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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: