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Title: Efficacy of Ozone in Killing *Listeria Monocytogenes* on Alfalfa Seeds and Sprouts and Effects on Sensory Quality of Sprouts

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Efficacy of Ozone in Killing *Listeria monocytogenes* on Alfalfa Seeds and Sprouts and Effects on Sensory Quality of Sprouts†

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ABSTRACT

A study was done to determine the efficacy of aqueous ozone treatment in killing *Listeria monocytogenes* on inoculated alfalfa seeds and sprouts. Reductions in populations of naturally occurring aerobic microorganisms on sprouts and changes in the sensory quality of sprouts were also determined. The treatment (10 or 20 min) of seeds in water (4°C) containing an initial concentration of 21.8 ± 0.1 μg/ml of ozone failed to cause a significant ($P \leq 0.05$) reduction in populations of *L. monocytogenes*. The continuous sparging of seeds with ozonated water (initial ozone concentration of 21.3 ± 0.2 μg/ml) for 20 min significantly reduced the population by 1.48 $\log_{10}$ CFU/g. The treatment (2 min) of inoculated alfalfa sprouts with water containing 5.0 ± 0.5, 9.0 ± 0.5, or 23.2 ± 1.6 μg/ml of ozone resulted in significant ($P \leq 0.05$) reductions of 0.78, 0.81, and 0.91 $\log_{10}$ CFU/g, respectively, compared to populations detected on sprouts treated with water. Treatments (2 min) with up to 23.3 ± 1.6 μg/ml of ozone did not significantly ($P > 0.05$) reduce populations of aerobic naturally occurring microorganisms. The continuous sparging of sprouts with ozonated water for 5 to 20 min caused significant reductions in *L. monocytogenes* and natural microbiota compared to soaking in water (control) but did not enhance the lethality compared to the sprouts not treated with continuous sparging. The treatment of sprouts with ozonated water (20.0 μg/ml) for 5 or 10 min caused a significant deterioration in the sensory quality during subsequent storage at 4°C for 7 to 11 days. Scanning electron microscopy of uninoculated alfalfa seeds and sprouts showed physical damage, fungal and bacterial growth, and biofilm formation that provide evidence of factors contributing to the difficulty of killing microorganisms by treatment with ozone and other sanitizers.

Outbreaks of *Salmonella* and *Escherichia coli* O157:H7 infections associated with eating seed sprouts have increased in frequency in recent years *(24, 32)*. Alfalfa sprouts have been implicated most often. *Listeria monocytogenes* has been isolated from commercially produced sprouted seeds, but no cases of human listeriosis have been linked to sprouts *(24)*. Pathogens that may be present on seeds or that may contaminate sprouts during production can grow to populations of 10^6 to 10^8 CFU/g on mature sprouts *(14, 19, 31)*. The growth of *Salmonella* and *E. coli* O157:H7 on alfalfa sprouts during production can be detected by their presence in spent irrigation water collected from rinsed sprouts *(9, 29)*, which provides a method for monitoring the safety of sprouts before their release for sale to consumers.

The treatment of seeds intended for sprout production with 20,000 μg/ml of calcium hypochlorite has been recommended to reduce the risk of pathogens in seed sprouts *(24)*. This treatment, however, cannot be relied on to eliminate *Salmonella* and *E. coli* O157:H7 from alfalfa seeds. Although treatment reduces populations, both pathogens may survive, as evidenced by detection and growth on sprouts subsequently produced from treated seeds *(19, 29, 30)*. Numerous other aqueous chemicals, including organic acids *(19, 30, 34)*, alkali *(1–3, 34)*, acidified sodium chlorite *(30, 31)*, and hydrogen peroxide *(30, 34)*, as well as physical treatments *(13, 27, 35)*, are not totally effective in eliminating *Salmonella* and *E. coli* O157:H7 on inoculated alfalfa seeds. Gaseous acetic acid *(6, 34)*, ammonia *(12)*, and volatile compounds naturally occurring in plants *(25, 34)* have been shown to reduce populations but not eliminate pathogens on alfalfa seeds or mung beans.

The treatment of raw fruits and vegetables with aqueous or gaseous ozone has proven effective in killing several naturally occurring microorganisms *(16, 18, 21)*. The lethality of ozone to foodborne pathogens varies, depending on treatment conditions. Several in vitro studies show that treatment with 1 to 1.5 μg/ml of ozone kills up to 10^7 CFU/ml of the suspension *(5, 15, 26, 33)*. The efficacy of ozone in killing pathogens on raw produce is generally less *(17)*, probably because of the inaccessibility of ozone to sites harboring targeted cells.

Recent studies have shown that ozone is relatively in-

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effective in killing *E. coli* O157:H7 on inoculated alfalfa seeds *(28)*. However, the efficacy of ozone in killing *E. coli* O157:H7 and aerobic microorganisms on alfalfa sprouts was not determined. The study reported here was undertaken to determine the effectiveness of aqueous ozone treatment in killing *L. monocytogenes* on alfalfa seeds and sprouts. Reductions in populations of naturally occurring aerobic microorganisms on sprouts and changes in the sensory quality of sprouts were also determined.

MATERIALS AND METHODS

Strains. Five strains of *L. monocytogenes* isolated from raw fruits and vegetables were used in this study. Strain codes, serotypes, and sources, respectively, were F8027 (4b, celery), F8255 (1/2b, peach/plum), F8369 (1/2a, corn), F8385 (1/2b, carrot), and G1091 (4b, coleslaw). All strains were obtained from the Centers for Disease Control and Prevention, Atlanta, Ga.

Preparation of cells for inocula. All strains were grown in tryptose phosphate broth (pH 7.3; BBL/Difco, Sparks, Md.) supplemented with nalidixic acid (50 μg/ml) (TPBN; Sigma Chemical Co., St. Louis, Mo.). Cultures were incubated at 37°C and transferred (one loopful to 10 ml) three times at 24-h intervals immediately preceding use as inocula for alfalfa seeds or sprouts.

