Applied and Environmental Microbiology

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Appl. Environ. Microbiol. **2013, 79(15):4613. DOI: 10.1128/AEM.00443-13.**
Published Ahead of Print 24 May 2013.

AEM
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Evaluation of Aerated Steam Treatment of Alfalfa and Mung Bean Seeds To Eliminate High Levels of *Escherichia coli* O157:H7 and O178:H12, *Salmonella enterica*, and *Listeria monocytogenes*

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Sprouts contaminated with human pathogens are able to cause food-borne diseases due to the favorable growth conditions for bacteria during germination and because of minimal processing steps prior to consumption. We have investigated the potential of hot humid air, i.e., aerated steam, to treat alfalfa and mung bean seeds which have been artificially contaminated with *Escherichia coli* O157:H7, *Salmonella enterica* subsp. *enterica* serovar Weltevreden, and *Listeria monocytogenes* Scott A. In addition, a recently collected *E. coli* O178:H12 isolate, characterized by a reduced heat sensitivity, was exposed to the treatment described. Populations of *E. coli* O157:H7 and *S. enterica* on alfalfa and mung bean seeds could be completely eliminated by a 300-s treatment with steam at 70 ± 1°C as revealed by enrichment studies. *L. monocytogenes* and *E. coli* O178:H12 could not be completely eliminated from artificially inoculated seeds. However, bacterial populations were reduced by more than 5 log CFU/g on alfalfa and by more than 4 log CFU/g on mung bean seeds. The germination rate of mung beans was not affected by the 300-s treatment compared to the germination rate of untreated seeds whereas that of alfalfa seeds was significantly lower by 11.9%. This chemical-free method is an effective alternative to the 20,000-ppm hypochlorite treatment presently recommended by the U.S. Food and Drug Administration (FDA).

Rising awareness about healthy diets in today's society has led to an increased consumption of fresh fruits and vegetables. Global distribution and trading chains as well as product varieties, especially of minimally processed produce, have been extended. However, fresh produce consumed raw is a potential source of human pathogens (1). This is particularly evident for sprouts, since the warm, humid conditions during sprouting and the availability of sugars diffusing from germinating seeds allow growth of pathogens to numbers that may cause disease in humans after consumption of the contaminated sprouts. Cases of sprout-related outbreaks have been reported from Canada, Denmark, Finland, France, Germany, Japan, The Netherlands, Norway, Sweden, United Kingdom, and the United States (2). A large outbreak in Japan in 1996 related to sprouts contaminated with *Escherichia coli* O157:H7 caused more than 9,000 cases of illness and 12 deaths (3). The *E. coli* O104:H4 outbreak in Germany in 2011 caused disease in more than 4,000 people, including 908 cases of hemolytic uremic syndrome and 50 deaths (4).

Sprouts are produced from a variety of different seeds, with alfalfa and mung bean seeds being the most commonly consumed and most frequently implicated ones in outbreaks in the European Union, the United States, and Asian countries. Mung bean sprouts are consumed in larger quantities in Asian than in Western countries and are most often served after at least light cooking in any country, whereas alfalfa sprouts are mostly eaten raw (2, 5, 6). *E. coli* O157:H7 and *Salmonella* are the leading pathogens in sprout-related outbreaks, but *Listeria monocytogenes*, *Staphylococcus aureus*, *Yersinia enterocolitica*, and *Bacillus cereus* have also been involved to a minor extent (2). The seeds used for sprouting are the major source of contamination (7). They can become contaminated by several potential routes such as contaminated irrigation water, insufficiently composted manure used as fertilizer, dust, fecal contamination transmitted through animals, and inadequate hygiene of workers and equipment during production, harvest, storage, and processing (1). Numbers of total mesophilic aerobic bacteria on alfalfa and mung bean seeds intended for sprout production have been revealed by several studies to be in the range of 3 to 5 log CFU/g, with fecal coliforms comprising up to 10% of the bacterial population (8–11). Total populations of mesophilic aerobic bacteria on commercially available alfalfa and mung bean sprouts range between 7 and 9 log CFU/g (12, 13). Increases of populations of *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* by up to 100,000-fold during sprouting, whereby the major growth occurs within the first 2 days of the sprouting process, have been previously described (13–19). Therefore, sanitizing treatments should be able to completely eliminate any existing pathogens from seeds used for sprouting.

