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Efficacy of Electrolyzed Oxidizing Water in Inactivating *Salmonella* on Alfalfa Seeds and Sprouts

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ABSTRACT

Studies have demonstrated that electrolyzed oxidizing (EO) water is effective in reducing foodborne pathogens on fresh produce. This study was undertaken to determine the efficacy of EO water and two different forms of chlorinated water (chlorine water from Cl_2 and $Ca(OCl)_2$ as sources of chlorine) in inactivating *Salmonella* on alfalfa seeds and sprouts. Ten-gram sets of alfalfa seeds inoculated with a five-strain cocktail of *Salmonella* (6.3×10^4 CFU/g) were subjected to 90 ml of deionized water (control), EO water (84 mg/liter of active chlorine), chlorine water (84 mg/liter of active chlorine), and $Ca(OCl)_2$ solutions at 90 and 20,000 mg/liter of active chlorine for 10 min at 24 ± 2°C. The application of EO water, chlorinated water, and 90 mg/liter of $Ca(OCl)_2$ to alfalfa seeds for 10 min reduced initial populations of *Salmonella* by at least 1.5 $\log_{10}$ CFU/g. For seed sprouting, alfalfa seeds were soaked in the different treatment solutions described above for 3 h. $Ca(OCl)_2$ (20,000 mg/liter of active chlorine) was the most effective treatment in reducing the populations of *Salmonella* and non-*Salmonella* microflora (4.6 and 7.0 $\log_{10}$ CFU/g, respectively). However, the use of high concentrations of chlorine generates worker safety concerns. Also, the $Ca(OCl)_2$ treatment significantly reduced seed germination rates (70% versus 90 to 96%). For alfalfa sprouts, higher bacterial populations were recovered from treated sprouts containing seed coats than from sprouts with seed coats removed. The effectiveness of EO water improved when soaking treatments were applied to sprouts in conjunction with sonication and seed coat removal. The combined treatment achieved 2.3- and 1.5-$\log_{10}$ CFU/g greater reductions than EO water alone in populations of *Salmonella* and non-*Salmonella* microflora, respectively. This combination treatment resulted in a 3.3-$\log_{10}$ CFU/g greater reduction in *Salmonella* populations than the control (deionized water) treatment.

Several outbreaks of *Salmonella* infection have been associated with sprouted alfalfa seeds in various parts of the world *(16, 24).* In addition, outbreaks of food poisoning caused by *Escherichia coli* O157:H7 have been associated with the consumption of raw sprouts *(11, 19).* Contaminated seeds were the most likely source of the pathogens in these outbreaks *(10).* These pathogens could be introduced into sprouts from the seeds or in the water used during production; they could also be the result of improper sanitation during production and marketing *(18).*

Populations of *Salmonella* exceeding 10^6 CFU/g can occur on alfalfa sprouts produced from contaminated seeds *(11);* several aqueous chemicals (ClO_2, NaOCl, $Ca(OCl)_2$, H_2O_2, Tsunami and Vortexx [Ecolab, St. Paul, Minn.], ethanol, and trisodium phosphate) have been examined for their efficacy in inactivating pathogens on alfalfa seeds *(26).* However, none of the chemicals tested was capable of completely eliminating pathogens from alfalfa seeds *(23).* The chlorine treatment of alfalfa seeds or sprouts at various stages of production has been suggested as an intervention method to reduce populations of natural microflora or to eliminate pathogenic microorganisms, such as *Salmonella,* without adversely affecting germination *(1, 2, 11).* Jaquette et al. *(11)* studied the effectiveness of chlorine in eliminating *Salmonella* Stanley inoculated onto alfalfa seeds at populations of 10^1 to 10^2 CFU/g. The treatment of seeds with 2,040 mg/liter of active chlorine derived from $Ca(OCl)_2$ reduced populations of bacteria to less than 1 CFU/g. Beuchat et al. *(4)* also studied the efficacy of chemical treatments in eliminating *Salmonella* and *E. coli* O157:H7 on alfalfa seeds. The treatment of alfalfa seeds containing 6.5 to 6.6 $\log_{10}$ CFU *Salmonella* per g with 20,000 mg/liter of active chlorine (as $Ca(OCl)_2$) for 15 and 30 min has been shown to reduce populations to less than 1 CFU/0.25 g; however, the pathogen could be detected by enrichment *(4).* Although the National Advisory Committee on Microbiological Criteria for Foods *(16)* recommends the use of 20,000 mg/liter of active chlorine (as $Ca(OCl)_2$) for sanitizing seeds intended for sprout production, there are concerns that this chemical may be hazardous to workers and the environment; additionally, it may be capable of forming organochlorine compounds during seed treatment *(3).*