Inoculation of alfalfa seeds. Seeds from different lots obtained from two commercial seed suppliers were inoculated and used in some experiments for determining differences in the susceptibility of *L. monocytogenes* to ozone treatment as potentially influenced by seed variety and extent of mechanical damage. Ten milliliters of 24-h TPBN cultures of five strains of *L. monocytogenes* containing approximately equal populations were combined with 1 liter of sterile deionized water (22 ± 1°C) and mixed thoroughly. Alfalfa seeds (1 kg, 22 ± 1°C) were combined with the five-strain cell suspension and gently stirred for 1 min. The seeds were then separated from the inoculum by pouring the mixture over a double layer of cheesecloth supported by a wire screen elevated approximately 5 cm above the work surface of a laminar flow hood. Seeds were spread in a layer approximately 0.5 cm thick and allowed to dry for 48 to 72 h in the hood at 22 ± 1°C. Inoculated, dried seeds were sealed in stomacher 400 bags (Seward Medical, Ltd., London, UK) and stored at 5°C for 1 week. Seeds were then subdivided into 5-g quantities, sealed in plastic bags, and stored at 5°C for up to 3 weeks before use in ozone treatment experiments. Populations of *L. monocytogenes* did not change significantly between 1 and 4 weeks of postinoculation storage at 5°C.

Inoculation of alfalfa sprouts. Cells in 24-h cultures of five strains of *L. monocytogenes* were collected by centrifugation (2,000 × *g*, 15 min). Cells of each strain were resuspended in 5 ml of sterile deionized water, which was followed by combining suspensions of all strains to obtain a mixed-strain inoculum containing approximately equal populations of each strain.

Alfalfa sprouts were purchased from a retail supermarket in Griffin, Ga. One milliliter of inoculum was deposited on either 25 g of sprouts in a stomacher 400 bag (Seward) or 10 g of sprouts in a 500-ml fleaker, depending on use in subsequent experiments. The inoculum was distributed on the surface of sprouts by gently tumbling. Inoculated sprouts were stored at 4°C for 24 h before being subjected to treatment with ozonated water.

Preparation of ozonated water. A laboratory scale ozone generator (Model H-50, Hess Machine International, Ephrata, Pa.) equipped with an oxygen concentrator (Model AS-12, AirSep, Buffalo, N.Y.) was used to produce ozone gas. Gas was delivered through an inlet line and a stainless steel sparger (10-μm pore size) to sterile deionized water (4°C) in 4-liter Erlenmeyer flasks or 500-ml fleakers. Water (2 liters) containing ozone at concentrations up to 23 μg/ml was produced within 60 min using this system. Gaseous ozone released from the water was passed through 2% potassium iodide solution to prevent release into the environment. Except for the ozone generator, the apparatus used to prepare ozonated water, as well as seed and sprout treatments, was a chemical fume hood.

The concentrations of ozone in water used to treat seeds and sprouts were determined by a spectrophotometric method *(10, 11)*. The absorbance of water and ozonated solutions at 258 nm was measured with a spectrophotometer (Genesys 5, Spectronic, Rochester, N.Y.). The formula used to calculate the ozone concentration (in micrograms per milliliter) was $C = A \cdot b/\epsilon$, where A = absorbance at 258 nm, b = length (in centimeters) of the path of light (width of quartz cuvette), and ϵ = molar absorptivity (2,900 M^{-1} cm^{-1}). Substituting values and conversion factors yields the equation $C = (A \times 48{,}000)/2{,}900$.

Treatment of seeds with ozonated water. Water (80 ml, 4°C) containing 0 or 21.9 ± 0.2 μg/ml of ozone was combined with 5.0 g of inoculated alfalfa seeds in a stomacher 80 bag. The bag was sealed, placed on a platform shaker, and agitated at 22 ± 1°C for 10 or 20 min. The ozonated water was decanted, and 20 ml of Dey-Engley neutralizer broth (DE broth) (BBL/Difco) was added to the seeds. The seed and broth mixture was pummelled in a stomacher at medium speed for 1 min. DE wash broth was analyzed for populations of *L. monocytogenes*.

A second experiment was performed in which inoculated seeds were treated by continuous sparging with ozonated water or immersed with gentle agitation in sterile deionized water (control) for 5, 10, or 20 min. Seeds (5 g) and sterile deionized water (80 ml) at 4°C were placed in a stomacher 80 bag. The stainless steel sparger was immersed in the water, and gaseous ozone was purged (0.34 m^3/h) through the seed and water mixture for up to 20 min. After treatment, ozonated water or deionized water was decanted, 20 ml of DE broth was added to the seeds, and the mixture was pummelled in a stomacher for 1 min. After treatment and pummelling, ozonated water, deionized soak water, and DE wash broth were analyzed for populations of *L. monocytogenes*.

Treatment of alfalfa sprouts with ozonated water. Inoculated sprouts (25 g) in a stomacher 400 bag were combined with 100 ml of sterile deionized water (4°C) containing 0, 5.0 ± 0.5, 9.0 ± 0.5, or 23.3 ± 1.6 μg/ml of ozone. The mixture was gently agitated for 2 min before water or ozonated water was decanted; 100 ml of DE broth was immediately added to the bag containing the sprouts, and the mixture was pummelled for 2 min. Stomachates were analyzed for populations of *L. monocytogenes* and aerobic microorganisms. Inoculated sprouts not subjected to water or ozone treatment for 2 min, but washed with water or DE broth for 2 min, were analyzed for populations of *L. monocytogenes*. This was done to determine if washing sprouts with DE broth had an effect on the number of *L. monocytogenes* recovered from washed sprouts.

