pathogens when chemical treatments are applied to alfalfa seeds.

The purpose of this study was to investigate the effects of dry heat on the viability of E. coli O157:H7 and Salmonella, the two most frequent pathogens that have been responsible for the widespread outbreaks associated with alfalfa sprout consumption, and the effect on the viability of alfalfa seeds with various heat treatments.

**MATERIALS AND METHODS**

**Cultures and media.** Five serovars of Salmonella enterica and five different strains of E. coli O157:H7 were used as inocula: Salmonella Gaminara, Salmonella Hartford, Salmonella Typhimurium, Salmonella Rubislaw, and Salmonella Montevideo; E. coli O157:H7 ATCC 43889 (clinical isolate), ATCC 43894 (clinical isolate), ATCC 43895 (raw hamburger meat isolate), 933 (raw hamburger meat isolate), provided by M. P. Doyle, Center for Food Safety, University of Georgia, and ATCC 35150 (clinical isolate) provided by J. Russell, Department of Microbiology, Cornell University. To facilitate the enumeration of colonies on tryptic soy agar (TSA), all of the strains were transformed by electroporation with the plasmid encoding for green fluorescence protein (pGFP) and ampicillin antibiotic resistance.

**Inoculation and treatment.** For high inoculation levels on alfalfa seeds, each serovar of S. enterica or each strain of E. coli O157:H7 was loop inoculated into 50 ml of tryptic soy broth (TSB; Criterion, Hardy Diagnostics, Santa Maria, Calif.) containing ampicillin at 100 mg/liter (Fisher Scientific, Pittsburgh, Pa.). Each 50 ml of TSB with inoculum was incubated at 37°C with 250-rpm shaking for 18 h (Braun 4230, New Brunswick Scientific, Edison, N.J.). After inoculation, five 50-ml strains of E. coli O157:H7 or five serovars of S. enterica were mixed to make a cocktail of 250 ml of inoculum. The cocktail was combined with 200 ml of sterile deionized distilled water (Milli-Q RG, Millipore, Bedford, Mass.) and 60 g of alfalfa seeds of the Lucerne variety (Springwater Sprouts Inc., Honeoye Falls, N.Y.). Seeds were soaked in a 500-ml sterile beaker for 20 min at 20°C with occasional agitation.

For low inoculation levels, each serovar of S. enterica or each strain of E. coli O157:H7 was loop inoculated into 5 ml of TSB containing ampicillin (100 mg/liter). The cultures were incubated as described above. After incubation, every culture of E. coli O157:H7 was serially diluted in TSB 1,000-fold and Salmonella by 10,000-fold. One milliliter of each diluted strain was combined as a mix of all five strains or serovars for making a 5-ml cocktail of S. enterica or E. coli O157:H7. Sixty grams of seeds was soaked in 1 liter of sterile deionized distilled water inoculated with 5 ml of S. enterica or E. coli O157:H7 cocktails for 20 min at 20°C with occasional agitation.

After soaking, the seeds were poured onto two layers of sterile cheesecloth, which was laid on a raised stainless steel mesh (10 by 25 cm; Hoeltge, Inc., Cincinnati, Ohio). Seeds were evenly spread to form a uniform thickness of approximately 5 mm and allowed to dry overnight in a biological safety cabinet (Nuaire Inc., Plymouth, Minn.).

Eight grams of dried inoculated seeds was placed into 15-ml sterile plastic centrifuge tubes (Fisherbrand, Fisher) and heated at 55°C for 4, 6, and 8 days in the same incubator described above. The capped tubes were placed horizontally on a rack to allow the seeds to spread evenly over the tube interior, ensuring equal heating.

