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Reduction of *Salmonella* and Shiga toxin-producing *Escherichia coli* on alfalfa seeds and sprouts using an ozone generating system

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ABSTRACT

Several outbreaks of illness have been associated with consumption of alfalfa sprouts contaminated with Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella*. The ozone application was investigated as an intervention. Alfalfa seeds were inoculated with cocktails of 3 *Salmonella* strains, including serotypes Typhimurium, Agona and Saintpaul, and 3 strains of Shiga toxin-producing *Escherichia coli* (STEC) including serotypes O104:H4, O157:H7 and O121:H19 with a final load of 7.0 log CFU/ml. Then, the inoculated seeds, and the sprouts obtained from these seeds were separately subjected to aqueous ozone treatment containing (5 mg/L) ozone for varied times of exposure. The mean log reductions for *Salmonella* achieved on seeds after 10, 15, and 20 min of ozone exposure were 1.6 ± 0.5, 1.7 ± 0.3, 2.1 ± 0.5, respectively and 1.5 ± 0.4, 1.6 ± 0.4, 2.1 ± 0.5 for STEC, respectively. For sprouts obtained from the inoculated seed, the mean log reductions for *Salmonella* after 10, 15, and 20 min exposure times were 0.7 ± 0.2, 1.1 ± 0.4, 3.6 ± 0.2, respectively, whereas the mean log reductions for STEC were 0.7 ± 0.1, 1.2 ± 0.3 and 1.8 ± 0.2, respectively. At each contact time, there were no differences in log reductions between pathogens on seeds ($P > 0.05$), whereas on sprouts, the reductions obtained at 20 min were significantly greater ($P < 0.05$) for *Salmonella* than for STEC. On both seeds and sprouts, the exposure time had significant ($P < 0.05$) effects on log reductions of *Salmonella* and STEC. The weight, color properties and shelf life of ozonated sprouts were also tested. The ozonation did not have negative effects on germination (%), color and mass of sprouts in comparison with the controls. This study confirmed that it is possible to substantially reduce *Salmonella* and STEC by using a low ozone concentration (5 mg/L) and reduce food safety risk with less concern about the safety for processing workers of this treatment, this without affecting seed germination. This procedure may be a promising intervention to reduce *Salmonella* and STEC from alfalfa seeds and sprouts.

1. Introduction

Foodborne illness is one of the worldwide concerns, and sprouted seeds have been commodities frequently found to transmit bacterial pathogens foodborne illness. In the United States, at least 48 foodborne outbreaks have been linked to consumption of sprouts since 1996 (Gensheimer and Gubernot, 2016). *Salmonella* and STEC are among the pathogens that are implicated in many of these outbreaks (Scallan et al., 2011). Particularly, alfalfa sprouts are commonly consumed due to their nutritional value (Bari et al., 2011; Donaldson, 2004; Kurtzweil, 1999), which make them a high risk commodity (Bari et al., 2011; Breuer et al., 2001; CDC, 1997; Mohle-Boetani et al., 2001; Pönkä et al., 1995; Scallan et al., 2011; Taormina et al., 1999). Although different sources can contaminate alfalfa sprouts, contaminated seeds are known to be the main source of pathogens in sprouts (Buck et al., 2003; NACMCF, 1999). Seeds can potentially be contaminated from a number of sources, including irrigation water, improperly composted manure, agriculture fields in close vicinity of animal farms, dirty harvesting or processing equipment and poor hygiene of workers (Bari et al., 2011). Numerous studies have been conducted to evaluate chemical and non-chemical intervention to inactivate *Salmonella* and STEC on the surface of seeds and sprouts (Bari et al., 2011; Buchholz and Matthews, 2010; Darmon et al., 2005;, Kumar et al., 2006; Montville and Schaffner, 2004; Neetoo et al., 2009; Neetoo and Chen, 2010; Scouten and Beuchat, 2002; Sharma et al., 2002, 2003, 2004; Singh et al., 2003; Tomás-Callejas et al., 2012; Zhao et al., 2010). However, the results obtained have resulted either in significant negative effects on the quality of sprouts and seed germination, or in a very low reduction of

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the pathogens.

The sprouting process fosters a conducive growth environment for pathogens, since sprout production requires high moisture and warmth (Gabriel, 2005; Howard and Hutcheson, 2003; Stewart et al., 2001; Taormina and Beuchat, 1999b). In addition, some treatments may negatively impact seed germination (sprouting) and may leave chemical residuals, which pose public health hazards to consumers (Neetoo and Chen, 2010; Taormina and Beuchat, 1999a). Non-chemical methods including natural antimicrobials, irradiation, or high-pressure processing, to decontaminate seeds and their sprouts have not shown success in completely eliminating pathogens from seed sprouts (Bari et al., 2003; Fett and Cooke, 2003; C. Kim et al., 2003; Neetoo and Chen, 2010; Thayer et al., 2003). Bari et al. (2008) tested the efficacy of hot water treatments and found that hot water was very effective in eliminating pathogens from mung bean seeds. However, hot water treatments can result in detrimental changes in sensory properties of sprouts (Muñoz et al., 2007).