Inoculated sprouts were also treated by continuous sparging with ozonated water. Water (200 ml) at 4°C was added to a 500-ml fleaker containing 10 g of inoculated sprouts. A stainless steel sparger connected to an inlet line from the ozone generator was positioned in the bottom of the fleaker to introduce gaseous ozone (0.34 m^3/h). Sprouts were treated with continuously sparged ozonated water or immersed in deionized water (control), with gentle agitation, for 5, 10, or 20 min. Treatment water was decanted, and

TABLE 1. *Populations of* L. monocytogenes *recovered from inoculated alfalfa seeds treated with ozone*

Source of seeds	Treatment		Population	
	Medium	Time (min)	($\log_{10}$ CFU/g)[a]	Change[b]
Supplier 1	Water	0	2.27 B	
		10	2.06 B	−0.21
		20	2.59 A	+0.32
	Ozone[c]	0	2.08 B	
		10	1.82 B	−0.26
		20	2.58 A	+0.50
Supplier 2	Water	0	2.65 B	
		10	2.72 B	+0.07
		20	3.24 A	+0.50
	Ozone[c]	0	2.65 B	
		10	2.38 B	−0.27
		20	3.37 A	+0.72

[a] Within the same seed supplier and treatment medium, means with different letters are significantly different ($P \leq 0.05$).
[b] Within the same seed supplier and treatment medium, a decrease (−) or an increase (+) in population ($\log_{10}$ CFU/g) compared to the control (0-min treatment).
[c] Seeds (5 g) were immersed in 80 ml of deionized water (4°C) initially containing 21.8 ± 0.1 μg/ml of ozone and were agitated without sparging with ozone for 10 or 20 min.

TABLE 2. *Populations of* L. monocytogenes *recovered from inoculated alfalfa seeds soaked in water or continuously sparged with ozone*[a]

Treatment		Population		
		Treatment medium	Seeds	
Medium	Time (min)	($\log_{10}$ CFU/ml)[b]	Log_{10} CFU/g[b]	Change ($\log_{10}$ CFU/g)[c]
Water	0		2.99 B	
	5	0.82 B	2.47 B	−0.52
	10	1.22 AB	3.04 B	+0.05
	20	1.66 A	4.80 A	+1.81
Ozone	0		2.99 A	
	5	0 A	0.60 B	−2.39
	10	0 A	1.03 B	−1.96
	20	0 A	1.51 B	−1.48

[a] Seeds (5 g, supplier 2) were immersed in 80 ml of deionized water (4°C) containing 21.3 ± 0.2 μg/ml of ozone and were continuously sparged with ozone for up to 20 min.
[b] Within the same treatment medium, means with different letters in the same column are significantly different ($P \leq 0.05$).
[c] Within the same treatment medium, a decrease (−) or an increase (+) in population ($\log_{10}$ CFU/g) compared to the control (0-min treatment).

200 ml of DE broth was immediately added to the sprouts. The mixture was pummelled in a stomacher for 2 min at medium speed. Ozonated treatment water, deionized soak water, and the sprout/DE broth stomachate were analyzed for populations of aerobic microorganisms and *L. monocytogenes.*

Microbiological analyses. Populations of *L. monocytogenes* in 24-h cultures, mixed-strain inocula used to inoculate seeds and sprouts, water and DE broth used to wash seeds and sprouts, and sprout/DE stomachates were determined by surface plating undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) and duplicate samples (0.1 ml) serially diluted in sterile 0.1% peptone on modified Oxford medium (Oxoid, Basingstoke, UK) supplemented with nalidixic acid (50 μg/ml). Plates were incubated at 37°C for 48 ± 2 h before presumptive colonies were counted. Two or three colonies from each sample were randomly picked and subjected to confirmation with API *Listeria* assay strips (BioMérieux, Hazelwood, Mo.).

Populations of aerobic microorganisms in wash solutions and stomachates in selected experiments using sprouts were determined by surface plating serially diluted samples (0.1 ml in duplicate) on plate count agar (BBL/Difco). Plates were incubated at 30°C for 48 h before colonies were counted.

Scanning electron microscopy. Alfalfa seeds and sprouts were obtained from sources different from those from which seeds and sprouts were obtained for the ozone treatment studies. Specimens were fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 50 mM 1,4-piperazinediethanesulfonic acid buffer (PIPES, pH 7.4) for 2 h at 22 ± 1°C, followed by 16 to 18 h at 4°C. Specimens were then rinsed in 50 mM PIPES buffer and postfixed in 2% osmium tetroxide in 50 mM PIPES buffer at 4°C for 20 to 24 h. This was followed by rinsing specimens twice in 50 mM PIPES buffer at 4°C and four times in distilled water at 22 ± 1°C. Specimens were dehydrated in ethanol by 10% (ethanol/water) increments (15 min each on a rotator) to 100% ethanol and rinsed three times with molecular-sieve-dried ethanol. Critical point drying was carried out in a Polaron Critical Point Dryer (Quarum Technologies, New Haven, East Sussex, UK) using ethanol as a transitional solvent to carbon dioxide. Specimens were mounted on aluminum mounts with contact adhesive pads and sputter coated in a Polaron E5100 Series II sputter coater (Quarum) for 2 min at 2.2 kV, 0.1 torr (13-mA current). Samples were observed and photographed with a JEOL JSM-S400 scanning electron microscope (JEOL USA, Peabody, Mass.).

Percentage of germination. Untreated and treated seeds were analyzed for their ability to germinate. Approximately 100 control or treated seeds were placed between two pieces of water-saturated 90-mm-diameter Whatman no. 4 filter paper (Whatman International Ltd., Maidstone, UK) in a plastic 90-mm-diameter petri dish and placed in the dark at 25°C for 72 h, with periodic applications of water. The number of germinated seeds was counted, and the percent that germinated was calculated.

Sensory analysis of sprouts. Fifty grams of uninoculated sprouts was placed in a 1-liter fleaker and stored at 4°C for 24 h. Sterile deionized water (800 ml, 4°C) containing 20 μg/ml of ozone was poured into the fleaker, and this was followed by continuous sparging with ozone for 5 or 10 min. Controls consisted of combining 50 g of sprouts and 800 ml of sterile deionized water, which was followed by immediately decanting the water or allowing the sprouts to soak with gentle agitation for 5 or 10 min. Treated and control samples were stored in sealed plastic Zip-lock (S. C. Johnson and Sons, Inc., Racine, Wis.) bags at 4°C for 0 (within 30 min), 3, 7, or 11 days before being subjected to sensory analysis.