Although seed soaking treatments in chlorinated water have been used by traditional sprout producers, the generation and use of electrolyzed oxidizing (EO) water has been applied to various fields ranging from agriculture *(5)* to food sanitation *(17).* Major advantages of using EO water for the inactivation of bacteria are (i) it is produced using

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pure water with no added chemicals, except for low levels of NaCl (0.05 to 0.1%), and (ii) there is no need for the handling and storing of potentially dangerous chemicals. Therefore, it has a less harmful effect on the environment, which is an increasingly important concern according to a review by Kroyer *(14).* Detailed descriptions of EO water generation and properties are provided by Kim et al. *(12).*

Recently, the effectiveness of EO water in inactivating *E. coli* O157:H7 and *Listeria monocytogenes* on lettuce *(17)* and the inactivation of *E. coli* O157:H7, *L. monocytogenes, Salmonella,* and *Bacillus cereus* by EO water were reported *(13, 25).* Park et al. *(17)* reported that a 3-min treatment with EO water could reduce populations of *E. coli* O157:H7 and *L. monocytogenes* on lettuce by 2.4 and 2.7 $\log_{10}$ CFU/g, respectively.

The effectiveness of EO water against *Salmonella* on alfalfa seeds and sprouts has not been reported. The present study was undertaken to compare the efficacy of EO water and other chemicals in inactivating *Salmonella* and non-*Salmonella* microflora commonly found on alfalfa seeds and sprouts. Additional applications of sonication and seed coat removal to enhance the effectiveness of the EO water treatment as well as the effects of different treatments on alfalfa seed germination rate were also investigated.

MATERIALS AND METHODS

Strains and preparation of inoculum. Five *Salmonella* serotypes isolated from outbreaks of salmonellosis associated with raw vegetables were used in this study: *Salmonella* Montevideo (G4639), from a tomato-associated outbreak, and *Salmonella* Infantis (H3517), *Salmonella* Anatum (H3536), *Salmonella* Cubana (H7976), and *Salmonella* Stanley (H1256), all from alfalfa sprout–associated outbreaks. All serotypes were obtained from the Center for Food Safety, University of Georgia Agricultural Experiment Station (Griffin, Ga.). All serotypes were grown in tryptic soy broth (TSB; Difco, Sparks, Md.) at 37°C and were transferred three times at 24-h intervals. Twenty-four-hour cultures of *Salmonella* were then centrifuged for 10 min at maximum speed (1,800 × *g,* 24°C) in a clinical centrifuge (Model IEC 809, International Equipment Co., Boston, Mass.). The pellets were resuspended in 5 ml of 0.1% (wt/vol) sterile peptone water (Difco), centrifuged at 1,800 × *g* for 10 min, and then resuspended in 5 ml of 0.1% sterile peptone water. An equal volume (2 ml) of five cultures was mixed to obtain an inoculum containing approximately 10^9 CFU/ml of *Salmonella.*

Inoculation of alfalfa seeds with pathogens. Ten-milliliter mixtures of 24-h TSB cultures of the five bacterial strain mixtures (2 ml per each bacterial strain) were combined with 1 liter of 0.1% sterile peptone water (24 ± 2°C) and mixed gently for 30 s at 100 rpm. One kilogram of Australian nonscarified alfalfa seeds obtained from a commercial seed supplier (International Specialty Supply Co., Cookeville, Tenn.) was added to the diluted cell suspension and gently stirred at 100 rpm for 1 min (24 ± 2°C). The seeds were separated from the cell suspension by pouring the mixture over a double layer of sterile cheesecloth supported by a wire screen under a laminar flow hood. The seeds were then spread in a single layer and allowed to dry for 72 h under the hood at 24 ± 2°C. After drying, 10-g quantities of seeds per bag were sealed in stomacher bags and stored at 4°C until used. After storing seeds at 4°C for 2 days, three 10-g samples were analyzed to obtain initial populations of *Salmonella* on seeds. To each bag, 90 ml of the neutralizing buffer solution (5.2 g/liter, neutralizing buffer, Difco) containing a mixture of 0.0043% monopotassium phosphate, 0.016% sodium thiosulfate, and 0.5% aryl sulfonate complex was added prior to stomaching for 60 s at medium speed. The neutralizing buffer solution was then serially diluted in 0.1% sterile peptone water and surface plated in duplicate (0.1 ml) on xylose-lysine-deoxycholate agar (XLD; BBL, Becton Dickinson, Cockeysville, Md.). After incubating at 37°C for 24 h, black colonies were counted.