The application of ozone has been tested as an intervention for eliminating pathogens from the surface of different seeds and sprouts (Sharma et al., 2002, 2003, 2004). Since ozone leaves no residues on food products and is recognized as a powerful antimicrobial agent (Güzel-Seydim et al., 2004; Sharma et al., 2004), its application is approved in the United States to be used as an antimicrobial agent for treatment, storage and processing of foods (J.G. Kim et al., 2003). Researchers have studied the efficacy of ozone treatment in inactivating *E. coli* O157:H7 and *Listeria monocytogenes* on alfalfa seeds and sprouts as well as its effect on sensory attributes and found that ozone treatment did not exhibit any obvious adverse effects on alfalfa quality (Sharma et al., 2004; Wade et al., 2003). There appears to be no published study on the efficacy of ozone in reduction or inactivation of *Salmonella* on alfalfa seeds and sprouts. Accordingly, the objective of this study was to use the ozone treatment and evaluate its ability to eliminate or reduce *Salmonella* from the surface of alfalfa seeds and sprouts and compare with the inactivation/reduction of STEC. The effect of ozone treatment on the sensory and quality attributes of alfalfa sprout was also determined.

2. Material and methods

2.1. Seed preparation

Certified organic alfalfa seeds (http://shop.eatorganicbuffalo.com/Certified-Organic-seed_c2.htm) were purchased from Brazos Natural Food Store (College Station, Texas) and Thiensville Farms. These seeds had not been subjected to an abrasion treatment. Upon receiving in the laboratory, the seeds were stored at 5 °C in a sealed box until the experiment started. The water activity was measured with an AquaLab water activity meter (Aqua Lab Series 3, Decagon Devices, Inc., Pullman, WA) immediately after seeds were obtained and recorded as a baseline water activity.

2.2. Revival of microorganisms

Three isolates of *Salmonella enterica* (serovars Typhimurium, Agona, and Saintpaul) and three isolates of Shiga toxin-producing *E. coli* (STEC, serotypes O157:H7, O104:H4 and O121:H19), were used. For differentiation purposes, rifampin-resistant (Rif +) variants of these bacteria had been previously selected from the original isolates by the procedure of Kaspar and Tamplin (1993) and were stored in vials at −80 °C in tryptic soy broth (TSB; Difco, Sparks, MD) with 15% (v/v) glycerol (Sigma-Aldrich Co. St. Louis, MO). All These cultures were obtained from the Food Microbiology Laboratory, Texas A&M University (College Station, TX) culture collection and a description and original sources of these isolates is shown in Table 1. Prior to use, each strain was individually grown by transferring cryopellets to 9 mL TSB broth followed by incubation at 35 °C for 18–24 h. Thereafter, a loopful of each 18–24 h culture was transferred into 9 mL TSB and incubated for another 18–24 h at 35 °C. Subsequently, each strain was aseptically streaked onto tryptic soy agar (TSA; Difco, Sparks, MD) plates and incubated aerobically for 18–24 h at 35 °C. Next, well-grown isolated colonies of each strain were picked from the plates and aseptically re-streaked onto TSA slants. These inoculated TSA slants were incubated at 35 °C for 24 h and stored at 4–5 °C during the course of the study.

2.3. Inoculum preparation

Prior to an experiment, two consecutive 18–24 h transfers in 9 mL TSB tubes incubated at 35 °C for 18–24 h were performed for each strain from previously grown TSA slants of *Salmonella* and STEC. Cells were harvested by centrifugation (3500 × *g* for 15 min at 4 °C) and were re-suspended in 10 mL phosphate buffer saline (PBS) solution. Then, equal aliquots of three strains of *Salmonella* and three strains of STEC were combined to make one bacterial cocktail. Preliminary studies confirmed that these strains maintained the same concentration as the individual concentration when combined together and they did not interact with each other. The resulting bacterial cocktail ($8 \log^{10}$ CFU/mL) was enumerated by spread plating appropriate dilutions in 0.1% (wt/vol.) sterile peptone water (PW; Difco), in duplicate, onto plates of lactose sulfite phenol red rifampicin (LSPR), with a rifampin (Sigma-Aldrich, St. Louis, MO, USA) concentration of 100 μg/mL (Castillo et al., 1998). This medium differentiates *Salmonella* and *E. coli*, enabling simultaneous enumeration of both pathogens in the same experiment. The plates were incubated at 35 °C for 18–24 h prior to colony counting.

