Efficacy of Ozone Against *Escherichia coli* O157:H7 on Apples

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ABSTRACT: Apples were inoculated with *Escherichia coli* O157:H7 and treated with ozone. Sanitization treatments were more effective when ozone was bubbled during apple washing than by dipping apples in pre-ozonated water. The corresponding decreases in counts of *E. coli* O157:H7 during 3-min treatments were 3.7 and 2.6 $\log_{10}$ CFU on apple surface, respectively, compared to $< 1 \log_{10}$ CFU decrease in the stem-calyx region in both delivery methods. Optimum conditions for decontamination of whole apples with ozone included a pretreatment with a wetting agent, followed by bubbling ozone for 3 min in the wash water, which decreased the count of *E. coli* O157:H7 by 3.3 $\log_{10}$ CFU/g.

Keywords: ozone, apple, *E. coli* O157:H7

Introduction

ESCHERICHIA COLI O157:H7 HAS EMERGED IN THE PAST TWO decades as an important cause of foodborne illness with symptoms ranging from hemorrhagic colitis to hemolytic uremic syndrome (Doyle 1991). Consumption of unpasteurized contaminated apple juice or cider has been linked to outbreaks of diseases caused by this pathogen (Besser and others 1993; CDC 1996). These disease outbreaks prompted the United States Food and Drug Administration (FDA) to regulate the production of cider with recommendations for the pasteurization of apple cider and other juice products or the use of alternative processing steps to reduce the counts of the pathogen in question by 5 $\log_{10}$ /mL (FDA 1998).

Alternative methods to cider pasteurization have been investigated. These methods include high pressure processing, pulsed electric field, ultraviolet irradiation, and addition of organic acid preservatives (Applebaum 1998; Evrendilek and others 1999; Uljas and Ingham 1999; Zhao and others 1993). Use of effective sanitizers on whole apples prior to pressing is a feasible option that may improve the safety of cider. Chlorine, hydrogen peroxide, combinations of hydrogen peroxide with surfactants, and isothiocyanate have been investigated (Beuchat and others 1998; Sapers and others 1999; Lin and others 2000). Heated solutions of hydrogen peroxide with acidic surfactants reduced the bacterial population by 3 to 4 logs (Sapers and others 1999) on whole apples. At present, it is not obvious whether any of these treatments can be used commercially to substitute the pasteurization process of the cider.

Ozone is an effective sanitizer with superior disinfecting properties when applied for the treatment of water and wastewater (Kessel and others 1943; Korich and others 1990; Scarpino and others 1972). Moreover, rapid decomposition of ozone to oxygen and lack of toxic residues make it a favorable environment-friendly sanitizer. Kim and others (1999) tested ozone in lettuce processing and reported that bubbling ozone reduced counts of natural microflora in the range of 2 to 3 $\log_{10}$ CFU/g. However, use of ozone to sanitize apples has not been explored. The objectives of this study are to define conditions for effective ozonation processes of whole apples inoculated with *E. coli* O157:H7, and enhance the effectiveness of ozone through selected pretreatments.

Materials and Methods

Organism and culture media

Escherichia coli O157:H7 (ATCC 35150), was obtained from the Department of Microbiology at The Ohio State University, Columbus. Stock cultures were maintained on slants of Trypticase soy agar (TSA) (Difco Laboratories, Detroit, Mich., U.S.A.) at 4 °C with occasional transfers. The bacterium was propagated by making 2 successive transfers in 10 mL Trypticase soy broth (TSB; Difco). The inoculated broth was incubated at 37 °C for 20 to 22 h before using the inoculum in these experiments. A portion (35 μl) of the resulting culture was transferred into 100 mL TSB and the mixture was incubated at 37 °C for 18 to 19 h with agitation; count at the end of the incubation period was ~10^9 CFU/mL. This culture was appropriately diluted, using 0.85% NaCl solution, and used for inoculation of apples.

Preparation and inoculation of apples

Unwaxed Red Delicious or Jonathan apples were purchased from a local store, refrigerated, and held at 22 to 25 °C for 24 h immediately prior to use. Whole, sound apples (100 to 120 g each) were washed with a 0.5% aqueous solution of a detergent (BacDown, Fisher Scientific, Pittsburgh, Pa., U.S.A., rinsed in tap water and wiped dry. Each apple was dipped in the diluted bacterial suspension (22 to 25 °C), in a 700-mL beaker, stirred gently for 1 min, drained, and placed on a sterile tray. Inoculated apples were incubated for 2.5 h at 22 to 25 ° C prior to the sanitization treatments.