Preparation of treatment solutions. The following chemical treatments were evaluated for their efficacy in inactivating *Salmonella* on alfalfa seeds: EO water (produced from an ROX-20TA EO water generator with 0.1% NaCl solution at a setting of 20.0 ± 0.2 A, Hoshizaki Electric Inc., Toyoake, Aichi, Japan), chlorine water (as Cl_2, certified for biological work, Fisher Scientific Co., Fair Lawn, N.J.) with an active chlorine concentration similar to the EO water, 90 mg/liter of active chlorine (as $Ca(OCl)_2$, certified for biological work, Fisher) solution, 20,000 mg/liter of active chlorine (as $Ca(OCl)_2$) solution, and deionized water as a control. All chemical treatment solutions were prepared within 30 min of use. Concentrations of chlorine in EO water, chlorine water, and $Ca(OCl)_2$ solutions were determined by total chlorine test kits (Hach Co., Loveland, Colo.). For each treatment solution, pH and oxidation-reduction potential were monitored after preparation with a pH/ion/conductivity meter (Accumet Model 50, Fisher) with a pH electrode (Combo pH Sealed Electrode, A. Daigger & Co., Lincolnshire, Ill.) and an oxidation-reduction potential electrode (Platinum AG/AGCI Combination Electrode with a BNC connector, Fisher). The temperature of treatment solutions and seeds was 24 ± 2°C when treatments were applied.

Chemical treatment of seeds. A 10-g set of inoculated seeds was treated with 90 ml of the chemical treatment solutions or deionized water (control) for 10 min on a platform shaker (Model C10, New Brunswick Scientific Inc., Edison, N.J.) at 100 rpm. A separate study was conducted to investigate the effect of soaking seeds in different chemical solutions for the inactivation of microorganisms. Ten grams of inoculated seeds was soaked for 3 h in 90 ml of different chemical treatment solutions. After soaking, the soaking solution was decanted, and the seeds were treated for 10 min with 90 ml of the same kind of treatment solution as that used for soaking. After the predefined treatment time, the treatment solution was decanted, and 90 ml of the neutralizing buffer solution was added before stomaching the seeds for 60 s at medium speed. The neutralizing buffer solution was then serially diluted (1:10) in 0.1% sterile peptone water, and appropriate diluents were surface plated in duplicate (0.1 ml) on XLD agar for *Salmonella* as described above. Plates were incubated for 24 h at 37°C before colonies were counted. For enrichment, a volume of 1 ml of each sample solution after treatment was also transferred to 10 ml of selenite cystine broth (Difco) and incubated at 37°C for 24 h. After the enrichment procedure, selenite cystine broth was surface plated in duplicate (0.1 ml) on XLD agar in an attempt to detect the presence of low numbers of *Salmonella* that had not been detected by previous direct plating. Since *Salmonella* shows red colonies with black centers on XLD medium incorporating lysine in xylose, we designated the red colonies with black centers "*Salmonella* counts" and other yellow and opaque colonies "non-*Salmonella* microflora" in this study. The whole-seed treatment experiment was replicated three times.