Thirteen technicians and graduate students in the Center for Food Safety and Department of Food Science and Technology at the University of Georgia served on a panel to subjectively evaluate untreated and ozone-treated sprouts for appearance, color, aroma, and overall quality. Prior to evaluation, panelists attended a 30-min presentation describing the sensory qualities of alfalfa sprouts. On each day of analysis, five 10-g samples (three control

TABLE 3. *Populations of aerobic microorganisms and* L. monocytogenes *recovered from untreated and ozonated alfalfa sprouts*

Treatment	Ozone concn. (μg/ml)[a]	Population: Aerobic microorganisms: Log_{10} CFU/g[b]	Aerobic microorganisms: Change[c]	*L. monocytogenes*: Log_{10} CFU/g[b]	*L. monocytogenes*: Decrease[c]
None					
Water wash		9.93 A		7.67 A	
DE broth wash		9.78 A		7.59 A	
Ozone (2 min)	0	9.41 A		6.95 A	
	5.0 ± 0.5	9.29 A	−0.12	6.17 B	−0.78
	9.0 ± 0.5	9.61 A	+0.20	6.14 B	−0.81
	23.3 ± 1.6	9.29 A	−0.12	6.04 B	−0.91

[a] Initial concentration in 100 ml of deionized water used to treat 25 g of sprouts inoculated with *L. monocytogenes* for 2 min.
[b] Within treatments (none or ozone) and within microorganisms (aerobic microorganisms or *L. monocytogenes*), means with different letters are significantly different ($P \leq 0.05$).
[c] A decrease (−) or an increase (+) in population (log_{10} CFU/g) compared to the control (0 μg/ml of ozone).

and two treated) at 4°C were placed on white 10-cm-diameter Styrofoam plates coded with random three-digit numbers. Plates were placed approximately 40 cm apart on a black laboratory bench. Panelists were instructed to examine the sprouts for appearance, color, aroma, and overall quality using a nine-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely) and to record their ratings accordingly.

Statistical analysis. All experiments were replicated three times. Data were subjected to the Statistical Analysis System (SAS Institute, Cary, N.C.) for analysis of variance and to Duncan's multiple range tests to determine statistical differences ($P \leq 0.05$) in populations of *L. monocytogenes* detected on alfalfa seeds or sprouts subjected to various treatments. Mean ratings for sensory qualities of untreated and treated sprouts were also analyzed to determine if differences were significant.

RESULTS AND DISCUSSION

A previous study *(28)* showed that ozone treatments of alfalfa seeds inoculated with *E. coli* O157:H7 were minimally effective in killing the pathogen. Less than 2 log_{10} CFU/g was killed on seeds treated with water containing an initial concentration of 21 μg/ml of ozone for up to 64 min. Populations of *L. monocytogenes* recovered from inoculated alfalfa seeds treated with water initially containing 21.8 ± 0.1 μg/ml of ozone for up to 20 min are shown in Table 1. Tests were performed with two different lots of seeds obtained from two suppliers to determine the potential influence of seeds from the two different lots on the efficacy of ozone as a sanitizer. Treatments with ozone did not significantly ($P > 0.05$) reduce the population of *L. monocytogenes,* regardless of the time of treatment (10 or 20 min) or the source of seeds. To the contrary, the treatment of seeds for 20 min with water containing 0 or 21.8 ± 0.1 μg/ml of ozone significantly ($P \leq 0.05$) increased the number of *L. monocytogenes* recovered compared to populations recovered from seeds treated for 0 or 10 min. The slight reduction (0.21 to 0.27 log_{10} CFU/g) during the first 10 min of treatment is attributed to the death of a portion of *L. monocytogenes* on the surface of the seeds. Apparently, the concentration of ozone is reduced to an ineffective level at some point during the 20-min treatment. Subsequently, soaking apparently facilitates the release of viable *L. monocytogenes* from the surface and, particularly,

TABLE 4. *Populations of aerobic microorganisms and* L. monocytogenes *recovered from alfalfa sprouts soaked in water or continuously sparged with ozone*[a]

Treatment: Medium	Treatment: Time (min)	Population: Aerobic microorganisms: Treatment medium (log_{10} CFU/ml)[b]	Aerobic microorganisms: Sprouts (log_{10} CFU/g)[b]	Aerobic microorganisms: Decrease[c]	*L. monocytogenes*: Treatment medium (log_{10} CFU/ml)	*L. monocytogenes*: Sprouts (log_{10} CFU/g)	*L. monocytogenes*: Decrease
Water	5	7.33 AB	8.44 A		6.64 A	6.74 A	
	10	7.30 B	8.43 A		6.65 A	6.64 A	
	20	7.37 A	8.42 A		6.70 A	6.63 A	
Ozone	5	0 C	7.89 B	−0.55	0 B	6.09 B	−0.65
	10	0 C	7.83 B	−0.60	0 B	5.86 B	−0.78
	20	0 C	6.74 B	−1.68	0 B	5.69 B	−0.94

[a] Sprouts (10 g) were immersed in 200 ml of deionized water containing 19.0 ± 1.7 μg/ml of ozone and were continuously sparged with ozone for up to 20 min.
[b] Means with different letters in the same column are significantly different ($P \leq 0.05$).
[c] A decrease in population (log_{10} CFU/g) compared to treatment in water for the same length of time (5, 10, 20 min).

