

Research Note

A Novel Aqueous Ozone Treatment as a Spray Chill Intervention against *Escherichia coli* O157:H7 on Surfaces of Fresh Beef

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ABSTRACT

This experiment determined the efficacy of using a novel aqueous ozone treatment as a spray chill intervention to reduce *Escherichia coli* O157:H7 on surfaces of fresh beef compared with traditional water spray chill. Cutaneous trunci muscles were obtained from a local beef cattle processing plant. Muscles were divided into sixteen 25-cm² sections, and each section was individually inoculated with *E. coli* O157:H7 to the final concentration of approximately 10⁵ CFU/cm². Muscle sections were collected and tested before and after simulated spray chill treatments of water or the novel aqueous ozone solution. Enumeration of survivors of each treatment was compared with that of the untreated muscle sections. Water spray chill reduced ($P \leq 0.05$) *E. coli* O157:H7 0.60 log, whereas the aqueous ozone spray chill reduction ($P \leq 0.05$) was 1.46 log on surfaces of fresh beef. Aqueous ozone spray chill reduced ($P \leq 0.05$) aerobic bacteria 0.99 log, but water spray chill did not significantly ($P > 0.05$) reduce aerobic bacteria on surfaces of fresh beef. The findings indicate that aqueous ozone can be used as a spray chill intervention to enhance the safety of beef.

HIGHLIGHTS

- Aqueous ozone spray chill could be an effective antimicrobial intervention.
- Nanobubble technology may enhance the efficacy of ozone application.
- These results need to be validated under commercial conditions.

Key words: Aqueous ozone; Beef; *Escherichia coli* O157:H7; Spray chill intervention

Most strains of *Escherichia coli* are harmless and live in the intestines of humans and other animals, *E. coli* O157:H7, however, produces Shiga toxin(s) that can cause severe illness. *E. coli* O157:H7 can potentially contaminate beef carcasses during hide and intestine removal processes. Subsequent fabrication and processing steps may spread bacteria to other products. An estimated 265,000 Shiga toxin-producing *E. coli* infections occur in the United States (10), whereas *E. coli* O157:H7 is also responsible for an estimated 96,000 cases of infection, 3,700 hospitalizations, and 31 deaths in the United States each year (11, 39). From 2003 to 2012, approximately 25 outbreaks of *E. coli* O157:H7 were attributed to beef consumption (23). To reduce the risk of contaminated beef, processors implement a comprehensive, multiple-hurdle food-safety system. One of those hurdles involves spraying hot carcasses with antimicrobial solutions. The effectiveness of antimicrobial interventions with hot water, diluted organic acids and their salts, and oxidizer solutions in reducing *E. coli* O157:H7 on carcass surfaces is well documented (2, 3, 5, 13, 18, 19, 27–

29, 31, 38). These antimicrobial interventions have been implemented for carcasses to reduce the pathogen before entering the hot box for chilling. The carcasses are chilled for 24 to 48 h before fabrication. During the chilling, potable water or an antimicrobial is lightly sprayed intermittently to speed chilling and to prevent or reduce moisture loss from the carcasses but also to provide another antimicrobial intervention step. However, the spray chill antimicrobial interventions currently used are expensive to maintain and may be highly corrosive.

Ozone is a naturally occurring, water-soluble gas that acts as a strong oxidizer. Ozone was approved by the U.S. Food and Drug Administration in June 2001 (45) as a sanitizer for food contact surfaces and for direct application on food products. Two major advantages of ozone are as follows: (i) ozone attacks the cellular membrane of bacterial cells, making it an effective germicide; and (ii) ozone autodecomposes rapidly to produce oxygen, thus leaving no residue in food (16). These two advantages of ozone make it an attractive, potential antimicrobial technology for the food industry. Several studies have been conducted with ozone as an antimicrobial intervention to reduce bacteria on the surfaces of fresh meat. However, the efficacy of the ozone

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treatment has been highly variable. Aqueous ozone had a 2.5-log reduction in aerobic bacteria on beef tissue with an 0.5% ozone concentration (22). Another study reported that gaseous ozone reduced aerobic bacteria and coliforms on pork by 0.45 to 1.04 log and 0.26 to 0.30 log, respectively (26). Beef trimmings dipped in 1% ozone solution for 7 or 15 min effectively reduced *E. coli* and *Salmonella* Typhimurium (41). However, other studies reported that aqueous ozone treatment did not reduce *E. coli* O157:H7 or *Salmonella* on the surface of beef carcasses and bovine head and cheek meat (8, 28). Information on the use of ozonated water as a spray chill in the hot box to control pathogens is limited. Conventional aqueous ozone is produced by passing oxygen through a high-voltage field (corona discharge) to add an extra oxygen atom to the oxygen molecule creating ozone. That ozone is infused into cold tap water to form aqueous ozone (34). In addition, new nanobubble technology is available that creates a more stable suspension of ozone in solution with smaller and fewer ozone bubbles than conventional ozonated water. The aqueous ozone solution, which contains a greater amount of ozone and has greater oxidation reduction potential, provides a more effective cleaning and sanitizing system (34). Therefore, the objective of this study was to evaluate the efficacy of aqueous ozone spray chill produced from nanobubble technology for reducing *E. coli* O157:H7 contamination on fresh beef.