Determination of seed germination percent. Approximately 100 treated or control seeds were placed on sterile deionized water-saturated no. 1 filter paper (90 mm in diameter, Whatman,

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TABLE 1. *Populations of* Salmonella *on alfalfa seeds after treatment with various sanitizer treatments*[a]

Treatment (10 min)	Chlorine (mg/liter)	ORP (mV)	pH	Surviving population ($\log_{10}$ CFU/g)	% germination
Deionized water	0	240	5.2	4.18 A[b]	91
Chlorine water	84	1,074	2.6	3.05 B	91
EO water	84	1,081	2.4	3.09 B	91
$Ca(OCl)_2$	90	632	9.0	3.26 B	92
	20,000	576	11.3	NE C	94

[a] ORP, oxidation-reduction potential; NE, negative by an enrichment procedure and no detectable survivors by a direct plating procedure.
[b] Values with the same letter are not significantly different ($P > 0.05$); the initial population on alfalfa seeds was 4.75 $\log_{10}$ CFU/g.

Maidstone, UK) in a plastic 90-mm-diameter petri dish. The petri dish with the alfalfa seeds was then placed in a plastic Ziplock bag in the dark at 24 ± 2°C for 3 days, with 12-h periodic applications of 10 to 15 ml of sterile deionized water. The number of germinated seeds was counted, and the percent that germinated was calculated.

Seed sprouting. Each 10-g set of seeds was presoaked by immersion into each 90-ml chemical treatment solution or deionized water (control) on a platform shaker at 100 rpm for 3 h. After the presoak, seeds were drained over a double layer of sterile cheesecloth and aseptically transferred into plastic boxes (30 by 16.5 by 10 cm) with drainage holes in the bottom. Seeds were spread evenly on sterile deionized water-saturated Whatman no. 1 filter paper in plastic boxes that were covered with a sheet of perforated plastic bag and stored in a dark incubator at 24 ± 2°C to germinate. Every 12 h, 10 to 15 ml of sterile deionized water was applied. After 48 h, boxes with germinated seeds were placed under fluorescent light for an additional 144 h. Every 12 h, 10 to 15 ml of sterile deionized water was evenly sprayed onto the surface of the sprouts. Mature sprouts (144 h after germination) were removed from the sprouting chamber.

Procedures for treating sprouts and microbiological analysis. Each 10-g set of sprouts from seeds soaked in sterile deionized water for 3 h was treated for 10 min with 90 ml of sterile deionized water, EO water, chlorine water with active chlorine concentration similar to the EO water, and $Ca(OCl)_2$ with concentrations of 90 and 20,000 mg/liter of active chlorine. Another 10-g set of sprouts from seeds, which had been soaked in different chemical treatment solutions for 3 h, was treated with 90 ml of the same kind of treatment solution for another 10 min. After treatment, drained sprouts were combined with 90 ml of the neutralizing buffer solution in a stomaching bag and pummeled for 60 s at medium speed with a stomacher (Model 400, Dynatech Laboratories Inc., Alexandria, Va.). The buffer solution was then serially diluted (1:10) in 0.1% sterile peptone water, and appropriate diluents were surface plated in duplicate (0.1 ml) on XLD agar for the detection of both *Salmonella* and non-*Salmonella* microflora. Although XLD agar is a *Salmonella*-selective medium, it also supports the growth of non-*Salmonella* microflora. Microbial enrichment for the samples after treatment was performed by the enrichment procedure described previously.

Additional treatment for eliminating *Salmonella* on sprouts. Since the main focus of the current study was to evaluate the effectiveness of EO water for the inactivation of *Salmonella* on alfalfa seeds and sprouts, only EO water was used with other additional treatments. To investigate sonication treatments to inactivate microorganisms on alfalfa sprouts, each 10-g set of sprouts from seeds soaked in sterile deionized water during germination was treated with 90 ml of sterile deionized water (control), EO water, or EO water with the application of sonication (Model FS60, 44 to 48 kHz, Fisher) for 10 min. To evaluate potential harborage of microorganisms in or on the seed coat during treatment, 90 ml of each treatment solution was also applied to 10 g of sprouts with the seed coat removed by sterile tweezers after sprouting.

Data analysis. Three replicate trials for each treatment were conducted. Data from each treatment were subjected to SAS *(21)* for analysis of variance and Duncan's multiple range tests to determine the effect of sanitizer and additional treatments (sonication and seed coat removal) on the survival of bacteria on alfalfa seeds/sprouts. All of the statistical analysis was done at the 0.05 level of significance.