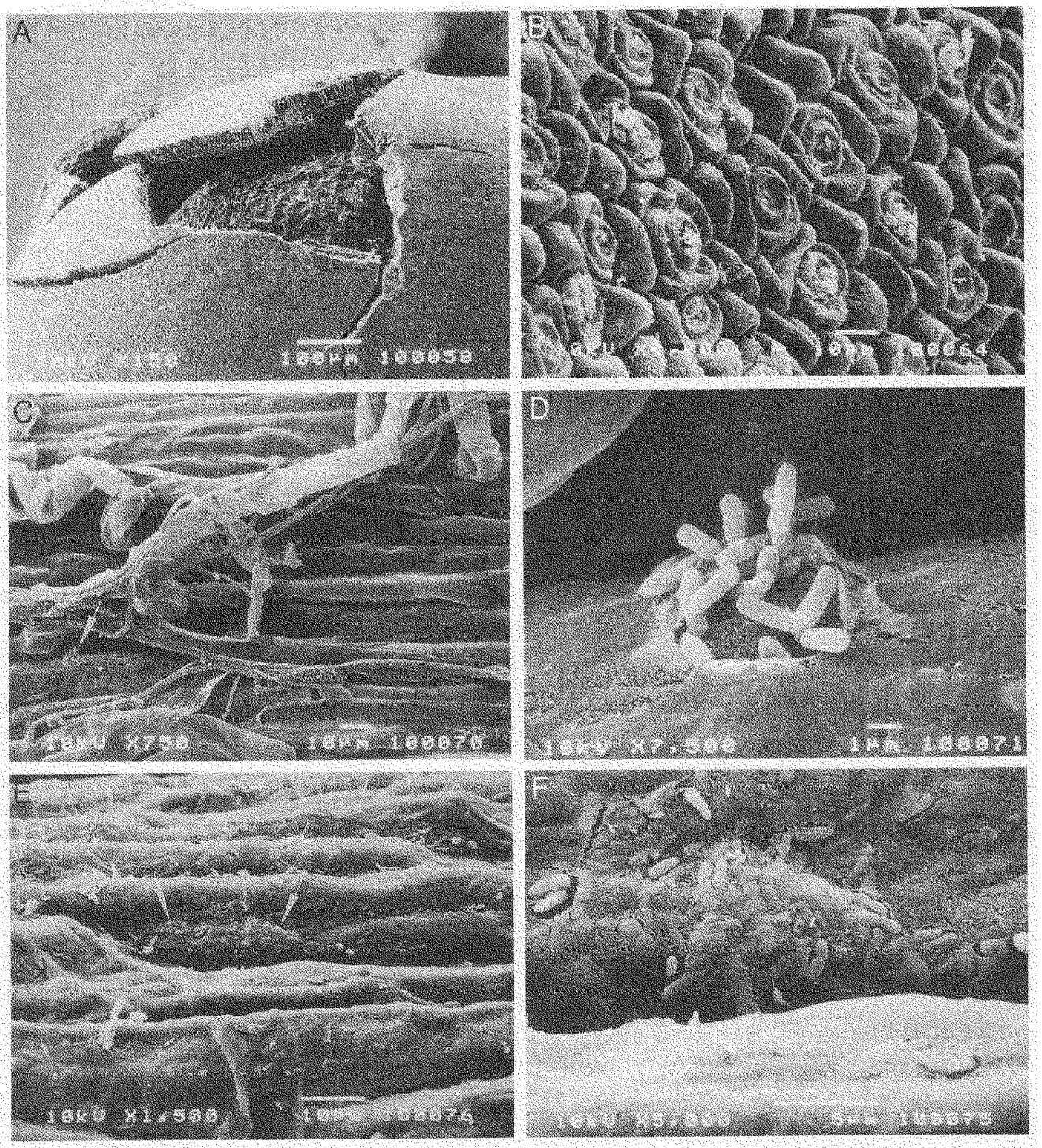

FIGURE 1. *Scanning electron photomicrographs of alfalfa seeds and sprouts. (A) Seeds with cracked testa showing cotyledon underneath; (B) surface of cotyledon with stomata; (C) root hair zone of sprout showing fungal hyphae and bacterial colony (arrow); (D) magnified view of bacterial colony shown in (C); (E) area on sprout several millimeters from the root tip showing bacterial cells and biofilm (arrows); (F) magnified view of biofilm shown in (E). Bars represent 1 μm (D), 5 μm (F), 10 μm (B, C, and E), and 100 μm (A).*

the subsurface areas, resulting in significant increases in the number of *L. monocytogenes* recovered after treatment for 20 min compared to 10 min. The concentration of ozone in treatment water was not measured during the 20-min treatment because of the interference of soluble and insoluble materials released during treatment with optical density readings. The germination percentages of seeds subjected to treatments shown in Table 1 ranged from 97.3 to 99.0% and were not significantly affected by ozone treatment. This concurs with general observations from a study reported by Sharma et al. *(28)* in which the germination percentage o alfalfa seeds obtained from a third source was not signifi cantly affected by treatment with water initially containing 21 μg/ml of ozone for 8, 16, or 64 min.

The next series of studies was designed to maintai ozone at a higher concentration during the treatment of in oculated seeds for 10 or 20 min. The initial ozone concen tration in water in which seeds were immersed was 21.3 ± 0.2 μg/ml. Gaseous ozone was continuously sparge through the seed-in-water mixture for up to 20 min. Pop

ulations of *L. monocytogenes* recovered from seeds are shown in Table 2. The treatment of seeds for 20 min in water containing no ozone significantly ($P \leq 0.05$) increased the number of *L. monocytogenes* recovered compared to treatments for 10 min or less. Treatments of seeds in water initially containing 21.3 ± 0.2 μg/ml of ozone and continuously sparged with ozone for 5, 10, or 20 min caused significant reductions in populations compared to the population recovered from untreated (0 min) seeds. Although the number of *L. monocytogenes* recovered from seeds treated 5, 10, or 20 min was not significantly different ($P > 0.05$), the highest reduction (2.39 $\log_{10}$ CFU/g) compared to the control (0 min) was in seeds treated for 5 min. This reduction was reduced to 1.96 and 1.48 $\log_{10}$ CFU/g after treatment for 10 and 20 min, respectively. This suggests that although the concentration of ozone in the continuously sparged water was sufficient to kill *L. monocytogenes* on the surface of seeds or on those released during sparging, increased soak times enhanced the release of viable *L. monocytogenes* from subsurface areas during post-treatment pummelling in DE broth. This observation, as well as observations from experiments in which seeds were not continuously sparged with ozone, indicates that prolonged soaking in water, with or without ozone but with alternate rinsing, may contribute to a substantial release of *L. monocytogenes.* The continuous sparging of seeds with ozone for up to 20 min did not significantly reduce the percentage of germination.