2.4. Inoculation of alfalfa seeds

Alfalfa seeds (400 g) were weighed and placed in polyethylene bags (36 cm × 40 cm, Pactiv Corporation, Lake Forest, IL), inoculated with 25 mL of the 6-strain cocktail to obtain approximately 6 log CFU/g and mixed thoroughly by manually shaking the bags for 2 min to result in homogeneous distribution of bacteria. Inoculated seed samples of 5 and 10 g were removed from the bag for water activity (a_w) and population density measurements, respectively. The remaining inoculated seeds were placed onto two sheets of paper towels layered inside a sterile plastic tub partially covered with aluminum foil and were allowed to dry for 6 h at 23 ± 2 °C. After drying, the a_w of the seed was measured using an AquaLab water activity meter (Aqua Lab Series 3, Decagon Devices, Inc. Pullman, WA). Then, the dried seeds (aw 0.63) were divided into three batches. The first batch was used to test the effect of ozone treatment on seeds, whereas the second and third batch was used to test the effect of ozone treatment during and after sprouting. Non-inoculated seeds were tested to ensure absence of Rif + bacteria.

2.5. Preparation of ozonated water

Ozone gas (O_3) was generated using an ozone generator (Model VMUS-4S, Azco Industries Ltd., College Station, TX). One liter of cold (~5 °C) sterilized distilled water (SDW) was poured into a 2 L glass Erlenmeyer flask fitted with a silicon stopper with 2 holes – one for an inlet line for incoming ozone and the other for an exit line for releasing extra ozone gas to maintain gas pressure at a certain level. The SDW was infused with ozone gas for 1 h to obtain an aqueous ozone concentration of 5 mg/L. Ozone bubbled during the treatment at 10 psi outlet pressure. Excess ozone was passed through another flask containing a 2% potassium iodide solution to prevent ozone from being released into the environment.

The concentrations of ozone in water used to treat alfalfa seeds and sprouts were determined by the indigo spectrophotometric method (Bader and Hoigné, 1981). The absorbance of water and ozonated solutions at 600 ± 10 nm was measured with a spectrophotometer (Thermo Scientific, BioMate™ 3S Waltham, MA). The ozone concentration was determined using the following formula:

Table 1
Sources of *Salmonella* and Shiga toxin-producing *Escherichia coli* used in this study.

Pathogen	Serotype	Strain code	Origin	Source
Salmonella	Typhimurium	ATCC 13311	Human feces from food poisoning case, 1911	Texas A&M Collections
	Agona	ATCC 51957	Deposited by Kauffmann & Edwards	Texas A&M Collections
	Saintpaul	476,398	Human stools from pepper outbreak linked to hot peppers	FDA/CFSAN
STEC	O104:H4	ATCC BAA-2326	Human stool from European outbreak linked to sprouts	USDA-ARS
	O157:H7	K3999	Spinach outbreak 2006	FDA/CFSAN
	O121:H19	CDC 97-3068	Human stool	USDA-ARS

O_3 concentration (mg/L) = 100 Δ (ΔA)/(f)(v)(b)

where ΔA is the difference in absorbency between sample and blank solutions, b is path length (cm) of cell in spectrophotometer, v is the volume (mL) of sample or blank and f is the factor that corresponds to an absorption coefficient for aqueous ozone = 0.42.

2.6. Germination of inoculated alfalfa seeds

For the second batch, three inoculated and dried seed samples (20 g each) were placed into glass jars and covered with a mesh to germinate and produce sprouts. After placing the mesh, the glass jars then were filled with 500 ml sterile distilled water; the seeds were left to soak for 8 h and then drained. The drained alfalfa seeds remained in the glass jars overnight at 23 ± 2 °C in a dark place and were then rinsed with distilled water and drained again. This rinse-and-drain procedure was repeated every day for six days until the sprouts were ready for harvesting. No testing was conducted in the rinse water, since bacterial reductions were not expected. All sprouts were harvested using tongs, which were sterilized by dipping in ethanol and flaming and were placed into sterile plastic plate, then weighed it. Three samples of 10 g sprouts then were collected from each jar and used for ozone treatment. Controls consisted of Another set of three sprout samples was washed with distilled water for 0 (before washing), 10, 15, and 20 min and was used as control. The samples then were followed by microbiological analysis.

For the third batch, a set of three inoculated seed samples (20 g each) was collected and placed in glass jars and covered with a mesh. Glass jars were halfway filled with ozonated (5 mg/L) water (500 mL); the seeds were left to soak for 8 h and then drained. After leaving the drained ozone-treated seeds inside the glass jars overnight at 23 ± 2 °C in a dark place, they were rinsed with ozonated water instead of distilled water and drained again. This procedure was repeated until the sprouts were ready for harvesting. To determine the number of bacteria that were removed from the germinated seeds after rinsing with ozonated water, 1 ml of drained water was taken after rinsing with ozone every day until the sprouts were ready for harvest and subjected to microbiological analysis. The sprouts then were harvested using alcohol flame-sterilized tongs and transferred to sterile plastic plate, then were weighed it, three samples of 10 g sprouts and the same number of control samples were collected from each jar for ozone treatment.