RESULTS AND DISCUSSION

The efficacy of EO water, chlorine water having active chlorine closely matching EO water, and $Ca(OCl)_2$ solutions with 90 and 20,000 mg/liter of active chlorine in reducing the populations of *Salmonella* on alfalfa seeds is shown in Table 1. No detectable *Salmonella* was found on the alfalfa seeds purchased from the supplier. Treatments of seeds with EO water, chlorine water, and 90 mg/liter of active chlorine from a $Ca(OCl)_2$ solution for 10 min reduced the populations of *Salmonella* on the seeds by at least 1.5 $\log_{10}$ CFU/g, except for the 20,000-mg/liter active chlorine treatment from $Ca(OCl)_2$, which eliminated the pathogen from three replicates as determined by the direct plating of peptone wash water. None of the treatments demonstrably affected the rate of germination of alfalfa seeds after treatment (Table 1).

Our results are in agreement with those of Beuchat et al. *(4),* who showed that treatment with 200 mg/liter of active chlorine for 15 min resulted in a 1.7-$\log_{10}$ CFU/g reduction in populations of *Salmonella* on alfalfa seeds. Results of experiments evaluating the application of 20,000 mg/liter of active chlorine from $Ca(OCl)_2$ likewise agreed with those of Taormina and Beuchat *(23)* (i.e., the application of 20,000 mg/liter of active chlorine [as $Ca(OCl)_2$] for 10 min completely eliminated *Salmonella* on alfalfa seeds). However, in another study, Weissinger and Beuchat *(26)* observed that 20,000 mg/liter of active chlorine derived from $Ca(OCl)_2$ failed to completely eliminate *Salmonella* on seeds after a 10-min treatment. These authors reported that differences in the efficacy of some of the tested sanitizers such as Tsunami, NaOCl, $Ca(OCl)_2$, and H_2O_2 in killing *Salmonella* on alfalfa seeds are likely the result of differences in seed cultivars, hardness of the seed coat,

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TABLE 2. *Populations of* Salmonella *and non-*Salmonella *microflora on alfalfa seeds after a soaking treatment with different sanitizers, followed by another 10-min treatment in the same chemical*[a]

Soaking treatment (3 h)	Chlorine (mg/liter)	ORP (mV)	pH	Populations (log_{10} CFU/g)		% germination
				Salmonella	Non-*Salmonella* microflora	
Deionized water	0	240	5.2	4.12 A[b]	6.28 AB	92.3 A
Chlorine water	84	1,074	2.6	3.61 B	6.61 A	90.4 A
EO water	84	1,081	2.4	3.51 B	5.88 B	96.3 A
$Ca(OCl)_2$	90	632	9.0	4.04 A	6.48 A	89.8 A
	20,000	576	11.3	NE C	NE C	70.7 B

[a] ORP, oxidation-reduction potential; NE, negative by an enrichment procedure and no detectable survivors by a direct plating procedure.
[b] Values with the same letter in the same column are not significantly different ($P > 0.05$); initial populations were 4.61 log_{10} CFU/g for *Salmonella* and 6.99 log_{10} CFU/g for non-*Salmonella* microflora.

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and seed coat damage, as well as the type and amount of organic material surrounding the target cells.

Because soaking seeds in chlorine solutions has been used by sprout producers as an intervention strategy, a 3-h soaking of seeds in different chemical solutions was also evaluated. The results of an additional 10-min chemical treatment after a 3-h soaking of seeds in EO water, chlorine water, and $Ca(OCl)_2$ solutions with 90 and 20,000 mg/liter of active chlorine are presented in Table 2. Treating seeds soaked for 3 h with chlorine water and EO water for an additional 10 min with fresh solutions significantly reduced populations of *Salmonella* by 1.0 and 1.1 log_{10} CFU/g, respectively; treatment with 90 mg/liter of $Ca(OCl)_2$ resulted in only a 0.6-log_{10} CFU/g reduction. These results are in agreement with those of Jaquette et al. *(11),* who showed that treating alfalfa seeds with 100 or 290 mg/liter of chlorine reduced populations of *Salmonella* Stanley by 0.3 or 0.6 log_{10} CFU/g, respectively, and that treatment with 1,010 mg/liter of chlorine resulted in a 1-log_{10} CFU/g reduction.