Ozone generation and measurement

Ozone (12 to 14% O_3 in the gaseous output mixture, 1.45 L/min total O_2/O_3 gas output) was produced on site by an electrochemical process using an ozone generator (Lynntech, Inc., College Station, Tex., U.S.A.). The electrochemical process of ozone generation includes splitting water molecules into hydrogen and oxygen atoms and recombination of the oxygen atoms to form ozone and oxygen. These gases are phase-separated from the water and deliv-

ered as a gas or dissolved into water as indicated later.

Ozone gas was bubbled into a beaker containing deionized water (1000 mL) for a specified time. Fine bubbles were obtained using a stainless steel sparger with 10 μm pore size (Solvent inlet Filter, Fisher Scientific, Fairlawn, N.J., U.S.A.). The entire experimental setup was placed in a chemical hood and all necessary safety precautions were followed. Excess ozone gas was passed into an ozone-decomposing column containing a heated catalyst (Lynntech, Inc.).

Measurement of ozone

For preparation of aqueous ozone, concentration was monitored continuously by measuring absorbance at 258 nm (A_{258}), using a UV spectrophotometer (Spectronic 1201, Milton Roy Co. Rochester, N.Y., U.S.A.), as described in a previous study (Kim and Yousef 2000). A chemical procedure (Bader and Hoigne 1981) using the indigo indicator (Aldrich Chemical Co., Inc., Milwaukee, Wis., U.S.A.), was used to measure the concentration of ozone in water during or after the washing process (residual ozone). This method is suitable for measurements of residual ozone since the presence of extraneous substances in apple wash water may affect the accuracy of the spectrophotometric method. Ozone decolorizes indigo trisulfonate and the resulting color changes are measured at A_{600}.

Treatments

Each treatment group, within an experiment, was comprised of 3 apples (individually treated and analyzed) and each experiment was run 2 or 3 times. Apples in the control group were inoculated with *E. coli* O157:H7, but they were not subjected to any treatment (unwashed) or washed in water under conditions similar to those of ozone treatments. The following treatments were carried out.

Evaluation of ozone delivery methods. Dipping inoculated apples in ozonated water was compared to washing the apples in bubbling ozone water. For dipping, apples were individually immersed in water (1000 mL at 22 to 24 °C) that had been previously ozonated to achieve an aqueous ozone concentration of 24 to 25 mg/L, and agitated for 1, 3, or 5 min using a magnetic stirrer. Agitation speed was adjusted to ensure that the apple was fully immersed and exposed to ozonated water. For bubbling ozone wash, individual apples were placed in water and ozone was bubbled continuously for 1, 3, or 5 min with agitation as described earlier. The residual ozone concentrations at the end of the bubbling treatments (1, 3, and 5 min) were measured and were ~21, 25, and 28 mg/L, respectively. To observe the influence of temperature on the ozonation process, water at 4, 22, or 45 °C was used. Apples were placed in bubbling ozone water at these temperatures for 3 min. The residual ozone concentration following the treatments at these temperatures were 36, 22, and 18 mg/L, respectively.

Pretreatments to enhance the effectiveness of ozone. Inoculated apples were subjected, individually, to 1 of the following pretreatments before they were washed in aqueous bubbling ozone for 3 min at 22 to 24 °C.

- A spray bottle, filled with sterile distilled water, was used to spray apples before the ozone treatment. The water spray (~ 4-5 mL/apple) was directed for 10 sec at the calyx and the stem ends of inoculated apples.
- Apples were cored, before or after the ozone treatment, to physically remove the hard-to-reach *E. coli* O157:H7 contaminants.
- The apples were dipped in a 0.1% solution of the wetting agent, tetrasodium pyrophosphate, (Sigma Chemical Co., St. Louis, Mo., U.S.A.) at 22 to 24 °C and agitated for 2 min. Apples that were rinsed in the wetting agent solution followed by a water wash were included as controls.

Enumeration of survivors.