To determine if the ozone was still active on the surface of alfalfa seeds or sprouts after treatment before microbiological analysis, which might lead to error in estimating contact time, the treated samples were washed with 50 mL warm water (~35 °C) for 5 min. Three samples of seeds or sprouts were washed with warm water immediately after treatment of 10 min exposure to ozonated water to neutralize any remaining ozone on the surface of seeds or sprouts, and then subjected to microbiological analysis.

2.7. Treatment of alfalfa seeds and sprouts with ozonated water

As mentioned above, the inoculated dried seeds from the first batch were separated in groups, and each group was immersed in 1 L of ozonated water (5 °C) containing 5 mg/L (5 ppm) ozone for 10, 15 or 20 min with continuous agitation. Control seeds were subjected to non-ozonated SDW (5 °C) for zero (before immersion), 10, 15 or 20 min. Three samples treated with ozonated water and 3 control samples (treated with DW) were collected at each contact time for conducting microbiological analysis. For ozone treatment, the sprouts were immersed in 1 L of ozonated water (5 °C) containing 5 mg/L (5 ppm) ozone and treated with ozone for 10, 15 and 20 min with continuous agitation. Control samples were tested the same as described above. The ozone concentration in the treatment water was again tested after each contact time and after sample collection.

2.8. Microbiological analysis

After validation that warm water rinse was sufficient to neutralize any residual ozone effect and therefore there was no need for other neutralizers, all ozone-treated and control samples (10 g) of alfalfa seeds or sprouts were immediately placed into sterile stomacher bags (BA 6041, 177 × 305 mm, VWR, Rochester, NY) containing 90 mL sterile PW (0.1%) and mixed for 1 min in a Stomacher Lab Blender 400 (Model BA6021, 34 × 29 × 32, A. J. Seward, London, UK). Populations of *Salmonella* and STEC were determined by surface plating serially diluted samples (0.1 mL in duplicate) on LSPR agar plates. Colonies were enumerated after 18–24 h of incubation at 35 °C in an incubator (460-ICE, Labline-Thermo Scientific). Because of the resistance of the growing colonies to rifampin (a rare trait in *Enterobacteriaceae*), the differential features of LSPR agar (which show important phenotypical characteristics of *Salmonella* and *E. coli*), and the lack of growth of colonies from non-inoculated seeds, no further confirmation of identity in colonies was needed.

2.9. Weight measurement procedure

The weight of all sprouts treated with ozone and controls was measured at the end of the sprouting time. Before harvesting, sprouts were allowed to drain for 3 h to eliminate as much of the water left from rinsing as possible. This draining time was tested previously to ensure that sprouts had no more running water on their surface. The sprout weight was obtained by subtracting the weight of the empty jar from the weight of the jar with sprouts using a precision laboratory scale (680 Mettler Toledo, Aldinger, USA). Weight measurement was performed for triplicate samples of control and ozonated sprouts. These measurements were repeated three times using triplicate samples on each repetition.

2.10. Color measurement of alfalfa sprouts

The color properties including lightness (L), yellowness (b), and redness (a) values of sprouts were measured immediately after harvesting to evaluate the effect of these treatments on their natural colors by comparing with control samples. Each sample of sprouts (~15 g) was placed onto a Petri dish to cover its surface evenly. A Spectra Photocolorimeter (Minolta CR −400; Tokyo, Japan) was used to measure the surface color of each ozonated sprout and control sprout sample. Color evaluation was conducted at three points or places on the surface of each sprout sample covered in a Petri dish and measurements on

triplicate samples were recorded. The results from both treated and control sprouts samples were analyzed and compared to each other.

2.11. Lactic acid bacteria testing

Lactic acid bacteria (LAB) are naturally present in alfalfa sprouts and evidence exists regarding correlation between the density of the LAB population and the time of storage (Axelsson and Ahrné, 2000). Therefore, LAB count was used as an indicator of shelf-life of the sprouts as affected by the ozone treatment. Six samples of the fresh sprouts produced with ozonated and non-ozonated water were plated onto De Man, Rogosa and Sharpe (MRS) agar immediately after harvesting, and incubated anaerobically for 24 h at 30 °C. The remaining ozonized and non-ozonized sprouts were stored at ~4 °C and on day 3 and 6 of storage, 6 samples were plated onto MRS, incubating anaerobically at 30 °C for 24 h. LAB populations on both ozonized and non-ozonized sprouts were recorded (as CFU/g) by counting colonies and compared.

2.12. Statistical analysis

All experiments were carried out in three replications each performed in triplicates. The results were analyzed using the general linear model approach in JMP (Version 13, SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) followed by the Tukey's Honestly Significant Difference test was applied to determine significant differences ($P \leq 0.05$) in populations of *Salmonella* and STEC on alfalfa seeds and sprouts subjected to different treatments.