After a 3-h soaking plus an additional 10-min treatment with fresh chemical solutions, the populations of *Salmonella* in both treated and untreated seeds (Table 2) were not significantly different from the corresponding seeds treated for 10 min (Table 1), except for the 90 mg/liter of active chlorine derived from $Ca(OCl)_2$ treatment and the chlorine water treatment, for which the populations of *Salmonella* were 0.8 and 0.6 log_{10} CFU/g higher, respectively (data not shown). In another study, Taormina and Beuchat *(23)* reported that populations of microorganisms recovered from seeds after a 1-h soak in sterile tap water were not significantly different from the initial population. However, other researchers *(22, 23)* reported that bacterial populations recovered from soaked seeds were greater and sometimes significantly higher than for the initial population of seeds. This can be attributed to the seeds imbibing water and releasing cells previously trapped in the cracks and crevices of the seeds. This also may be due to seed coats being softened and organic materials around seeds being washed off during the soaking treatment.

Even though no detectable *Salmonella* was found on the alfalfa seeds purchased from the supplier, the population of non-*Salmonella* microflora on the seeds was 5.26 log_{10} CFU/g as determined by plating on XLD agar. This result is similar to that reported by others, in which populations of non-*Salmonella* microflora ranged from 4.79 to 6.60 log_{10} CFU/g *(20).* Soylemez et al. *(22)* stated that microorganism populations on seeds varied because of differences in seed production, storage, and handling. The Food and Drug Administration *(9)* also reported that seeds could potentially become contaminated with microorganisms from animals housed near the production facility, manure fertilizer, contaminated water, and inadequate employee hygiene. Although a 10-min application of 20,000 mg/liter of active chlorine from $Ca(OCl)_2$ to seeds after 3 h of soaking reduced the population from an initial 6.99 log_{10} CFU of non-*Salmonella* microflora per g of alfalfa seeds to an undetectable level, treatment with chlorine water or 90 mg/liter of active chlorine from $Ca(OCl)_2$ reduced populations of non-*Salmonella* microflora by only 0.4 or 0.5 log_{10} CFU/g, respectively, and treatment with EO water reduced non-*Salmonella* microflora by 1.11 log_{10} CFU/g (Table 2).

Also listed in Table 2 are the percentages of seeds that successfully germinated after a 3-h soaking treatment, followed by another 10-min chemical treatment. Of the chemical treatments evaluated, except for the 20,000 mg/liter of active chlorine from $Ca(OCl)_2$ treatment, none significantly affected the percentage of seeds that germinated (Table 2). Our results also agreed with those of Taormina and Beuchat *(23),* i.e., at higher concentrations of $Ca(OCl)_2$ treatments, the rate of seed germination was reduced. Beuchat et al. *(4)* and Charkowski et al. *(6)* indicated that the sensitivity of alfalfa seeds to treatment with 20,000 mg/liter of chlorine might differ depending on various factors, e.g., the cultivar and whether the seed was scarified or not. Alfalfa seeds are sometimes scarified by seed suppliers to enhance rapid and uniform germination.

An evaluation of the efficacy of applying various chemicals in reducing the populations of *Salmonella* and non-*Salmonella* microflora on alfalfa sprouts after the sprouting process is presented in Table 3. The initial populations of *Salmonella* and non-*Salmonella* microflora on alfalfa seeds used for sprouting were 4.58 and 5.23 log_{10} CFU/g, respectively. The results presented in Table 3 show that, regardless of seed soaking treatment and treatment on sprouts, only a 20,000-mg/liter active chlorine (as $Ca(OCl)_2$) treatment significantly reduced populations of

TABLE 3. *Populations of* Salmonella *and non-*Salmonella *microflora on alfalfa sprouts after various treatments during the sprouting process*