In the United States, the FDA recommends soaking seeds in 20,000-ppm hypochlorite prior to sprouting in order to reduce the bacterial burden. Yet studies have shown that this results in a median reduction of only 2.5 log CFU/g (20). Other sanitizing treatments include ionizing radiation (21), high hydrostatic pressure (22) in combination with mild heat (23), supercritical carbon dioxide (24), hot water (25–27), and dry heat (28). There is, however, no decontamination method available to date to ensure elimination of pathogens in all types of seeds without reducing seed germination or sprout yields (2). The use of aerated steam to disinfect seeds is an easy-to-handle, inexpensive method that was

Received 10 February 2013 Accepted 19 May 2013
Published ahead of print 24 May 2013
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doi:10.1128/AEM.00443-13

already shown to be effective in eliminating seed-borne plant-pathogenic bacteria and fungi (29–32). Aerated steam is accomplished by passive aspiration of air through nozzles into the current of steam, and the temperature of the steam is controlled by opening or closing the air inlet nozzles.

The aim of the present study was to evaluate the use of aerated steam (70 ± 1°C) to eliminate *E. coli* O157:H7, *Salmonella enterica* subsp. *enterica* serovar Weltevreden, and *L. monocytogenes* Scott A on artificially contaminated alfalfa and mung bean seeds and to test the effect of the treatment on the germination capacity of the seeds. In addition, we tested the efficacy of the aerated steam treatment to eliminate *E. coli* O178:H12, an isolate from raw milk cheese which attracted attention because of its reduced heat sensitivity compared to that of the other generic and Shiga toxin-producing *E. coli* strains (33).

MATERIALS AND METHODS

Bacterial strains. *E. coli* O157:H7 (NCTC 12900) does not produce Vero cytotoxins and was chosen because of laboratory safety advantages. The *eaeA* gene coding for intimin, a protein involved in attachment, is still intact. *E. coli* O178:H12 (FAM 21843) is PCR negative for *stx*, *hlyA*, and *eaeA* (33). *Salmonella enterica* subsp. *enterica* serovar Weltevreden is an isolate from a gastroenteritis outbreak in Scandinavia associated with contaminated alfalfa sprouts and fresh salad (34). *Listeria monocytogenes* Scott A is a widely distributed serovar 4b clinical isolate from the 1983 listeriosis outbreak in Massachusetts (35, 36).

Culture and cell suspension. *S. enterica* and both strains of *E. coli* were cultured overnight in tryptic soy broth (Sigma-Aldrich, St. Louis, MO) at 37°C. *L. monocytogenes* was cultured in brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) at 37°C. The next morning, the overnight cultures were diluted 1:10 in fresh media and grown at 37°C to optical densities at 600 nm (OD_{600}) of 1.75, 1.65, 1.95, and 2.1 for *E. coli* O157:H7, *S. enterica*, *L. monocytogenes*, and *E. coli* O178:H12, respectively. The cells were then harvested by centrifugation at 4,500 × *g* for 5 min. The supernatant was discarded, and the cells were washed once with 0.1% sterile peptone solution (Merck, Darmstadt, Germany). The final pellet was again resuspended in sterile 0.1% peptone solution to a final concentration of ~10^9 CFU/ml. Each culture suspension was immediately used for further experiments.

Seed material and inoculation. Alfalfa and mung bean seeds were obtained from a commercial supplier (Eric Schweizer AG, Switzerland). Dry seeds were immersed in each bacterial suspension with occasional stirring for 5 min. The suspension was then discarded, and the seeds were spread on a sterile filter paper inside a biosafety hood, where they were dried for approximately 20 h at room temperature with periodical rotation for obtaining homogeneously dried seeds. This procedure provided completely dried seeds as shown by differential weighting. Dried seeds were divided in 2-g and 4-g portions for alfalfa and mung bean seeds, respectively, and were immediately used for further experiments.

Aerated steam treatment. Inoculated seed material was treated using a steaming device constructed in-house based on a commercially available steam generator with a power consumption of 2,000 W (Wagner GmbH, Germany) (Fig. 1). The modified device comprised a basic unit containing 3.5 liters of water, a mains lead, and a steam hose leading into a pipe with a diameter of 4 cm that was connected to a reversed funnel with a diameter of 25 cm. The pipe contained four air inlet nozzles where the steam hose was connected to the pipe and six additional inlet nozzles at the pipe base through which air was aspirated passively into the steam current. Plugs fitting into the nozzles were used to adjust the temperature of the steam to 70 ± 1°C. The temperature of the steam was measured using a thermocouple placed internally at the point of treatment, i.e., on the seeds. The water content of the aerated steam achieved as described was 33 g/liter. The seeds were placed evenly on a mesh that was put onto a stainless steel grid, and treatment was performed for 30 s, 90 s, 180 s, and 300 s. Control seeds were not treated with aerated steam but were otherwise handled as experimental seeds.