3. Results and discussion

When the potential effect of residual ozone in the samples, on the counts of *Salmonella* and STEC was tested, no significant differences ($P > 0.05$) in counts of *Salmonella* and STEC were found for samples that were plated after finishing the ozone treatment vs. samples that were subjected to a warm water rinse to neutralize ozone. These results indicated that sample neutralization after ozone treatment may not be necessary, and that the ozone molecule is probably decomposed promptly after mixing the sample with the dilution fluid. Nevertheless, rinsing with warm water was followed to accelerate the neutralization of residual ozone remaining on seed or sprout samples.

In another preliminary experiment, different times of exposure were tested for designing the experiment including; 5, 10, 15, 20, and 25 min. We decided to do 10, 15, and 20 min exposure time for the entire experiment. It was found that the 5 min exposure was close to initial bacterial concentration (control), therefore, the 5 min exposure time was not repeated for remaining replicates. Also noticed no statistical difference between 20 min exposure and 25 min exposure. Therefore, 25 min exposure was also removed from further trials. During the treatment, the ozone concentration was measured before and after treatment.

Ozone concentration at the end of each contact time was 4.9–4.4 mg/L. Although slightly less than its initial concentration of 5 mg/L, this reduction of ozone concentration was not significant ($P > 0.05$). Thus, ozone decrease was ignored as not a possible factor affecting the overall goal of the study, which is exposure to 5 mg/L ozone concentration.

When seeds were treated with ozonated water for 20 min, the mean log counts ± standard deviation of surviving *Salmonella* and STEC were 3.5 ± 0.1/g and 3.5 ± 0.2/g, respectively when the initial log population was 5.6 ± 0.2/g, resulting in 2.1 ± 0.5 log reduction of both pathogens. After 10 min exposure, the counts of *Salmonella* on alfalfa seeds were significantly reduced by 1.6 ± 0.5 in comparison with the initial counts ($P < 0.05$) (Table 2). However, after this initial reduction the counts of *Salmonella* were not significantly reduced between 10, 15 and 20 min ($P > 0.05$), even though the reduction after 20 min reached 2.1 ± 0.5 log. In contrast, the counts of STEC (initial count of 5.6 ± 0.2 log CFU/g) showed a continued decrease ($P < 0.05$) after each of the 10, 15 and 20 min times of contact with ozonated water reaching a 2.1 log reduction after 20 min contact with ozone.

In the case of sprouts, log reductions of *Salmonella* at 20 min exposure to ozonated water were significantly greater than the reductions observed on seeds. In contrast, a continued significant reduction along 10, 15 and 20 min treatment with ozonated water was observed for STEC showed, and this pattern was similar on sprouts and on seed (Table 3). Overall, exposure of seeds to ozonated water for 20 min caused a reduction of 2.1 log cycles on both *Salmonella* and STEC, whereas exposure of sprouts to ozonated water for 20 min resulted in a reduction of 3.6 and 1.8 log cycles for *Salmonella* and STEC, respectively. Although the alfalfa sprouts had initially higher counts of *Salmonella* and STEC than alfalfa seeds, the magnitude of the reduction on sprouts was significantly different from that on seeds for *Salmonella* counts; this comparison is shown in Figs. 1 and 2. The differences in reduction of *Salmonella* population between alfalfa seeds and sprouts, observed after 20 min contact time seemed atypical, since most of the results pointed towards a greater antimicrobial effect of ozonated water on seed than sprouts. A possible explanation could be a larger surface area in sprouts, which may allow more bacteria to be exposed. However, while reports exist about geometrical characteristics of sprouting seeds (Unal et al., 2008), no information was found about the

Table 2
Survival of *Salmonella* and STEC on alfalfa seeds after ozone treatments.

Pathogen	Treatment time (min)	Populations (Log CFU/g)	
		Distilled water[a]	Ozonated water[b]
Salmonella	0	5.6 ± 0.05AX[c]	5.6 ± 0.2AX
	10	5.5 ± 0.1AX	4.0 ± 0.2BY
	15	5.3 ± 0.3AX	3.9 ± 0.3BY
	20	5.2 ± 0.3AX	3.5 ± 0.1BY
STEC	0	5.6 ± 0.2AX	5.6 ± 0.2AW
	10	5.5 ± 0.2AX	4.1 ± 0.1BX
	15	5.5 ± 0.4AX	4.0 ± 0.4BX
	20	5.4 ± 0.2AX	3.5 ± 0.1BY

[a] Mean Log (CFU) of *Salmonella* or STEC survival on inoculated alfalfa seeds after soaked with distilled water for each time (n = 9).
[b] Mean Log (CFU) of *Salmonella* or STEC survival on inoculated alfalfa seeds after ozonated treatment for each time (n = 9).
[c] In the same row, means with the same letter A or B are not different; within columns, means with the same letter W, X, Y or Z are not significantly different ($P > 0.05$).

Table 3
Survival of *Salmonella* and STEC on alfalfa sprouts after ozone treatments.