MATERIALS AND METHODS

Bacterial strain, growth conditions, inoculum preparation. An *E. coli* O157:H7 strain that lacked both Shiga toxins (ATCC 43888) was used for inoculation. This strain was selected because it can be easily differentiated from background microflora and has well-defined characteristics as well as historical avirulence (24). The *E. coli* O157:H7 strain ATCC 43888 is also known as the strain B6914-MS1 (B6914), and its genomic sequence is similar to that of the *E. coli* O157:H7 outbreak strain (44). In some studies, the ATCC 43888 strain has been described to be more resistant than other wild-type strains (17, 44, 46), making it a good surrogate. The ATCC 43888 strain has been used extensively, alone or as part of a pool of strains, in several applied research studies because of its low potential pathogenicity (14, 17, 27, 32, 33, 46, 47). The ATCC 43888 strain was grown statically in nutrient broth for 16 to 18 h at 37°C. Bacterial cell concentration was adjusted to approximately 1×10^8 CFU/mL with a spectrophotometer at 600 nm to determine the optical density (36). The bacterial cells were further 10-fold diluted with sterile saline solution to serve as inoculum and kept in an ice bath before inoculation to prevent bacterial cell proliferation.

Fresh beef preparation for inoculation and aqueous ozone treatment. Frozen vacuum-packaged beef flanks (cutaneous trunci muscle; pH approximately 5.6 to 5.8) previously collected from a local beef packing plant were thawed at 4°C overnight and marked with edible ink to make four 100-cm² sections. Each 100-cm² section was divided into four 25-cm² sections making a total of sixteen 25-cm² sections for testing per beef flank. A 50- μ L aliquot of the inoculum was spotted individually on each 25-cm² section, spread with a sterile cell spreader, and held at room temperature for 15 to 20 min to allow bacterial cell attachment to the meat and to allow bacterial cell resuscitation from the cold stress. The holding time of 15 to 20 min was based on evidence

that most attachments of *E. coli* occur between 1 and 20 min over a wide temperature range (2.5 to 37°C) with little increase occurring after 20 min (7). Eight 25-cm² tissue sections were chosen randomly, recorded for location (top or bottom part of the beef flank), excised, and placed individually into eight sterile filter bags to serve as untreated controls for the initial population. A refrigerator was equipped with spray bars and three hooks to hang flanks to simulate carcass spray chill conditions. After sampling for controls, the inoculated, fresh beef flanks were hung vertically inside the refrigerator to simulate carcasses hanging in a hot box. Aqueous ozone or water control spray treatment was conducted inside the refrigerator, and two nozzles were used to spray each beef flank. The spray treatment (either chilled water or chilled aqueous ozone at 4.6 to 5.6°C) was 90 s of spray every 30 min for 12 h. Another set of eight 25-cm² tissue sections was chosen randomly, recorded for location, and excised after 12 h of Viriditec aqueous ozone (TetraClean/CleanCore Technologies, Omaha, NE) or water spray treatment. The Viriditec aqueous ozone system has been patented and uses a proprietary nanobubble technology to combine water and ozone to yield aqueous ozone. The concentration of aqueous ozone was 12 ppm with an oxidation-reduction potential of approximately 2.6 V measured with a digital dissolved-ozone monitor (Q45H, Analytical Technology, Inc., Collegeville, PA) and an oxidation-reduction potential monitor (Q46R, Analytical Technology, Inc.), respectively. Aqueous ozone was sprayed at 6 to 8 lb/in² at the nozzles (SSTG-1, Tee-Jet, Dultmeier Sales, Omaha, NE) with approximately 0.12 L/min per nozzle. Three beef flanks were used for each of three independent replications for the study.