The treatment of alfalfa seeds with exceptionally high concentrations (21 to 22 μg/ml) of ozone, even using a continuous sparging system, proved ineffective in killing more than ca. 1.48 $\log_{10}$ CFU of *L. monocytogenes* per g. Compared to the 20-min control (water) treatment, continuous sparging with ozone for 20 min resulted in an additional reduction of 3.29 $\log_{10}$ CFU/g. This is in contrast to observations by others reporting reductions of 7 $\log_{10}$ CFU/ml resulting from treatments of cell suspensions with 0.4 μg/ml of ozone for 13 min *(15),* 5.4 $\log_{10}$ CFU/ml with 0.25 μg/ml of ozone for 2 min *(8),* and 3.9 $\log_{10}$ CFU/ml with 1.0 μg/ml of ozone for ≤20 s *(33).* Gaseous ozone (2 μg/ml) reduced the population of *Listeria innocua* on a stainless steel coupon immersed in a one-fourth–strength Ringer solution by ca. 0.11 and 3.1 $\log_{10}$ CFU within 1 and 4 h, respectively *(22).* All of these studies, however, were performed using *Listeria* cell suspensions containing little or no organic material that would neutralize the bactericidal activity of ozone or limit accessibility of ozone to the cells. These inactivation kinetics cannot be applied to the *L. monocytogenes* on the surface or enmeshed in the organic matrix of alfalfa seeds.

The results of studies to determine the efficacy of ozone in killing aerobic microorganisms and *L. monocytogenes* on alfalfa sprouts are shown in Table 3. Sprouts were immersed in deionized water initially containing 0, 5.0 ± 0.5, 9.0 ± 0.5, or 23.3 ± 1.6 μg/ml of ozone for 2 min. Preliminary control treatments were performed to determine if water and DE broth used to wash sprouts have an effect on the number of microorganisms recovered. The type of wash fluid did not have a significant effect on populations of aerobic microorganisms or *L. monocytogenes* detected. The treatment of sprouts with up to 23.3 μg/ml of ozone did not result in a significant reduction in populations of aerobic microorganisms. Treatments with 5.0, 9.0, or 23.3 μg/ml of ozone significantly ($P \leq 0.05$) reduced the population of *L. monocytogenes,* unlike treatments in water, although reductions were ≤0.91 $\log_{10}$ CFU/g.

Populations of aerobic microorganisms and *L. monocytogenes* recovered from alfalfa sprouts soaked in water or continuously sparged in water containing an initial concentration of 19.0 ± 1.7 ppm of ozone for 5, 10, or 20 min are listed in Table 4. Populations of microorganisms recovered from water and ozonated water after the treatment of sprouts are also listed. Large numbers of aerobic microorganisms and *L. monocytogenes* were present in water after washing sprouts and on sprouts after washing. In contrast, no *L. monocytogenes* or aerobic microorganisms (<4 CFU/ml) were detected in ozonated water after treating sprouts; reductions, respectively, were 7.30 to 7.37 and 6.64 to 6.70 $\log_{10}$ CFU/ml of water after continuous sparging for 5 to 20 min. The maximum reduction of *L. monocytogenes* was 0.94 $\log_{10}$ CFU/g of sprouts sparged for 20 min. Significant reductions of up to 1.68 $\log_{10}$ CFU of aerobic microorganisms per g also resulted from continuous sparging with ozone for 20 min. Kim et al. *(16)* reported that mesophilic bacteria on shredded lettuce were reduced by 1.4 and 3.9 $\log_{10}$ CFU/g during sparging with ozone (1.3 mM) for 3 and 5 min, respectively. They concluded that the bactericidal activity of ozone is most efficient when the treatment consists of a combination of continuous ozone bubbling and high-speed stirring. Fisher et al. *(8)* studied the effectiveness of ozonated water in killing or removing *L. monocytogenes* inoculated onto cabbage leaves. Dipping leaves in water containing 1 μg/ml of ozone for 5 min reduced populations by 100%. The population recovered from inoculated cabbage not treated with ozone was not reported.

The relative inefficiency of ozone for controlling *L. monocytogenes,* as well as *E. coli* O157:H7 *(28),* on alfalfa seeds and sprouts is attributed in part to its inaccessibility to sites harboring these pathogens. Wrinkled alfalfa seeds have been reported to contain higher numbers of aerobic bacteria than smooth, healthy seeds *(4).* After inoculation with *Salmonella* Newport, significantly higher numbers of aerobic bacteria were recovered from wrinkled seeds than from smooth seeds. The pathogen on wrinkled seeds was more difficult to kill by treatment with 20,000 μg/ml of free chlorine. These observations support the hypothesis that microbial cells harbored in damaged tissues or lodged in otherwise inaccessible areas escape exposure to chemical disinfectants. Cells imbedded in biofilms known to be present on alfalfa sprouts *(7)* may also diminish the efficacy of sanitizers.