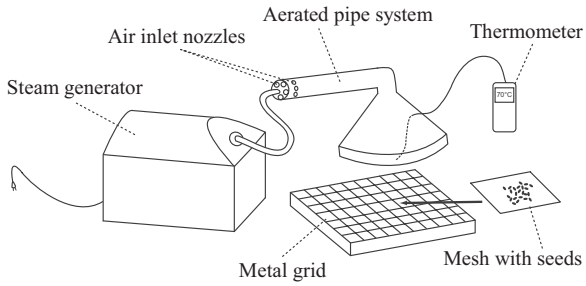

FIG 1 Scheme of the aerated steam treatment device.

Microbiological analysis. Treated and control seeds were collected in sterile 50-ml Falcon tubes. Sterile 0.1% peptone solution (10 ml and 20 ml, respectively) was added to alfalfa and mung beans. The tubes were then vortexed for 30 s in order to detach the surviving bacteria from the seeds. A serial dilution series was prepared in 0.1% peptone solution. *E. coli* O157:H7 and O178:H12 were plated (200 μl each) onto MacConkey-Sorbitol agar (Sigma-Aldrich, St. Louis, MO). *S. enterica* and *L. monocytogenes* were plated (200 μl each) onto *Salmonella-Shigella* agar (Difco/Becton, Dickinson, Sparks, MD), and Oxford agar (Sigma-Aldrich, St. Louis, MO), respectively. All plates were incubated at 37°C for ~22 h before enumeration.

For the enrichment studies, treated seeds were collected in 50-ml Falcon tubes containing 10 ml and 20 ml of sterile 0.1% peptone solution for alfalfa and mung bean seeds, respectively. The tubes were incubated shaking at 37°C for 24 h. Aliquots of 100 μl of the enrichment cultures were plated undiluted on selective agars as mentioned above as well as on non-selective agar (tryptic soy agar; Sigma-Aldrich, St. Louis, MO). The plates were incubated for 24 h at 37°C before being observed for the presence of colonies.

Seed germination. Uncontaminated seeds in portions of at least 100 seeds per plate were treated with aerated steam as described above and spread evenly on 2% H_2O agar plates. The plates in triplicates were kept at room temperature for 6 days and overlaid with a wet cloth every day for 4 h in order to provide humidity. The same plate was used to determine the germination percentage of mung bean seeds at days 3, 4, 5, and 6 post-treatment by counting at least 100 seeds per treatment condition. For alfalfa seeds, the germination percentage was also determined at days 3, 4, and 5 by counting at least 100 seeds per treatment condition. The germination percentage was calculated by dividing the number of germinated seeds by the total number of seeds.

Statistical analysis. The entire experiment was repeated at least three times. Analysis of variance (ANOVA) and Tukey's one-way multiple comparisons were performed using XLSTAT version 2011.2.04 to determine the differences in the germination percentages of seeds. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

RESULTS

Aerated steam treatment of *E. coli* O157:H7. Treatment of *E. coli* O157:H7-contaminated alfalfa seeds for 30 s resulted in a 2.58 log CFU/g decrease from the initial count of 6.21 ± 0.14 log CFU/g to 3.63 ± 0.38 log CFU/g. Already, a 90-s and longer treatment had reduced the initial *E. coli* O157:H7 population to below 1.40 log CFU/g, the detection limit of the experiment (Fig. 2A). For mung bean seeds, the 30-s treatment reduced the initial bacterial count of 5.36 ± 1.09 log CFU/g to 4.04 ± 0.41 log CFU/g, the 90-s treatment reduced it to 2.39 ± 1.04 log CFU/g, with one of the three replicates being below the detectable level, and the 180-s