Pathogen	Treatment time (min)	Populations (Log CFU/g)	
		Distilled water[a]	Ozonated water[b]
Salmonella	0	7.1 ± 0.05AX[c]	7.1 ± 0.06AX
	10	6.9 ± 0.1AX	6.5 ± 0.2AX
	15	6.7 ± 0.3AX	6.0 ± 0.2BX
	20	6.6 ± 0.3AX	3.5 ± 0.2BY
STEC	0	7.1 ± 0.2AX	7.1 ± 0.1AW
	10	6.9 ± 0.3AX	6.4 ± 0.1BX
	15	6.6 ± 0.4AX	5.9 ± 0.3BY
	20	6.7 ± 0.2AX	5.3 ± 0.2BZ

[a] Mean Log (CFU) of *Salmonella* or STEC survival on inoculated alfalfa seeds after soaked with distilled water for each time (n = 9).
[b] Mean Log (CFU) of *Salmonella* or STEC survival on inoculated alfalfa seeds after ozonated treatment for each time (n = 9).
[c] In the same row, Means with the same letter A or B are not different; within columns, means with the same letter W, X, Y or Z are not significantly different ($P > 0.05$).

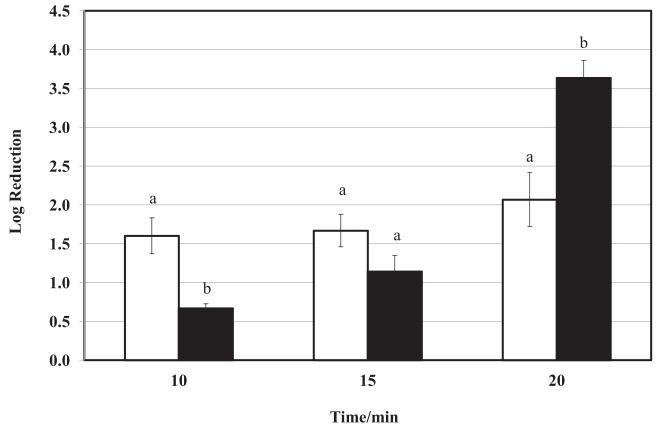

Fig. 1. Log Reduction of *Salmonella* on the surface of alfalfa seeds (white column) and sprouts (black column) after subjected to ozonated water 5 mg/L for 10, 15, 20 min. Significant differences ($P < 0.05$) in log reduction of *Salmonella* between seeds and sprouts by using ozone treatment for 20 min. Ozone resulted in 3.6 ± 2 log on sprouts, while resulted in 2.1 ± 0.1 log on the seeds. Bars on the top of column refer to standard deviation. Within each time, same letter (a or b) indicates no difference ($P > 0.05$) between seeds (white) and sprouts (black).

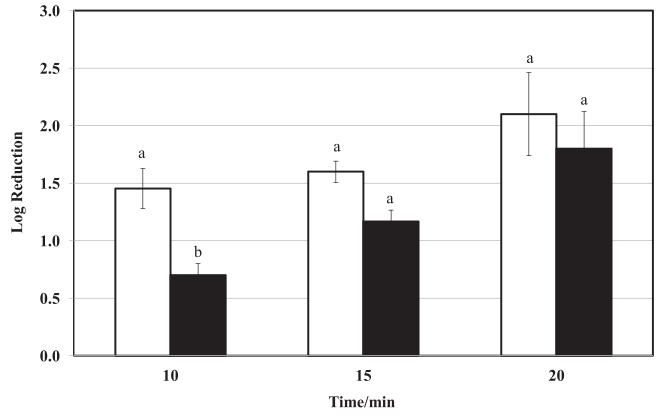

Fig. 2. Log Reduction of STEC on the surface of alfalfa seeds (white column) and sprouts (black column) after subjected to ozonated water 5 mg/L for 10, 15, 20 min. There are no significant differences ($P > 0.05$) in log reduction of STEC between seeds and sprouts by using ozone treatment for 20 min exposure. Ozone resulted in 1.8 ± 2 log on sprouts, and 2.1 ± 0.1 log on the seeds. Error bars indicate standard deviation. Within each time, same letter (a or b) indicates no difference ($P > 0.05$) between seeds (white) and sprouts (black).