Following the treatments, whole or portions (surface and stem-calyx regions) of apples were analyzed. In case of whole apples, a ~ 25-g wedge was aseptically cut, chopped with a sterile knife, mixed with 0.1% peptone water (1:10, w/v) in a blender jar, and homogenized at a medium speed for 45 sec. When portions were analyzed, each apple was cored aseptically using a sterile metal corer (15 mm diameter). The core, including the stem and calyx portion, was then weighed and blended in 0.1% peptone water as indicated earlier. Similarly, a 25 g portion of the rest of the apple (surface) was chopped using a sterile knife and blended with peptone water. Homogenized samples were serially diluted and pour-plated, in duplicate, using TSA (Difco) and Violet Red Bile Agar (VRBA, Difco) and plates were incubated at 37 °C for 24 h. Each apple was analyzed separately and average counts ($\log_{10}$ CFU/g) were calculated. Because of high inoculation rate, compared to the level of natural flora on apples, and lack of cell injury by the treatments, differences between counts on VRBA and TSA were minimal (data not shown); therefore, counts on VRBA only were reported. Selected colonies were confirmed as *E. coli* O157:H7 using a commercial test kit (Petrifilm for Hemorrhagic *E. coli* O157:H7, 3M Health Care, St. Paul, Minn., U.S.A.).

Statistical analysis

Counts ($\log_{10}$ CFU/g) of controls (inoculated unwashed or water-washed apples) and inoculated, ozone-treated apples were analyzed by the Minitab statistical analysis software (Minitab Inc., State College, Pa., U.S.A.). One-way analy-

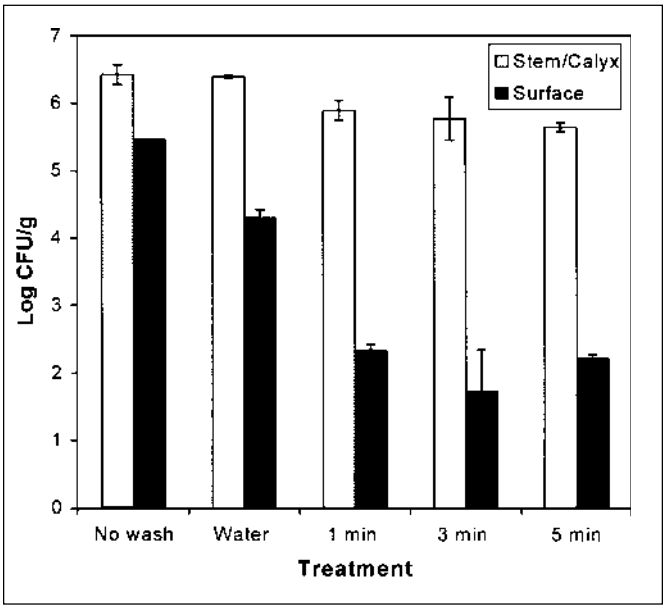

Figure 1—Counts of *Escherichia coli* O157:H7 ($\log_{10}$ CFU/g) on inoculated apples that were unwashed, water-washed, or treated with bubbling ozone in water at 22 to 25 °C; residual ozone concentration at 1 min: 20.8 mg/L, 3min: 24.5 mg/L, and 5 min: 27.7 mg/L. Error bars represent the standard deviation of the mean of 6 apples.

sis of variance was used to compare the effect of the pretreatments with regard to ozone efficacy. The effect of ozone treatments at different exposure times (1 to 5 min) was analyzed using the two-way analysis of variance. When significant, means were compared using Tukey's test.

Results and Discussion

Evaluation of ozone delivery methods

Populations of *E. coli* O157:H7 recovered from unwashed and water-washed inoculated apples were significantly different ($P < 0.05$) on the apple's surface region (Figure 1). Washing with water decreased the population of the pathogen 1.2 $\log_{10}$ CFU/g on the surface region, but no decrease in count was detected in the stem-calyx area. Sapers and others (1999), reported < 1 $\log_{10}$ CFU/g decrease on whole apples by water washing. Counts of *E. coli* O157: H7 on the surface region were significantly different ($P < 0.001$) between the control (unwashed or water washed) and the ozone treated apples (Figure 1).