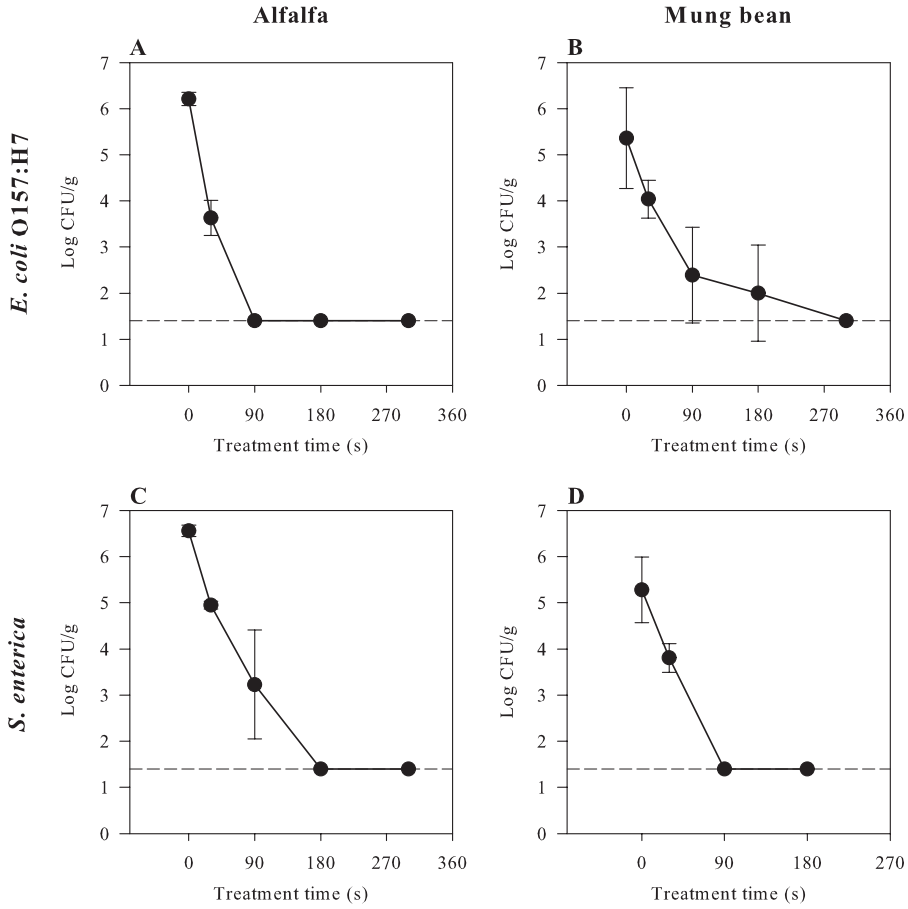

FIG 2 Effect of aerated steam treatment on population levels of *E. coli* O157:H7 (A and B) and *S. enterica* (C and D) on alfalfa (A and C) and mung bean (B and D) seeds. The detection limit is 1.4 log CFU/g (dashed line). Bars indicate standard deviations.

treatment reduced it to 2.00 ± 1.04 log CFU/g, with two replicates being below the detectable level. The 300-s treatment was able to decrease the populations to undetectable levels in all three replicates (Fig. 2B).

In general, alfalfa seeds had a markedly higher initial contamination level compared to mung bean seeds, which can be explained by the higher surface area-to-mass ratio of the alfalfa seeds.

Aerated steam treatment of *S. enterica*. The initial *S. enterica* population of 6.56 ± 0.12 log CFU/g on alfalfa seeds was reduced to 4.95 ± 0.08 and 3.23 ± 1.18 log CFU/g by the 30-s and 90-s treatments, respectively. The 180-s and 300-s treatments both produced results in all tested samples that were below detectable levels (Fig. 2C). Despite the use of the same bacterial suspension for artificial contamination of mung beans, the population level of the untreated control of the mung beans (5.28 ± 0.71 log CFU/g) was more than 1 log CFU/g below that of the alfalfa seeds. Treatment of mung beans for 30 s resulted in a 1.5 log CFU/g decrease to a bacterial count of 3.81 ± 0.31 log CFU/g. Treatments for 90 s and longer reduced the *S. enterica* population to undetectable levels (Fig. 2D).

Aerated steam treatment of *L. monocytogenes*. The initial count of *L. monocytogenes* on alfalfa seeds was determined to be 6.57 ± 0.14 log CFU/g. Treatment for 30 s and 90 s decreased the levels to 3.80 ± 0.4 and 1.90 ± 0.46 log CFU/g, respectively. The 180-s and 300-s treatments reduced levels of *L. monocytogenes* to below the detection limit of 1.40 log CFU/g (Fig. 3A). As with *E. coli* and *Salmonella*, the *Listeria* population level of 5.49 ± 0.38 log CFU/g on mung beans was remarkably lower than that on the alfalfa seeds. Treatment of the mung beans for 30 s reduced the population of *Listeria* by only 0.4 log CFU/g to 5.09 ± 0.97 log CFU/g. The 90-s and 180-s treatments led to bacterial counts of 3.29 ± 0.25 log CFU/g and 1.76 ± 0.32 log CFU/g, respectively. The 300-s treatment was able to diminish the population of *L. monocytogenes* to undetectable levels in two replicates, with the third replicate displaying a count of 1.40 log CFU/g (Fig. 3B).