geometrical sprouts. The fact that the inoculated seed was subjected to a drying process, resulting in a cross-protective mechanism as stress response (Rodriguez-Romo and Yousef, 2005), whereas bacteria growth during sprouting, possibly without any stress, may have resulted in the pathogens being more sensitive to long exposure to ozone when present in sprouts. This is difficult to prove, especially when STEC did not show the same response. In the absence of more available information, this result will lack a plausible explanation, and ongoing research is expected to provide clarification on this observation. Changes in the inactivation kinetics of ozone depending on the commodity have been reported (Sharma et al., 2003). For STEC, the log reduction was almost identical for sprouts and seeds, which indicates that the sensitivity of STEC to ozonated water may be similar on surface of both alfalfa seeds and sprouts extending the exposure time of seeds and sprouts to > 20 min did not increase the reduction of population of pathogens. This may be due to the short half-life of ozone, which is about 30 min and it starts fading after 20 min (Anonymous, n.d.). The microbiological tests in germinated seeds grown for sprouts showed that the differences in colony counts of *Salmonella* and/or STEC were not more than one log (CFU/ml) during the sprouting time. The seeds were rinsed and moistened with ozonated water for 6 sequential days, and the resulting drain water of every day was tested for numbers of *Salmonella* & STEC. The mean and standard deviation of surviving *Salmonella* and STEC in the drained water for 6 days were 3.1 ± 01 and 2.7 ± 0.3 respectively. This demonstrates the fact that even if a few cells are left after seed treatment, or possible cells in biofilms were not removed by rinsing, the microorganisms are still able to grow to significant numbers due to the ideal growing conditions (Castro-Rosas and Escartin, 2000; Palmai and Buchana, 2002) and will continue to pose a hazard to the consumers (Bridier et al., 2011). Therefore, the ozonizing treatment of germinating seeds during sprout production did not seem to be useful at reducing bacterial pathogens. This points to the possibility that effective seed disinfection may be a major factor in ensuring production of pathogen-free sprouts. However, the requirement for discarding the entire batch of sprouts if the spent water tests positive for pathogens (Federal register, 2015), increases the level of protection for consumers.

The effect of ozonated water on the quality properties of sprouts from germination to harvesting was also determined by comparing weight, color, and LAB counts (as indicators of shelf life) of treated samples with control samples. The mean and standard deviation of weight (g) of sprouts produced using ozonated water vs. control sprouts produced using distilled water were 70.6 ± 1.3 and 73.3 ± 1.3 respectively, and these means were not significantly different ($P > 0.05$). This indicated that the ozonizing treatment did not affect seed germination and sprout yield when treated with ozonated water for 20 min.

Similarly, no significant differences were observed in the color of ozonized sprouts at 20 min exposure time compared to non-ozonized sprouts. The mean and standard deviation of color values (Lightness, Redness, and Yellowness) is shown in (Table 4). The results indicated that ozone treatment did not affect the color of the sprouts. These results were in agreement with Sharma et al. (2002, 2003) who demonstrated that ozone treatments did not have a detrimental effect on quality of alfalfa seeds and sprouts.

When the populations of LAB were determined as a function of storage time, a continued significant increase ($P < 0.05$) was observed over 6 days of storage at 5 °C, from 4.0 ± 0.2 log CFU/g at day zero, to 6.8 ± 0.5 log CFU/g at day 6, and 4.2 ± 0.2 at day zero to 6.8 ± 0.4 log CFU/g at day 6 for controls and ozone-treated sprouts, respectively. However, no differences ($P > 0.05$) were observed between the LAB counts for control vs. ozonated sprouts (Table 5).

While treatment with ozonated water at a concentration of 5 mg/L resulted in a significant reduction of both *Salmonella* and STEC on the surface of alfalfa seeds and sprouts, a reduction of these pathogens to undetectable level on seeds or sprouts was not achieved. These results

Table 4
The effect of treatment with ozonated water on color values of alfalfa sprouts.

Color value	Mean color values[a]	
	Control	Ozone
Lightness, L[b]	64.62 ± 2.1[e]	66.01 ± 0.8
Redness, a[c]	−0.85 ± 0.2	−2.10 ± 0.6
Yellowness, b[d]	14.06 ± 1.3	14.66 ± 0.8

[a] Mean sprout color values after harvesting (n = 9).
[b] Mean of sprout samples of lightness (L) value from the alfalfa seeds that were treated with distilled water or treated with ozonated water.
[c] Mean of sprout samples of redness (a) value from the alfalfa seeds treated with distilled water or ozonated water.
[d] Mean of sprout samples of yellowness (b) value from the alfalfa seeds treated with distilled water or ozonated water.
[e] For each color value, no significant differences ($P > 0.05$) were found between control and ozone-treated sprouts.

Table 5
Counts of lactic acid bacteria (LAB) during storage of sprouts treated with ozonated water.

Day of storage	LAB Log CFU/g (sprouts)[a]	
	Control[b]	Ozone[c]
0	4.0 ± 0.2A[d]	4.2 ± 0.2A
3	5.7 ± 0.1B	5.7 ± 0.2B
6	6.8 ± 0.5C	6.8 ± 0.4C

[a] Mean of Log (CFU) of Lactic acid bacteria on the sprouts (n = 9).
[b] Sprout samples from the alfalfa seeds that were treated with distilled water.
[c] Sprout samples from the alfalfa seeds treated with ozonated water.
[d] Means within each row & column with same letters (A, B, C) are not different (P > 0.05).