Soaking treatment (3 h)	Chlorine (mg/liter)	Sprout treatment (10 min)	Populations ($\log_{10}$ CFU/g)[a]	
			Salmonella	Non-*Salmonella* microflora
Deionized water	0	Neutralizing buffer	5.99 A	8.40 AB
		Deionized water	5.39 A	8.07 ABCD
		Chlorine water	5.98 A	7.90 ABCD
		EO water	5.65 A	7.35 D
		90 mg/liter $Ca(OCl)_2$	5.33 A	7.47 D
		20,000 mg/liter $Ca(OCl)_2$	NE[b] B	NE E
Chlorine water	84	Neutralizing buffer	6.09 A	8.30 ABC
		Chlorine water	5.69 A	7.62 CD
EO water	84	Neutralizing buffer	6.24 A	8.31 ABC
		EO water	5.54 A	7.45 D
$Ca(OCl)_2$	90	Neutralizing buffer	6.13 A	8.25 ABC
		90 mg/liter $Ca(OCl)_2$	5.94 A	7.72 BCD
	20,000	Neutralizing buffer	6.01 A	8.55 A
		20,000 mg/liter $Ca(OCl)_2$	NE B	NE E

[a] Values with the same letter in the same column are not significantly different ($P > 0.05$); initial populations of *Salmonella* and non-*Salmonella* microflora on alfalfa seeds used for sprouting were 4.58 and 5.23 $\log_{10}$ CFU/g, respectively.
[b] NE, negative by an enrichment procedure; no detectable survivors by a direct plating procedure.

Salmonella and non-*Salmonella* microflora. In the production of sprouts, seeds are soaked in water and then held at 24 ± 2°C for several days while being intermittently sprayed with water during germination and growth. These factors, together with nutrients from the seeds and sprouts, provide good conditions for bacterial growth during the sprouting process. Even if a single pathogen cell is present on the seeds, it may grow extensively during germination and sprouting. The implications of this growth are clearly illustrated by the observation that although a 20,000-mg/liter active chlorine (as $Ca(OCl)_2$) treatment of seeds reduced *Salmonella* populations to an undetectable level (Table 2), an amount >6 $\log_{10}$ CFU/g of *Salmonella* was still recovered from sprouts generated from these seeds (Table 3). Only a 10-min application of 20,000 mg/liter of $Ca(OCl)_2$ reduced the *Salmonella* population of 5.99 $\log_{10}$ CFU/g (control treatment) to an undetectable level. No other treatment significantly reduced populations of *Salmonella*. The most likely reason for the inability of other treatments to achieve a significant destruction of microflora may be due to the physical protection that is provided for microflora cells from direct contact with aqueous sanitizers because of cracks and crevices in the seeds from which sprouts arise. Fett *(8)* also reported that biofilms could develop on alfalfa sprouts during the sprouting process, further diminishing the potential effectiveness of sanitizers.

Exposing alfalfa sprouts to 20,000 mg/liter of active chlorine derived from $Ca(OCl)_2$ for 10 min reduced populations of non-*Salmonella* microflora from 8.55 $\log_{10}$ CFU/g to an undetectable level. For the sprouts soaked in deionized water for 3 h after a 10-min neutralizing buffer solution treatment, EO water and 90 mg/liter of active chlorine from $Ca(OCl)_2$ reduced populations of non-*Salmonella* microflora by 1.1 and 0.9 $\log_{10}$ CFU/g, respectively, and chlorine water reduced such populations by 0.5 $\log_{10}$ CFU/g (Table 3). For sprouts generated from seeds

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TABLE 4. *Populations of* Salmonella *and non-*Salmonella *microflora on alfalfa sprouts after treatment with EO water in conjunction with sonication and physical removal of seed coats*

Solution	Additional treatments (10 min)		Surviving population ($\log_{10}$ CFU/g)[a]	
	Sonication[b]	Seed coat removal	*Salmonella*	Non-*Salmonella* microflora
Deionized water	No	No	5.27 A	7.56 A
EO water	No	No	4.31 A	7.08 AB
EO water	Yes	No	3.70 AB	6.90 B
Deionized water	No	Yes	4.20 AB	7.10 AB
EO water	No	Yes	2.71 BC	5.74 C
EO water	Yes	Yes	2.00 C	5.54 C

[a] Values with the same letter in the same column are not significantly different ($P > 0.05$); initial populations of *Salmonella* were 5.69 $\log_{10}$ CFU/g for sprouts with seed coats and 4.56 $\log_{10}$ CFU/g for sprouts with seed coats removed; initial populations of non-*Salmonella* microflora were 7.89 $\log_{10}$ CFU/g for sprouts with seed coats and 7.44 $\log_{10}$ CFU/g for sprouts with seed coats removed.
[b] Alfalfa sprouts were treated with electrolyzed oxidizing (EO) water for 10 min with the application of sonication.