Aerated steam treatment of *E. coli* O178:H12. With 7.17 ± 0.09 and 5.97 ± 0.08 log CFU/g on alfalfa and mung bean seeds, respectively, *E. coli* O178:H12 showed the highest initial contamination levels of the four strains tested. The initial load was reduced to 5.79 ± 0.18, 4.26 ± 0.38, and 2.08 ± 1.19 log CFU/g by the 30-s, 90-s, and 180-s treatments, respectively, for the alfalfa seeds. Two replicates were below detectable levels after the 180-s treatment. The 300-s treatment led to a reduction to below the detection limit in all replicates (Fig. 3C). The bacterial burden on mung bean seeds was reduced to 5.18 ± 0.18, 4.41 ± 0.14, 3.08 ± 1.68, and 1.50 ± 0.17 log CFU/g by the 30-s, 90-s, 180-s, and 300-s treatments, respectively, with one replicate of the 180-s treatment and two replicates of the 300-s treatment being below detectable levels (Fig. 3D).

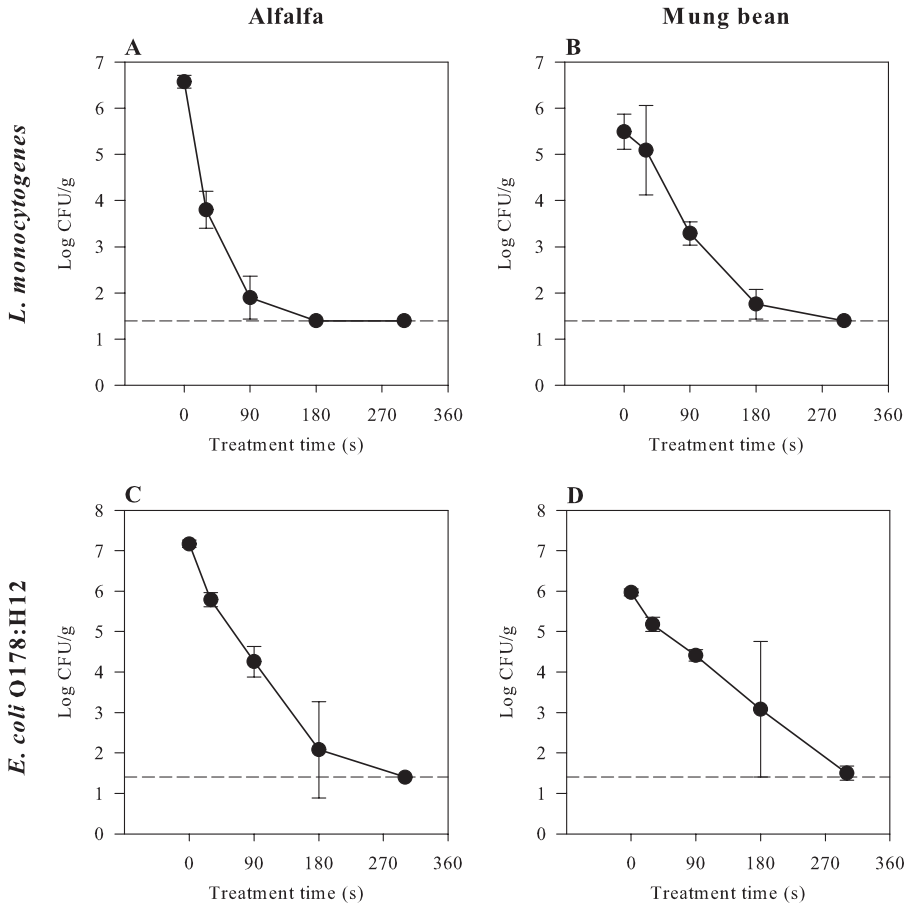

FIG 3 Effect of aerated steam treatment on population levels of *L. monocytogenes* (A and B) and *E. coli* O178:H12 (C and D) on alfalfa (A and C) and mung bean (B and D) seeds. The detection limit is 1.4 log CFU/g (dashed line). Bars indicate standard deviations.

Enrichment experiments. In a separate experiment, the presence of any surviving pathogens after aerated steam treatment was determined by culturing the treated seeds in nonselective broth and subsequent plating of the broth on selective and nonselective media. Since the treatment for 30 s was not able to reduce the population of pathogens to below detectable levels in any experiment (Fig. 2 and 3), only treatment times of 90 s, 180 s, and 300 s were tested in the enrichment studies. Results revealed that *E. coli* O157:H7 and *S. enterica* were completely eliminated from alfalfa and mung bean seeds by the 300-s treatment (Tables 1 and 2). The 180-s treatment of alfalfa seeds resulted in elimination of *E. coli* O157:H7 as well (Table 1). For the shorter treatment times, at least one of the three replicates was found to be positive for *S. enterica* or *E. coli* O157:H7 (Tables 1 and 2). *L. monocytogenes* and *E. coli* O178:H12 were eliminated from mung bean seeds after the 300-s treatment (Table 2) but not from alfalfa seeds, where at least one of the three replicates still tested positive (Table 1).