agree with Sharma et al. (2002, 2003) who demonstrated that ozone treatment could not completely inactivate or eliminate *Escherichia coli* O157: H7 from the surface of alfalfa seeds and sprouts. It is well documented that even low counts of pathogens in the seeds are likely to result in the presence of these pathogens in the sprouts due to the chances for microbial growth during sprouting (Gabriel, 2005; Howard and Hutcheson, 2003). The design of this study included high levels of initial contamination of seeds. This may be the reason for the countable populations of pathogens in the spent water even when using ozonated water. However, the antimicrobial treatment of seeds also impacts the prevalence of pathogens and therefore the magnitude of the risk for contaminated sprouts reaching the consumer. During a Monte Carlo modeling of behavior of pathogens in sprouts, Montville and Schaffner (2005) determined that although antimicrobial treatment of seeds did not guarantee absence of pathogens in the sprouts, it did reduce the risk for contaminated sprouts to reach the consumer, and that this reduction was affected by the initial levels of contamination of the seed. The 2.1 log reductions obtained in the present study for *Salmonella* and STEC on alfalfa seeds, coupled with 3.6 and 1.8 log reductions of these pathogens in sprouts, may result in a reduction in the risk of foodborne illness, especially regarding the reductions in seeds. A recent risk assessment for salmonellosis linked to consumption of alfalfa sprouts showed that each log reduction of seed treatment provided a greater risk reduction, and that the risk reduction was increased by seed treatment combined with spent water testing (Chen et al., 2018).

Almost all previous studies testing ozone treatments to reduce pathogens on alfalfa seeds or sprouts have been focused on inactivating *E. coli* O157: H7 (Sharma et al., 2002; Sharma et al., 2003; Singh et al., 2003; Sharma et al., 2004), may be overlooking *Salmonella* reduction on alfalfa seeds or sprouts. However, *Salmonella* has been the etiologic agent in most of the outbreaks associated with alfalfa sprouts. In fact, between 1988 and 2016, there were at least 32 outbreaks of *Salmonella* infection associated with alfalfa sprouts, while only few outbreaks of *E. coli* O157:H7 infection were reported in that period (Taormina et al., 1999; FSN, 2016; CDC, 2016). Therefore, this study focused on inactivation of *Salmonella* and STEC on alfalfa seeds and their sprouts. Based on the results obtained, it was noticed the *Salmonella* was more sensitive to ozone treatment on alfalfa sprouts than STEC. Since it appears that each pathogen responded differently to ozone treatment, it is imperative to continue research to determine the value of pathogen interventions such as ozone treatment, on managing food safety in alfalfa seed or sprouts. On the other hand, greater reductions of *Salmonella* and STEC were achieved on both alfalfa seeds and sprouts with lower ozone concentration (5 mg/L) compared to other studies. Sharma et al. (2002) Investigated the inactivation of *E. coli* O157:H7 on alfalfa seeds, and they were able to reduce a maximum of 2.2 log of *E. coli* 157: H7 with an ozone concentration of 21 mg/L. Singh et al. (2003) reduced *E. coli* populations by 1.75 log cycles with a maximum ozone concentration of 14.3 mg/L for 10 min exposure time. Finally, Sharma et al. (2003) were able to reduce the population of *E. coli* O157: H7 by 2.2 log after exposing alfalfa sprouts to ozonated water at a concentration of 20 mg/L for 64 min.

Since the efficacy of ozone is reduced in the presence of organic compounds (Güzel-Seydim et al., 2004), the organic matter present on alfalfa seeds and sprouts is the possible reason for incomplete pathogen inactivation within alfalfa seeds and sprouts. Under the conditions of this study, treatment with ozonated water was not successful at reducing pathogens to non-detectable levels on alfalfa seeds or sprouts. Given the ability of *Salmonella* and STEC, as well as other pathogens (Castro-Rosas and Escartin, 2000; Palmai and Buchana, 2002) to grow during sprouting, even one viable cell after seed treatment would represent a high risk of infection after sprouting. To achieve the reduction of human pathogens to undetectable levels on alfalfa seeds or sprouts, the use of combined interventions may be a good option. Numerous reports of research evaluating interventions that used combined treatments are available in the literature. In general, combined interventions have been found to be more effective than using an individual treatment (Bari et al., 2003; Bari et al., 2009; Neetoo et al., 2009; Scouten and Beuchat, 2002; Sharma et al., 2002; Singh et al., 2003). Since the current concentration of ozone treatment did not affect the quality of products, combining ozone treatment (at 5 mg/L) with other decontamination processes may result in a higher reduction of both pathogens that would be consistent with a sufficient reduction of the risk of foodborne illness.

The methods and procedures followed in this study were design to be applied in scenarios resembling current industry practices. The data generated in this study may be applied as an alternative for compliance with the recently released Food and Drug Administration standards for ensuring the safety of produce, including sprouts (Federal Register, 2015). This rule requires that seeds that are used to grow sprouts should be treated using a scientifically valid method to reduce microorganisms of public health significance, if it is known or there is reason to believe that a lot of seeds or beans may be contaminated with a pathogen. Furthermore, this rule also requires using clean water along the entire sprouting process. In these two cases, ozone treatment may be used as an intervention to eliminate the potential risk of pathogenic bacteria on the surface of alfalfa seeds and sprouts.

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