**Germination.** After 0, 4, 6, and 8 days of incubation at 55°C, 1 g of seeds (ca. 550 seeds) was placed into 50-ml sterile centrifuge tubes (Simport Plastics, Beloit, Quebec, Canada). The tubes were filled with sterile distilled water to 50 ml. Seeds were soaked for 4 h with occasional agitation. The seeds were poured onto a 3M filter paper (Fisher) in a sterile plastic container (12 by 8 by 6 cm) that was used to simulate the normal sprouting conditions. The filter paper was placed on top of a sponge inside the plastic container to provide humidity during germination. The seeds were evenly spread over the filter paper with a sterile glass rod to ensure every seed was in contact with the filter paper.

The seeds were incubated at 30°C for 3 days before the germination rate of the seeds, and pathogen population levels of the sprouts were determined. During the time of germination, sterile distilled water was sprayed on the seeds and sprouts twice daily (12 h apart) to maintain the amount of moisture the seeds require for sprouting. The percentage of germination was calculated by dividing the number of germinated seeds by the total number of seeds in the sample at the time of germination. The treatments and germinations were performed in triplicate in separate containers. Ruptured seeds with cotyledons still intact inside the cuticle were not considered germinated but were enumerated as part of the total seed numbers.

**Bacterial analysis.** After inoculation, drying, or heating, 1 g of the alfalfa seeds was sampled and mixed with 9 ml of 0.1% peptone water in a 60-ml sterile stomacher bag (Fisherbrand, Fisher). The bag was stomached at 260 rpm for 60 s (Stomacher 400, Seward Ltd., Basingstoke, UK). The homogenate was serially diluted in sterile 0.1% peptone water. For the seeds inoculated with Salmonella, duplicate 0.1-ml portions of each dilution and quadruplicate 0.25-ml portions of the undiluted mixture were surface plated onto TSA (Difco, Becton, Dickinson, Sparks, Md.) supplemented with ampicillin (100 mg/liter) and onto Hektoen enteric agar (HEA; Criterion, Hardy); for the seeds inoculated with E. coli O157:H7; the dilutions were surface plated by the same method as described above, except for the use of violet red bile agar with MUG (4-methylumbelliferyl-β-glucuronide) (VRBA; Difco, Becton, Dickinson) in lieu of HEA.

When the seeds had been sprouted for 3 days, 3 to 6 g of the sprouts was combined 10-fold with 0.1% peptone water. Three separate containers were sampled and assayed for surviving populations of Salmonella and E. coli O157:H7 on sprouts by the same method mentioned above.

TSA plus ampicillin medium and VRBA were incubated at 37°C for 24 h, and HEA was incubated for 48 h. The enumeration of the colonies on TSA plus ampicillin medium was done under UV light of 320 nm. Only the colonies that exhibited green fluorescence were counted on TSA plus ampicillin. Typical colonies on HEA and VRBA were also counted. Suspected colonies that appeared to be E. coli O157:H7 or Salmonella were confirmed with Chromagar O157 and Chromagar Salmonella (BBL, Becton Dickinson, Sparks, Md.).
TABLE 1. Thermal inactivation (at 55°C for up to 8 days) of Salmonella on alfalfa seeds at high and low inoculum levels of 8.00 (8.69) and 2.17 (2.63) log CFU/g, respectivelya

<table>
<thead>
<tr>
<th>Heating time (days)</th>
<th>High inoculum levels</th>
<th>Low inoculum levels</th>
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<tbody>
<tr>
<td></td>
<td>After heating</td>
<td>After 3 days of sprouting</td>
</tr>
<tr>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>2.14 ± 0.14</td>
<td>5.92 ± 0.14</td>
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<td>(1.49 ± 0.20)</td>
<td>(8.50 ± 0.12)</td>
</tr>
<tr>
<td>6</td>
<td>1.32 ± 0.28</td>
<td>5.82 ± 0.91</td>
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<td>(&lt;1)</td>
<td>(8.61 ± 0.36)</td>
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<td>8</td>
<td>&lt;1</td>
<td>5.73 ± 0.45</td>
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<td>(&lt;1)</td>
<td>(8.91 ± 0.09)</td>
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</table>

a After drying, the levels of Salmonella on seeds were 6.79 (7.19) and <1 (1) log CFU/g for high and low inoculum levels, respectively. Data were from TSA plus ampicillin and from HEA (in parentheses). Values are means of triplicates, except for those below the detection limit. NA, not applicable; ND, not determined.