Effect of aerated steam treatment on seed germination. Noninoculated seeds were treated with aerated steam to test whether the treatment had an effect on germination capacity. Table 3 shows that

TABLE 1 Bacterial load on artificially contaminated alfalfa seeds and numbers of enrichment cultures found to be positive after aerated steam treatment

Treatment status or duration	Mean ± SD population recovered from untreated seeds (log CFU/g) or no. of positive results/total no. of results after 24-h enrichment			
	E. coli O157:H7	*S. enterica*	*L. monocytogenes*	*E. coli* O178:H7
No treatment	5.79 ± 0.16	6.2 ± 0.32	6.64 ± 0.12	7.34 ± 0.13
Treatment time (s)				
90	1/3	3/3	3/3	3/3
180	0/3	2/3	2/3	3/3
300	0/3	0/3	2/3	1/3

TABLE 2 Bacterial load on artificially contaminated mung bean seeds and number of enrichment cultures found to be positive after aerated steam treatment

Treatment status or duration	Mean ± SD population recovered from untreated seeds (log CFU/g) or no. of positive results/total no. of results after 24-h enrichment			
	E. coli O157:H7	*S. enterica*	*L. monocytogenes*	*E. coli* O178:H7
No treatment	5.51 ± 0.62	4.27 ± 0.84	5.48 ± 0.54	5.71 ± 0.88
Treatment time (s)				
90	2/3	2/3	3/3	3/3
180	1/3	2/3	1/3	2/3
300	0/3	0/3	0/3	0/3

TABLE 3 Germination percentages of alfalfa seeds after aerated steam treatment[a]

Day	% germination after indicated duration of treatment				
	Control	30 s	90 s	180 s	300 s
3	90.4 ± 3.1^{A}	94.4 ± 1.0^{A}	92.9 ± 1.4^{A}	94.0 ± 0.6^{A}	74.5 ± 6.0^{B}
4	91.6 ± 2.6^{A}	94.7 ± 1.2^{A}	93.5 ± 1.8^{A}	94.7 ± 0.5^{A}	76.9 ± 5.2^{B}
5	91.6 ± 2.6^{A}	94.7 ± 1.2^{A}	94.1 ± 1.4^{A}	95.0 ± 0.5^{A}	79.7 ± 7.0^{B}

[a] Values represent means and standard deviations of the results determined for three replicates. Different superscript capital letters in each row indicate a significant difference ($P < 0.05$).

treatment of alfalfa seeds with aerated steam for up to 180 s did not significantly influence the germination rate at any of the times tested ($P > 0.05$). At days 3, 4, and 5 posttreatment, there was a significant difference observed between the 300-s treatment and the control as revealed by the ANOVA. At day 5, there was a mean difference of 11.9% between the control and the 300-s treatment results. The germination capacity of mung bean seeds was not significantly affected by the aerated steam treatment for any of the tested treatment times and days observed (Table 4).

DISCUSSION

The use of hot steam as a way to eliminate plant pathogens on various seeds and crops had already been investigated in the early 20th century (31, 37). Hot steam commonly applied to a field is the method employed for sanitation of soil intended for food production (38). In addition to eliminating undesired pests and weeds, hot-steam treatment induces the release of nutritive substances blocked within the soil (39). Nevertheless, to our knowledge there is no publication available so far which has reported the study of the application of aerated steam to sanitize seeds contaminated with human pathogens.

Our results show that aerated steam treatment is an effective method to reduce high bacterial loads on alfalfa and mung bean seeds. In general, reductions of bacterial populations (*E. coli* O157:H7, *S. enterica*, *L. monocytogenes*, and *E. coli* O178:H12) of approximately 5 log CFU/g on alfalfa seeds and 4 log CFU/g on mung bean seeds were observed after treatment with aerated steam for 300 s. The enrichment experiments performed to allow resuscitation of possible sublethally injured cells due to the heat treatment revealed that *E. coli* O157:H7 and *S. enterica* could be completely eliminated from both types of seeds by application of the 300-s treatment. In contrast, *L. monocytogenes* and *E. coli* O178:H12 could be completely eliminated from mung bean seeds but not from alfalfa seeds, where survivors were detectable after enrichment in at least one out of three replicates. It is possible that different factors led to the incomplete elimination of *L. monocytogenes* and *E. coli* O178:H12 from alfalfa seeds. It is noteworthy that the initial inoculation levels of alfalfa seeds were higher than those of mung bean seeds. Other specific factors, e.g., specific attachment properties of *L. monocytogenes* and *E. coli* O178:H12, might also have affected the result and have to be determined in further studies. In addition, during the initial 90 s of the aerated steam treatment, the population of the heat-tolerant strain *E. coli* O178:H12 was less reduced than that of *E. coli* O157:H7 on alfalfa seeds (2.91 log CFU/g versus 4.81 log CFU/g) (Fig. 3C and 2A) and, to a lesser extent, on mung bean seeds (1.56 log CFU/g versus 2.97 log CFU/g) (Fig. 3D and 2B). This may be a further indication of the importance of strain-specific factors and their role in the behavior of the contaminating bacteria on different seed surfaces.