Microbiological and statistical analyses. Enumeration of the survivors was determined by both nonselective and selective media. The rationale behind the use of the nonselective medium was that antimicrobial treatments not only kill but also inflict sublethal injury to microorganisms (28). Therefore, the use of selective medium only to enumerate microorganisms might lead to overestimation of the effectiveness of the antimicrobial compounds because sublethally injured cells might not be able to grow in the presence of selective agents. Both control and treated samples were also enumerated on a nonselective medium to allow injured cells to resuscitate and multiply. Each sample bag was vigorously homogenized with 75 mL of Dey/Engley neutralizing broth supplemented with 3.5 g/L potassium phosphate dibasic and 1.5 g/L potassium phosphate monobasic (Sigma-Aldrich, St. Louis, MO) for 1 min using a stomacher (BagMixer 400, Interscience, Rockland, MA). A 1-mL aliquot of homogenate was serially diluted 10-fold, with a maximum-recovery diluent (Difco, BD, Franklin lakes, NJ), and appropriate dilutions were surface plated on tryptic soy agar (Difco, BD; as a nonselective medium for aerobic plate count (21)) and on ChromAgar O157 (DRG International Inc., Springfield, NJ; as a selective agar medium for *E. coli* O157:H7 enumeration). Plates were incubated at 37°C for 24 h before counting the surviving colonies and reported as CFU per cubic centimeter. Presumptive colonies (two colonies per plate) on ChromAgar O157 were picked for dry spot latex agglutination test (Remel Inc., Lenexa, KS) and were confirmed as *E. coli* O157:H7 using a PCR assay for the combination of O157 (reverse followed by forward: 5'-GTGTCCATTATACGGACATCCATG-3', reverse followed by reverse: 5'-CCTATAACGTCATGCCAATATTGCC-3'), H7 flagella (FLIC_{H7} forward: 5'-GCGCTGTCGAGTTCTATCGAGC-3', FLIC_{H7} reverse: 5'-CAACGGTGACTTATCGCCATTCC-3'), intimin (Int forward: 5'-GACTGTGCATGCATCAGGCAAAG-3', Int reverse: 5'-TTGGAGTATTAACATTAACCCAGG-3'), Shiga

TABLE 1. Survival population of *E. coli* O157:H7 and aerobic bacteria on fresh beef after aqueous ozone spray chill application

Treatment ^a	n	Survivors (log CFU/cm ²) ^b	
		O157	APC ^c
Control	24	5.19 ± 0.18 A	5.40 ± 0.08 A
Water	24	4.54 ± 0.29 B (0.60) ^d	5.31 ± 0.15 A (0.09)
Control	72	5.24 ± 0.13 A	5.42 ± 0.10 A
Ozone	72	3.78 ± 0.38 C (1.46)	4.43 ± 0.24 B (0.99)

^a Control, no treatment of any kind; water, water spray chill for 90 s every 30 min for 12 h; ozone, treated with aqueous ozone for 90 s every 30 min for 12 h.

^b Means within the same column with no common letter are significantly different ($P \leq 0.05$).

^c APC, aerobic plate count.

^d Numbers in parentheses indicate log reduction.

toxin 1 (SLT-I forward: 5'-TGTAAGTGGAAAGGTGGAGTATAC-3', SLT-I reverse: 5'-GCTATTCTGAGTCAACGAAAAA-TAAC-3'), and Shiga toxin 2 (SLT-II forward: 5'-GTTTTTCTTCGGTATCCTATTCCG-3', SLT-II reverse: 5'-GATGCATCTCTGGTCATTGTATTAC-3') genes (25).

Colony counts from control and treated samples for both aqueous ozone and water treatments were transformed to log CFU per square centimeter values from 3 experimental replications × 3 beef flanks × 8 tissue sections ($n = 72$) for each treatment. For spray chill with water, the colony counts were transformed to log CFU per square centimeter values from 3 beef flanks × 8 tissue sections ($n = 24$). For evaluation of the effect of aqueous ozone spray on the top and the bottom sections of fresh beef, the data were sorted by location before statistical analysis. One-way statistical analysis (analysis of variance) was performed with JMP software (version 13.1.0, Statistical Discovery, SAS Institute, Cary, NC) for the main effects of treatment and location. Least-squares means were calculated, and pairwise comparisons of means were determined by the Tukey-Kramer test method with a probability level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Spray chill with cold water reduced ($P \leq 0.05$) *E. coli* O157:H7 by approximately 0.5 log, whereas cold water spray chill had no statistical effect ($P > 0.05$) on the reduction of aerobic bacteria (Table 1). Greater bacterial reductions on selective medium (ChromAgar O157), but not on nonselective medium (tryptic soy agar), indicated sublethal injury of bacterial cells during 12 h of spray chill treatment as well as during refrigeration. Typically, the injured bacterial cells cannot grow on medium with selective agents during enumeration (42). Chilling itself also causes a slight reduction in microbial count on carcasses and has been shown to reduce the microbial population on carcasses by only 0.3 to 0.7 log (37, 43).