All the experiments were performed in separate triplicate trials with duplicate plating.

Statistical methods. The percentages of germination from a series of treatment times were compared with the control (no heating time) to determine if heat treatments reduce the germination significantly by the Dunnett method. Analysis of variance between treatments with different heating times was used to compare mean significantly by the Dunnett method. Analysis of variance between treatments with different heating times was used to compare mean significantly by the Dunnett method. Analysis of variance between treatments with different heating times was used to compare mean significantly by the Dunnett method. Analysis of variance between treatments with different heating times was used to compare mean significantly by the Dunnett method. Analysis of variance between treatments with different heating times was used to compare mean significantly by the Dunnett method. Analysis of variance between treatments with different heating times was used to compare mean significantly by the Dunnett method.

RESULTS

Remaining Salmonella populations. As Table 1 indicates, the levels of Salmonella on seeds decreased compared with the untreated control seeds. However, Salmonella on sprouts from all treatments and control seeds rapidly increased to approximately the same levels during spraying, regardless of the treatment and Salmonella reduction achieved on the seeds. The increase in Salmonella levels on the sprouts is even much higher, considering the increase in weight and surface area that occurs during the spraying process.

It is notable that overnight drying of freshly inoculated seeds resulted in a 1-log reduction of Salmonella. Similar results were found in the subsequent experiments of this study. With the high inoculation level of 8.00 log CFU/g, Salmonella was inactivated by more than 5 log on seeds after 6 days of heating at 55°C (Table 1). After 8 days of heating, the population of Salmonella on seeds was below detection limits (1 log CFU/g or 10 CFU/g of seeds). After 3 days of spraying with favorable conditions for pathogen growth (incubation at 30°C and water spraying), the populations of the pathogens from different batches of sprouts treated for 4, 6, and 8 days were not significantly different from each other (P > 0.05, not marked in Table 1), suggesting that the final pathogen populations on the sprouts were not related to the different treatment times as long as there was Salmonella surviving on or inside the seeds. On TSA plus ampicillin, pathogen populations on the seeds were below the detection limit after 8 days of heating, while it was already undetectable on HEA, even after 6 days of heating. However, after 3 days of spraying, the pathogens grew quickly to high numbers on sprouts, even though it was below the detectable limit on the seeds. Although the numbers of Salmonella on sprouts were lower than in the control, it is obvious that with high inoculation levels, heating at 55°C had little effect on the reduction of the pathogen on sprouts, while it was easy to obtain a 5-log reduction on seeds. The likely reason for this observation is that there were surviving pathogens on seeds, although they were too few to be detected, and they could grow to high numbers on sprouts during the spraying process, which allows rapid pathogen growth. It was found that, normally, there was a difference in pathogen counts between TSA plus ampicillin and HEA, especially for sprouts. The difference could be attributed to the lack of recovery for heat-injured cells and a proportion of the total cells that lost the pGFP plasmid and, as a consequence, were no longer ampicillin antibiotic resistant or capable of producing green fluorescent protein. However, plasmidless cells grew on the selective medium HEA, resulting in an enumeration difference.

After the seed inoculation level was lowered to ~2 log CFU/g, the pathogen was still capable of growing to high numbers on sprouts after the spraying process from untreated seeds (control) (Table 1), although on TSA plus ampicillin, it was below detection limits prior to spraying. As the results of both TSA plus ampicillin and HEA show, the pathogen on all heat-treated seeds for 4, 6, and 8 days were not detected. Furthermore, no pathogens were detected on sprouts after 3 days of spraying at 30°C, indicating that the heating process is effective in eradicating the pathogens from the seeds.