soaked for 3 h in different chemical solutions, a 10-min EO water treatment on alfalfa sprouts reduced populations of non-*Salmonella* microflora by 0.9 $\log_{10}$ CFU/g. Other chemical treatments were not as effective as EO water (a 0.7-$\log_{10}$ CFU/g reduction by chlorine water and a 0.5-$\log_{10}$ CFU/g reduction by 90 mg/liter of active chlorine from $Ca(OCl)_2$), except for the 20,000-mg/liter active chlorine from $Ca(OCl)_2$ treatment, which achieved complete elimination.

Weissinger and Beuchat *(26)* reported that the inability of sanitizers to eliminate viable cells of pathogens on alfalfa seeds may be because cells were trapped in cracks or crevices and hence were not exposed directly to active sanitizer components. Another study *(7)* on mung bean seeds demonstrated that bacteria penetrated into the seeds through the stem scar. For the current study, alternative methods were investigated to enhance the bactericidal effect of EO water on alfalfa sprouts. Alfalfa sprouts from the seeds inoculated with *Salmonella* were subjected to EO water treatment along with applications of sonication and physical removal of seed coats from sprouts. Although the populations of *Salmonella* on untreated sprouts were 5.69 $\log_{10}$ CFU/g for sprouts with seed coats and 4.56 $\log_{10}$ CFU/g for sprouts with seed coats removed, the populations of non-*Salmonella* microflora were 7.89 and 7.44 $\log_{10}$ CFU/g. Results suggest that although there was no statistical difference among bacterial populations, seed coat removal alone reduced initial populations of *Salmonella* and non-*Salmonella* microflora by 1.1 and 0.5 $\log_{10}$ CFU/g, respectively (Table 4). Similar results were observed for deionized water–treated sprouts (a control treatment), i.e., populations of *Salmonella* and non-*Salmonella* microflora recovered from the sprouts with seed coats were 1.1 and 0.5 $\log_{10}$ CFU/g higher than the sprouts with seed coats removed.

It is not known how deep *Salmonella* cells penetrate into alfalfa seeds or sprouts during the sprouting process. The differences noted between the effectiveness of bactericidal treatments for *Salmonella* and non-*Salmonella* microflora when seed coat removal and sonication were employed may be due to the ability of non-*Salmonella* microflora to reside longer and more deeply in cracks, crevices, biofilms, or other protected sites. The treatment of alfalfa sprouts with EO water plus the application of sonication reduced populations of *Salmonella* and non-*Salmonella* microflora by 0.6 and 0.2 $\log_{10}$ CFU/g, respectively, compared to EO water alone. In the meantime, the simultaneous application of both sonication and physical removal of seed coats enhanced the effectiveness of the EO water treatment significantly and reduced initial populations of *Salmonella* and non-*Salmonella* microflora by 3.7 and 2.4 $\log_{10}$ CFU/g, respectively (Table 4). The approximate 1.6-$\log_{10}$ CFU/g reduction resulting from treatment in EO water with sonication is similar to that reported by Lillard *(15)* on the bactericidal effect of chlorine on attached *Salmonella* to broiler skin with sonication. She reported that a population of *Salmonella* of 5.2 $\log_{10}$ CFU/12 cm^2 was reduced to 3.1 $\log_{10}$ CFU/12 cm^2 after sonicating a broiler skin for 15 min in chlorine water containing 0.5 mg/liter of chlorine.

In summary, the persistent failure to significantly inactivate bacteria present on alfalfa seeds and sprouts could be due to the inability of the sanitizers to penetrate the seeds or sprouts to reach the *Salmonella*. The ability of EO water to more easily access microflora improved when seed coats attached to the sprouts were removed and when sprouts were subjected to sonication during treatment (reductions in *Salmonella* and non-*Salmonella* microflora were 2.3 and 1.5 $\log_{10}$ CFU/g, respectively, more than for EO water alone). Sonication and removal of seed coats may have detached cells that were attached or entrapped in sprouts, thus making *Salmonella* more susceptible to the EO water. This contributed to the enhanced efficiency of EO water in inactivating bacteria on sprouts.

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