These findings underline the importance of considering the presence of *L. monocytogenes* as well as of heat-tolerant pathogenic strains of *E. coli* with respect to sprouts and fresh produce in general, although *Listeria* has been associated with only very few cases of illness due to sprout consumption and strains of heat-tolerant pathogenic *E. coli* have not been associated with produce-related outbreaks to date.

Regarding the germination capacity of treated seeds, it was shown that the 300-s treatment did not affect germination of mung bean seeds compared to untreated seeds at all examination dates. For alfalfa seeds, however, the 300-s treatment significantly reduced the germination percentage by 11.9%, which still might be a commercially acceptable level for sprout producers in practice. The thick, robust seed coat and the high thermal mass of mung bean seeds protecting their embryos and inner storage tissues from harmful heat may explain why mung beans are more resistant to the heat treatment than alfalfa seeds with respect to the germination percentages. Since the tested 300-s treatment did not reveal any sign of a reduced germination capacity, even hotter steam or longer treatment times may be applied to mung bean seeds.

In several reported studies, treatments of sprout seeds artificially inoculated with human pathogens using hot water or dry heat have been examined. A population of *S.* Stanley, for example, was reduced by 2.5 log units after treatment of artificially inoculated alfalfa seeds with water at 60°C for 5 min without significant reduction of the germination rate (16). However, a longer treatment at 60°C (10 min) significantly reduced the germination rate

TABLE 4 Germination percentages of mung bean seeds after aerated steam treatment[a]

Day	% germination after indicated duration of treatment				
	Control	30 s	90 s	180 s	300 s
3	35.6 ± 4.2^{A}	34.1 ± 8.6^{A}	47.9 ± 7.7^{A}	43.9 ± 5.5^{A}	45.6 ± 12.3^{A}
4	68.6 ± 2.4^{A}	63.1 ± 9.7^{A}	69.1 ± 4.4^{A}	64.6 ± 4.5^{A}	69.3 ± 5.5^{A}
5	84.6 ± 3.3^{A}	78.9 ± 6.8^{A}	82.6 ± 3.5^{A}	79.0 ± 1.4^{A}	85.0 ± 2.9^{A}
6	93.9 ± 3.1^{A}	87.7 ± 2.4^{B}	92.9 ± 1.1^{A,B}	90.4 ± 1.7^{A,B}	92.0 ± 1.3^{A,B}

[a] Values represent means and standard deviations of the results determined for three replicates. Different superscript letters in each row indicate a significant difference ($P < 0.05$).

by 54% and a temperature of 71°C for 5 min reduced it by 80%. In another study, hot-water treatment of artificially contaminated mung bean seeds at 85°C for 40 s reduced populations of *S.* Enteritidis and *E. coli* O157:H7 by 3.7 and 3.8 log units, respectively. The combination of hot-water treatment (85°C for 40 s), dipping in cold water (30 s), and soaking in chlorine water (2,000 ppm, 2 h) was shown to completely eliminate *S.* Enteritidis and *E. coli* O157:H7 from mung bean seeds (25). In a study using dry heat as a sanitizing treatment for mung bean seeds, initial bacterial loads of 6 log CFU/g for *E. coli* O157:H7 and 4 log CFU/g for *S. enterica* were reduced below detectable levels (<1 log CFU/g) when the seeds were kept at 55°C for 4 and 5 days, respectively. Germination rates were not affected. However, germination rates of alfalfa seeds were significantly reduced after the same treatment (40). In a recent study, dry-heat treatments of artificially contaminated alfalfa seeds at 55°C and 60°C for 10 days achieved reductions of only 1.6 and 2.1 log CFU/g in a population of *Salmonella* spp., respectively. Temperatures of 65°C for 10 days or 70°C for 24 h resulted in a reduction from the initial ~5 log CFU/g to below the detection limit (<0.7 log CFU/g) for *Salmonella* and *E. coli* O157:H7. However, germination rates decreased significantly (28).