Scanning electron photomicrographs of alfalfa seeds and sprouts show the existence of sites that would potentially protect microorganisms against chemical decontamination treatments (Fig. 1). Observations of many lots of alfalfa seeds have revealed that 3 to 85% of the seeds are cracked, missing part of the testa, or both. One pound (453.6 g) of alfalfa seeds contains ca. 200,000 seeds. If 25

TABLE 5. *Mean hedonic ratings for sensory attributes of alfalfa sprouts treated by continuous sparging with water initially containing 20 μg/ml of ozone, followed by storage at 4°C for up to 11 days*[a]

Storage time (days)	Attribute	Treatment time (min)[b]				
		Water control			Ozone (20 μg/ml)	
		0	5	10	5	10
0	Appearance	5.5 B	6.1 AB	6.3 AB	6.5 A	6.0 AB
	Color	6.1 B	6.5 AB	6.6 AB	7.0 A	6.7 AB
	Aroma	6.2 A	6.7 A	6.6 A	6.4 A	6.3 A
	Overall	5.9 B	6.4 AB	6.4 AB	6.6 A	6.2 AB
3	Appearance	4.3 B	4.5 B	6.1 A	4.9 B	5.9 A
	Color	4.6 C	4.7 C	6.0 AB	5.3 BC	6.2 A
	Aroma	5.0 C	5.4 BC	6.2 A	5.6 AB	6.2 A
	Overall	4.3 C	4.7 BC	5.9 A	5.0 B	5.9 A
7	Appearance	4.1 AB	4.1 AB	4.8 A	3.2 C	3.5 BC
	Color	4.2 B	4.4 AB	5.1 A	3.9 B	4.1 B
	Aroma	5.0 A	5.1 A	5.3 A	3.7 B	3.8 B
	Overall	4.2 AB	4.5 A	4.7 A	3.3 B	3.5 BC
11	Appearnce	4.1 A	3.5 A	3.5 A	1.9 B	1.3 B
	Color	4.2 A	3.5 B	3.8 AB	1.9 C	1.3 D
	Aroma	4.4 A	4.7 A	4.2 A	2.0 B	1.5 B
	Overall	3.9 A	3.7 A	3.7 A	1.8 B	1.3 B

[a] Ratings were assigned by panelists using a nine-point hedonic scale, with 1 = dislike extremely, 5 = neither like nor dislike, and 9 = dislike extremely.

[b] Means with different letters in the same row are significantly different ($P \leq 0.05$).

lb (ca. 11.3 kg) of seeds are used to grow a batch of sprouts and half of the seeds are cracked or in some way damaged, 2,500,000 seeds are potentially difficult to sanitize. Cells of *L. monocytogenes* or other bacterial pathogens that may be lodged in areas between the testa and cotyledon (Fig. 1A) or within the stomata (Fig. 1B) would be inaccessible to ozone or other sanitizers. The surface area of the sprout root (Fig. 1C) is much greater than its overall cylindrical appearance might suggest. The mechanical damage or fungal invasion of sprouts would likely release nutrients that would support the growth of indigenous or pathogenic bacteria. The development of bacterial colonies (Fig. 1C and 1D) would ensue, with the potential development of biofilms (Fig. 1E and 1F) that would exacerbate attempts to remove cells or sanitize the sprouts. Indeed, the fungal hyphae and bacterial cells shown in Figure 1C through 1F resisted removal by the washing solutions used to prepare these specimens. The presence of naturally occurring biofilms on alfalfa and other types of seed sprouts *(7)* and the harboring of aerobic bacteria and wrinkled alfalfa seeds *(29)* have been described, with conclusions that these conditions interfere with effective sanitizing treatments.

Table 5 summarizes results from the sensory evaluations of alfalfa sprouts treated with 20 μg/ml of ozone for 5 or 10 min, followed by storage for up to 11 days at 4°C. Quality attributes of sprouts treated with deionized water or ozonated water for the same length of time were not significantly different on day 0 or 3 of storage. However, except for the color of sprouts stored for 7 days, the ratings of all sensory attributes were significantly lower ($P \leq 0.05$) for the sprouts treated with ozone than for the control (water) sprouts treated for the same length of time, at 7 and 11 days of storage. Sprouts treated with ozone appeared to darken in color and take on a limp, oily appearance during storage. Other researchers have reported changes in color of broccoli florets *(20)* and carrots *(21)* treated with ozone. Naito and Shiga *(23)* reported that an ozone treatment of bean sprouts resulted in the elongation of hypocotyls. We did not observe this phenomenon with ozonated alfalfa sprouts.

In summary, the ozone treatment of alfalfa seeds and sprouts inoculated with *L. monocytogenes* was minimally effective in killing the pathogen or naturally occurring aerobic mesophilic microorganisms. Although the treatment of seeds with up to ca. 21 μg/ml of ozone for 20 min did not reduce the percentage of germination, this treatment did adversely affect the sensory quality of sprouts. The use of ozone as a sanitizer to replace the treatment of seeds intended for sprout production with 20,000 μg/ml of chlorine cannot be recommended. More research to improve the effectiveness of ozone in killing *L. monocytogenes* and other foodborne pathogens is needed.

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REFERENCES

1. Beuchat, L. R. 1997. Comparison of chemical treatments to kill *Salmonella* on alfalfa seeds destined for sprout production. *Int. J. Food Microbiol.* 34:329–333.
2. Beuchat, L. R., and A. J. Scouten. 2002. Combined effects of water activity, temperature, and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. *J. Appl. Microbiol.* 92:382–395.
3. Beuchat, L. R., T. E. Ward, and C. A. Pettigrew. 2001. Comparison

of chlorine and a prototype produce wash product for effectiveness in killing *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. *J. Food Prot.* 64:152–158.