In this study, aqueous ozone spray chill significantly reduced ($P \leq 0.05$) both *E. coli* O157:H7 and aerobic bacteria on the surfaces of fresh beef. Compared with the spray chill of cold water, the spray chill with aqueous ozone at 12 ppm reduced *E. coli* O157:H7 ($P \leq 0.05$) 1.46 log (Table 1), which was an addition of a 0.86-log reduction more than by that of water alone. Similarly, spray chill with aqueous ozone reduced aerobic bacteria ($P \leq 0.05$) by 0.99

TABLE 2. Effect of sampling location on the reduction of *E. coli* O157:H7 and aerobic bacteria on fresh beef after aqueous ozone spray chill application

Sampling location	n	Log reduction (CFU/cm ²) ^a	
		O157	APC
Top section	36	1.57 ± 0.39 A	1.03 ± 0.26 A
Bottom section	36	1.35 ± 0.40 B	0.94 ± 0.24 A

^a Means within the same column with no common letter are significantly different ($P \leq 0.05$).

log, which was an additional 0.90-log reduction more than that of water alone.

Because spray chill nozzles in hot boxes are located on top of the rails, the cold-water rinsing action is from the top to the bottom of the carcasses. To simulate spray chill in the beef hot box, this study used nozzles installed at the top part of the refrigerator. This allowed a comparison of the efficacy of aqueous ozone in reducing bacterial cells on the top and bottom of beef flanks from the treatment cascading down from the top to bottom of the flanks. Ozone spray chill reduced *E. coli* O157:H7 more ($P \leq 0.05$) on the top sections than it did on the bottom sections, whereas no location difference for aerobic bacteria was observed (Table 2). The greater reduction of *E. coli* O157:H7 on the top section was due to contacting fresh aqueous ozone before it lost efficacy because of organic matter as it cascaded down to the bottom section. However, the reduction in aerobic bacteria was the same ($P > 0.05$) on the top and the bottom sections of beef flanks. This result was because the bacterial cells injured by the aqueous ozone treatment repaired themselves on nonselective bacterial medium and proliferated during enumeration, as previously reported (28). These findings indicate that the ozone treatment may have had reduced efficacy because of the decomposition of ozone by the time it reaches the lower portions of carcasses. Fresh beef is rich in heme iron, which is an initiator of ozone decomposition (6, 40) by selectively adsorbing the iron atoms of the heme prosthetic groups of hemoglobin (9). Thus, the aqueous ozone treatment should be tested in commercial applications to evaluate its efficacy on the lower portions of carcasses.

Aqueous ozone acts as a dipolar ion (6) with electrophilic and nucleophilic properties. Ozone reacts with organic and/or inorganic compounds in one of the following pathways (40): direct reaction with molecular ozone, or decomposition of ozone into a radical before reacting with the compound. Previous results on the application of ozone as an intervention for fresh meat are conflicting, basically because of factors changing reactivity and antimicrobial efficacy. For example, ozone treatment has not been effective when applied to hot carcasses because the high-temperature exchange reduces the solubility and stability of ozone (1, 4). The surface pH of hot carcasses (prerigor, pH 6.2 to 6.8) is closer to neutral-causing ozone to be less stable after contact with meat compared with lower surface pH of postrigor meat (pH 5.5 to 5.8). Kim (30) reported that the stability of ozone in solution was the greatest when pH was 5.0, whereas ozone stability decreased as pH increased,

and no ozone was detected in a buffer with a pH of 9.0. Because ozone is a strong oxidizer, the presence of organic substances with high ozone reactivity may compete with microorganisms for ozone (15). Therefore, the temperature and pH of fresh meat, as well as the ozone concentration, should be considered before the application of ozone.

In conclusion, the experiment was designed to treat fresh beef with a high concentration of a novel aqueous ozone as a low-pressure spray treatment to reduce ozone off-gassing (release of excess ozone into the work environment) and a low-temperature treatment to limit ozone decomposition in a simulated beef carcass spray chill application. The proprietary technology to generate high quantity and stability of the aqueous ozone as well as the longer contact time on fresh beef through a spray chill application produced $\leq 90\%$ inactivation of *E. coli* O157:H7 and aerobic bacteria. Aqueous ozone not only inactivated the target bacteria but also inflicted sublethal injury to the bacterial cells. The injured bacterial cells cannot proliferate under stressed conditions of low-temperature storage and usually subsequently die under those conditions (12, 20, 35). The study was performed under controlled conditions, rather than in a commercial operation; therefore, the validation of the novel aqueous ozone spray chill treatment under actual, in-plant conditions will ultimately be necessary.

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