As shown in the present study, aerated steam treatment at 70°C for 300 s was sufficient to reduce *E. coli* O157:H7, *S. enterica*, *L. monocytogenes*, and *E. coli* O178:H12 populations to a degree comparable to or even higher than those reported in the studies mentioned above. In addition, the results indicate that survival rates of *E. coli* O157:H7 and *S. enterica* decrease more rapidly under conditions of aerated steam treatment than under conditions of dry-heat treatment. Thus, aerated steam is a promising and efficient alternative to the hot-water and dry-heat treatment methods that are already in use by several sprout producers in Japan, Europe, and the United States for sanitation of seeds.

In numerous sprout-related outbreaks, seeds have been revealed to be the origin of the pathogens on the basis of microbiological or epidemiological evidence (2). However, for only a few outbreaks, the pathogen could be isolated from the seeds, suggesting that the contamination is not homogeneous in the seed lots and that contamination may be at low levels. There are no data on the natural occurrence of *E. coli*, *Salmonella* spp., and *L. monocytogenes* on alfalfa and mung bean seeds used for sprouting outside outbreak analyses. Analysis of alfalfa seed lots implicated in two outbreaks of salmonellosis revealed 0.1 to 0.6 CFU of *S.* Newport per 25 g of seeds associated with one outbreak and *S.* Tennessee, *S.* Cubana, and *S.* Havana at levels of approximately 4 CFU/kg of seeds related to the other outbreak (7). The artificial contamination procedure used in the present study, i.e., immersion of dry seeds in bacterial suspensions of *E. coli*, *S. enterica*, and *L. monocytogenes*, resulted in contamination levels of dry seeds that were high compared to the reported naturally occurring contamination levels of seeds. Additionally, the physiological state of the bacteria at the time points of inoculation and treatment with aerated steam may have contributed to an enhanced robustness of resistance to heat treatment compared to the level occurring naturally in contaminating bacteria. Since low water activity of seeds has been reported to be linked with higher heat tolerance (28, 41), the use of completely dried seeds in the present study might has supported thermal tolerance as well. In conclusion, it is suggested that the aerated steam treatment is suitable as a treatment to reduce and eliminate naturally occurring contaminations in a manner that is at least similar to that seen with the artificial contamination tested. This view is supported by studies in which a hypochlorite treatment has been applied to alfalfa seeds naturally contaminated with *S.* Mbandaka of around 1 most probable number (MPN)/100 g or *S.* Muenchen (42, 43). Both studies revealed complete elimination of the pathogens.

Aerated steam treatment might in addition offer a major advantage over chemical decontamination due to heat transfer to inner parts of the seed. The bacteria which can be present there (44) are not accessible to various chemical disinfectants such as, e.g., hypochlorite, since these disinfectants are not able to penetrate the seed coat (45–47). The extent to which aerated steam can be applied to eliminate bacterial loads not only on the outer seed surface but also in the inner parts of the seed needs to be studied in future work.

The high log reductions of 4 to 5 log CFU/g seeds to below the detection level (1.40 log CFU/g seeds) achieved in the present study are attributed to the high initial bacterial loads of 5 to 6 CFU/g seeds. This is noteworthy in comparisons of log reductions to the results of other studies in which low initial bacterial loads were used. Future experiments will therefore also include assessing the efficacy of sanitizing naturally contaminated seeds by aerated steam. Differences in the bacterial loads of samples, possible heterogeneous distribution of pathogens within the seed lot, the type of seeds, and the location of bacteria on the seed need to be considered carefully in such experiments. Scale-up equipment where seeds are carried along on a moving belt and treated with aerated steam from the top is under construction at our institute and will be evaluated for large-scale seed sanitation. The application of the aerated steam treatment immediately before seeds are sprouted by the sprout producers is recommended as a risk mitigation measure in practice.

In conclusion, the use of aerated steam is an effective method to ensure the microbiological safety of seeds used in sprouting regarding contamination by *E. coli* O157:H7 and *S. enterica*, which are the leading bacteria associated with sprout-related outbreaks. A strong advantage, in addition to the short treatment time and the ease of handling, is the lack of any chemical substances, which reduces the danger of the presence of harmful residues on the final food product. It is therefore a promising and attractive alternative for use in combination with good manufacturing practices and end product testing in both conventional and organic sprout production.

ACKNOWLEDGMENTS

We are indebted to Martin J. Loessner for supplying *Listeria monocytogenes* Scott A, to Brion Duffy and Kerstin Brankatschk for supplying *Salmonella enterica* subsp. *enterica* serovar Weltevreden, and to Monika Volkan for technical assistance.

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