Remaining E. coli O157:H7 populations. E. coli O157:H7 remained on the sprouts at almost the same level of cell concentrations as on the seeds that did not undergo heat treatment (Table 2). For the seeds with high inoculation levels, the pathogens on the seeds were detected at low numbers on TSA plus ampicillin, while on VRBA, no cells were detected on the seeds after 4 days of heating at 55°C. The reduction of more than 5 log on seeds was achieved by heating the seeds at 55°C for 4 days or more. However, during spraying, E. coli O157:H7 quickly grew to high levels, even if it was below the detection limits on VRBA from the seeds that underwent 4 days of heating. After 6 days of treatment, the pathogens were below detectable limits on the seeds. After spraying, E. coli O157:H7 was still below detectable limits on the sprouts. Therefore, the temperature and time regime of this heat treatment was effective in killing high levels of E. coli O157:H7 on sprouts.

The effect of heat treatment on low levels of inoculation was also investigated. Seeds were inoculated with E. coli O157:H7 cocktail at the level of ~3 log CFU/g (Table
TABLE 2. Thermal inactivation (at 55°C for up to 8 days) of E. coli O157:H7 on alfalfa seeds at high and low inoculum levels of 8.75 (8.68) and 3.15 (3.00) log CFU/g, respectively

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<th>Heating time (days)</th>
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<th>Low inoculum levels</th>
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<tr>
<td></td>
<td>After heating</td>
<td>After 3 days of sprouting</td>
</tr>
<tr>
<td>0</td>
<td>NA</td>
<td>8.59 ± 0.08 (NA)</td>
</tr>
<tr>
<td>4</td>
<td>1.6</td>
<td>6.96 ± 0.27 (1.00)</td>
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<td>6</td>
<td>&lt;1</td>
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<td>8</td>
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*After drying, the levels of E. coli O157:H7 on seeds were 7.29 (6.82) and 2.33 (2.02) log CFU/g for high and low inoculum levels, respectively. Data were from TSA plus ampicillin and from VRBA (in parentheses). Values are means of triplicates, except for those below the detection limit, NA, not applicable.

Germination percentages of treated seeds. After inoculation with high levels of Salmonella, drying in the laminar flow biological safety cabinet, heating at 55°C for up to 8 days, and sprouting the seeds for 3 days, the percentages of germination were obtained by counting the sprouts from the different treatments. It was found that the germination of the seeds decreased significantly (P < 0.05) from 82.0 to 62.0, 59.7, and 47.0% for 4, 6, and 8 days of heating at 55°C, respectively, compared with the control (Table 3).

The germination of the seeds that underwent heat treatment without inoculation procedures was investigated to determine the extra effects, if any, of the inoculation on seed viability. The percentage of germination of the heat-treated seeds remained at 78.7 and 76.8% with 4 and 6 days of heating, respectively. The percentages of germination of the seeds that were heated for 4 and 6 days were not significantly different from the control, 82.8% (P > 0.05). After 8 days of heating, the germination decreased to 74.60%, which was significantly different (P < 0.05) from the control. The overall appearance of the treated sprouts was not different from the control.

DISCUSSION

From the results obtained in this study, it was possible to achieve a 5-log reduction of both Salmonella and E. coli O157:H7 on alfalfa seeds without significantly affecting the seed viability. However, after the sprouting process, both Salmonella and E. coli O157:H7 were still observed after sprouting. For 6 days or more of heating, no pathogens were observed for TSA plus ampicillin or VRBA media. Furthermore, pathogens were under detection limits on sprouts, suggesting that the treatment of 55°C for 6 days had eradicated the pathogens from the seeds.

Seeds inoculated at high levels of E. coli O157:H7 and heat treated at 55°C for ≥4 days resulted in alfalfa sprouts that were below detectable levels. However, only seeds inoculated with low inoculum levels of Salmonella and heat treated at 55°C for ≥4 days resulted in sprouts that were below detectable levels. These results suggest that the higher heat resistance of the Salmonella strains allowed lower inactivation rates in seeds and consequently the presence of Salmonella in sprouts.

It has been reported that Salmonella grows faster (6) and attaches more tightly to the surfaces of the alfalfa sprouts (2) than does E. coli O157:H7 during the sprouting process. In this case, Salmonella might recover more quickly from heat stress than E. coli O157:H7. Therefore, Salmonella is more recalcitrant for the elimination from alfalfa seeds.

It was observed that with alfalfa sprouts, there was a large gap in the numbers of typical colonies of Salmonella formed on TSA plus ampicillin and HEA media, while the numbers of E. coli O157:H7 on TSA plus ampicillin were in close agreement with those on VRBA. This observed difference might be attributable to the instability of the plasmid (pGFP) in Salmonella cells. Current studies are being performed to confirm this hypothesis.

In this study, heating alfalfa seeds at 55°C for 4 or more days was capable of achieving a >5-log reduction in both Salmonella and E. coli O157:H7 populations, which greatly enhanced the safety of alfalfa sprouts. This method
is effective only in removing low levels of *Salmonella* contamination on seeds, while it is effective in eradicating high contamination levels of *E. coli* O157:H7 at more than 8-log CFU/g contamination levels. Under normal conditions, the pathogens contaminate the seeds at very low numbers before harvest. The effectiveness of this method to decontaminate pathogens at artificially high pathogen levels should be sufficient for naturally contaminated seeds at much lower pathogen contamination levels. Higher temperatures were attempted and showed faster kill rates, but the germination rates were significantly reduced. The germination of alfalfa seeds was greatly reduced at temperatures higher than 55°C after several days of holding (data not shown).

The difference in the percentages of germination of heated seeds with and without inoculation could be attributed to the inoculation effect on the viability of the seeds. To be inoculated, the seeds were soaked in the inoculum for 20 min and then dried overnight in the laminar flow biological safety cabinet. These procedures could add to the effects of heat in reducing the germination rate of the alfalfa seeds. The combined effects of inoculation and heating resulted in the significant reduction of seed viability. The results from the heated seeds without inoculation indicated that heat treatment at 55°C for up to 6 days did not reduce the percentage of germination significantly, and even after 8 days of heating, the germination was still acceptable. The percentages of germination obtained in this study showed that dry heat at 55°C could be an effective treatment for ensuring the safety of the alfalfa sprouts without affecting the viability of the alfalfa seeds significantly.

Dry heat is more practical and promising, because the germination of alfalfa seeds appeared to be less sensitive to dry heat than to heat in hot water. Hot water treatment for alfalfa seeds at around 55°C or more than 55°C was previously reported to be detrimental to seed viability, even for several minutes of treatment (13, 18, 23). However, this study indicates that the germination of alfalfa seeds was stable for several days of dry heat at 55°C, suggesting that dry heat is applicable to the sprout industry both in terms of safety assurance and of maintaining an acceptable percentage of germination for the producers.

It is the wrinkled surface seed structure of alfalfa seeds that makes the eradication of pathogens from seeds difficult. Some pathogens remain inside the seed coat, wrinkled grooves, or crevices that are not accessible to chemical sanitizers; hence, there is incomplete access to the contaminating pathogens by chemical sanitizers that largely work as surface sanitizers. However, heat transfer can penetrate the food matrix, regardless of the intricate structure of the seeds. Therefore, heat treatment can be an effective treatment for removing even internalized or protected pathogens from alfalfa seeds.

The results of this study showed that heat treatment of seeds at 55°C for up to 8 days can be an effective method for ensuring the safety of the alfalfa sprouts without reducing the germination of the seeds significantly. Heat treatment may be a promising alternative to current ineffective methods because the treatment can easily be applied to large quantities of seeds prior to selling them to sprout producers. In addition, the application is easily monitored and recordable to ensure the proper treatment was applied.

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