4. Charkowski, A. O., C. Z. Sarreal, and R. E. Mandrell. 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. *J. Food Prot.* 64:1292–1298.
5. Dave, S., J. G. Kim, Y. Lou, and A. E. Yousef. 1998. Kinetics of inactivation of *Salmonella enteritidis* by ozone, p. 15. Institute of Food Technologists Annual Meeting, Book of Abstracts.
6. Delaquis, P. J., P. L. Sholberg, and K. Stanich. 1999. Disinfection of mung bean seeds with gaseous acetic acid. *J. Food Prot.* 62:953–957.
7. Fett, W. F. 2000. Naturally occurring biofilms on alfalfa and other types of sprouts. *J. Food Prot.* 63:625–632.
8. Fisher, C. W., D. Lee, B. Dodge, K. M. Hamman, J. B. Robbins, and S. E. Martin. 2000. Influence of catalase and superoxide dismutase on ozone inactivation of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 66:1405–1409.
9. Fu, T., D. Stewart, K. Reineke, J. Ulaszek, J. Schesser, and M. Tortorello. 2001. Use of spent irrigation water for microbiological analysis of alfalfa sprouts. *J. Food Prot.* 64:802–806.
10. Gordon, G., G. E. Pacey, W. J. Cooper, and R. G. Rice. 1988. Current state-of-the-art measurements of ozone in the gas phase and in solution. *Ozone Sci. Eng.* 10:353–366.
11. Grunwell, J., J. Benga, H. Cohen, and G. Gordon. 1983. A detailed comparison of analytical methods for residual ozone measurement. *Ozone Sci. Eng.* 5:203–223.
12. Himathongkham, S., S. Nuanualsuwan, H. Riemamn, and D. O. Cliver. 2001. Reduction of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in artificially contaminated alfalfa seeds and mung beans by fumigation with ammonia. *J. Food Prot.* 64:1817–1819.
13. Holliday, S. L., A. J. Scouten, and L. R. Beuchat. 2001. Efficacy of chemical treatments in eliminating *Salmonella* and *Escherichia coli* O157:H7 on scarified and polished alfalfa seeds. *J. Food Prot.* 64: 1489–1495.
14. Jaquette, C. B., L. R. Beuchat, and B. E. Mahon. 1996. Efficacy of chlorine and heat treatment in killing *Salmonella* Stanley inoculated onto alfalfa seeds and growth and survival of the pathogen during sprouting and storage. *Appl. Environ. Microbiol.* 62:2212–2215.
15. Kim, J. G., and A. E. Yousef. 2000. Inactivation kinetics of foodborne spoilage and pathogenic bacteria by ozone. *J. Food Sci.* 65: 521–528.
16. Kim, J.-G., A. E. Yousef, and G. W. Chism. 1999. Use of ozone to inactivate microorganisms on lettuce. *J. Food Saf.* 19:17–34.
17. Kim, J. G., A. E. Yousef, and S. Dave. 1999. Application of ozone for enhancing the microbiological safety and quality of foods: a review. *J. Food Prot.* 67:1071–1087.
18. Kim, M. J., Y. A. Oh, M. H. Kim, M. K. Kim, and S. D. Kim. 1993. Fermentation of Chinese cabbage kimchi inoculated with *Lactobacillus acidophilus* and containing ozone-treated ingredients. *J. Korean Soc. Food Nutr.* 22:165–174.
19. Lang, M. M., B. H. Ingham, and S. C. Ingham. 2000. Efficacy of novel organic acid and hypochlorite treatments for eliminating *Escherichia coli* O157:H7 from alfalfa seeds prior to sprouting. *Int. J. Food Microbiol.* 58:73–82.
20. Lewis, L., H. Zhuang, F. A. Payne, and M. M. Barth. 1996. Beta-carotene content and color assessment in ozone-treated broccoli florets during modified atmosphere packaging, p. 99. Institute of Food Technologists Annual Meeting, Book of Abstracts.
21. Liew, C. L., and R. K. Prange. 1994. Effect of ozone and storage temperature on postharvest diseases and physiology of carrots (*Caucus carota* L.). *J. Am. Soc. Hortic. Sci.* 119:563–567.
22. Moore, G., C. Griffith, and A. Peters. 2000. Bactericidal properties of ozone and its potential application as a terminal disinfectant. *J. Food Prot.* 63:1100–1106.
23. Naito, S., and I. Shiga. 1989. Effect of ozone treatment on elongation of hypocotyl and microbial counts of bean sprouts. *Nippon Shokuhin Kogyo Gakkaishi* 36:181–189.
24. National Advisory Committee on Microbiological Criteria for Foods. 1999. Microbiological safety evaluation and recommendations on sprouted seeds. *Int. J. Food Microbiol.* 52:123–153.
25. Park, C. M., P. J. Taormina, and L. R. Beuchat. 2000. Efficacy of allyl isothiocyanate in killing enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. *Int. J. Food Microbiol.* 56:13–20.
26. Restaino, L., E. W. Frampton, J. B. Hemphillo, and P. Palnikar. 1995. Efficacy of ozonated water against various food-related microorganisms. *Appl. Environ. Microbiol.* 61:3471–3475.
27. Scouten, A. J., and L. R. Beuchat. 2002. Combined effects of chemical, heat, and ultrasound treatments to kill *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. *J. Appl. Microbiol.* 92:668–674.
28. Sharma, R. R., A. Demirci, L. B. Beuchat, and W. F. Fett. 2002. Inactivation of *Escherichia coli* O157:H7 on inoculated alfalfa seeds with ozonated water and heat treatment. *J. Food Prot.* 65:447–451.
29. Stewart, D. S., K. F. Reineke, J. M. Ulaszek, and M. L. Tortorello. 2001. Growth of *Salmonella* during sprouting of alfalfa seeds associated with salmonellosis outbreaks. *J. Food Prot.* 64:618–622.
30. Taormina, P. J., and L. R. Beuchat. 1999. Comparison of chemical treatments to eliminate enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. *J. Food Prot.* 62:318–324.
31. Taormina, P. J., and L. R. Beuchat. 1999. Behavior of enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa sprouts during the sprouting process as influenced by treatments with various chemicals. *J. Food Prot.* 62:850–856.
32. Taormina, P. J., L. R. Beuchat, and L. Slutsker. 1999. Infections associated with eating sprouts: an international concern. *Emerg. Infect. Dis.* 5:626–634.
33. Unal, R., J. G. Kim, and A. E. Yousef. 2001. Inactivation of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Lactobacillus leichmannii* by combinations of ozone and pulsed electric field. *J. Food Prot.* 64:777–782.
34. Weissinger, W. R., and L. R. Beuchat. 2000. Comparison of aqueous chemical treatments to eliminate *Salmonella* on alfalfa seeds. *J. Food Prot.* 63:1475–1482.
35. Wu, F. M., L. R. Beuchat, J. G. Wells, L. Slutsker, M. P. Doyle, and B. Swaminathan. 2001. Factors influencing the detection and enumeration of *Escherichia coli* O157:H7 on alfalfa seeds. *Int. J. Food Microbiol.* 71:93–99.