Two ozone delivery methods that are potentially applicable to commercial sanitization of apples were compared in this study: (1) dipping in ozonated water (22 to 24 mg O_3/L), and (2) washing in bubbling ozone water (~21, 25, and 28 mg O_3/L residual ozone). In both methods, apples were treated for 1, 3, and 5 min. Maximum decreases in surface counts of *E. coli* O157:H7 were 3.7 and 2.6 $\log_{10}$ CFU/g when apples were treated for 3 min by washing in water with bubbling ozone or dipping in ozonated water, respectively (Figure 1 and 2), compared to unwashed controls. In both delivery methods, counts of *E. coli* O157:H7 in the stem-calyx region did not decrease appreciably; the bubbling ozone wash and dip method decreased these populations 0.6 and 0.5 $\log_{10}$ CFU/g, respectively. Differences in counts among the 3 exposure times, in both the delivery methods, were not significant ($P > 0.1$). Kim and others (1999) reported that increasing the time of treating lettuce with bubbling ozone increased the inactivation rates of microorganisms. This is contrary to the results observed in the current study. Moreover, a 5-min exposure time in washing apples is likely to be impractical in industrial facilities. An earlier study (Farooq and others 1990) suggested that ozone concentration in the liquid film at the gas liquid interface is higher than in the surrounding solution, which may account for the greater efficacy of bubbling, compared to dipping treatments. Therefore, ozone bubbling was used in the remainder of the study.

Effect of water temperature during ozonation

When apples were treated with bubbling ozone for 3 min at 4, 22, and 45 °C, counts of *E. coli* O157:H7 decreased 3.3, 3.7, and 3.4 at the surface, and 0.4, 0.4, and 0.2 $\log_{10}$ CFU/g on the stem-calyx regions, respectively (Figure 3). Statistical analysis, however, showed no significant difference between the 3 treatments ($P > 0.05$). The residual ozone concentration was greatest at the lowest temperature (4 °C) and decreased with increasing temperature (Figure 3). At colder temperatures ozone is relatively stable, but as the temperature increases the decomposition rate increases (Sease 1976). Additionally, efficacy of ozone should increase when treatment temperature increases. It appears that when treatment temperature was increased in this study, the increase in ozone reactivity compensated for the decrease in its stability, and thus no appreciable change in efficacy was observed. On the contrary, Kim (1998) observed that ozone reduced more contaminants when it was applied at higher than refrigeration temperatures.

Pretreatments to enhance ozone effectiveness.

Ozone inactivated *E. coli* O157: H7 effectively on the surface, but its efficacy was limited in the inaccessible areas (the stem and the calyx regions) of the apple. Therefore, inoculated apples were subjected to pretreatments to help ex-

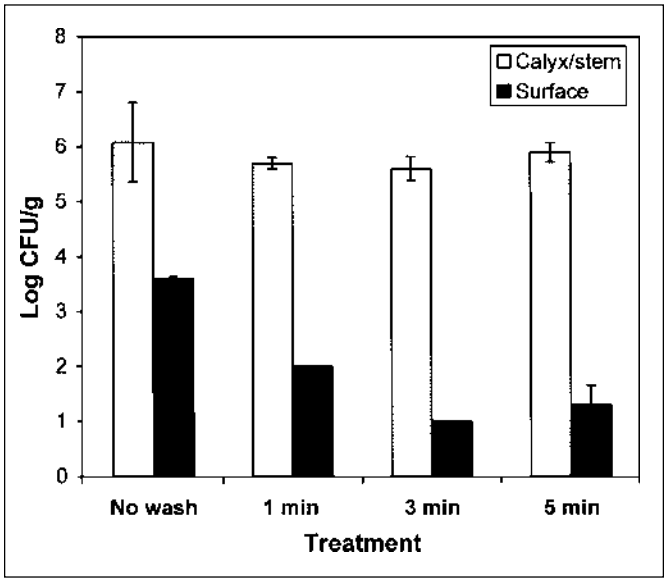

Figure 2—Counts of *Escherichia coli* O157:H7 ($\log_{10}$ CFU/g) on inoculated apples that were dipped in ozonated water (22 to 24 mg O_3/L) at 22 to 25 °C for up to 5 min. Error bars represent the standard deviation of the mean of 6 apples.

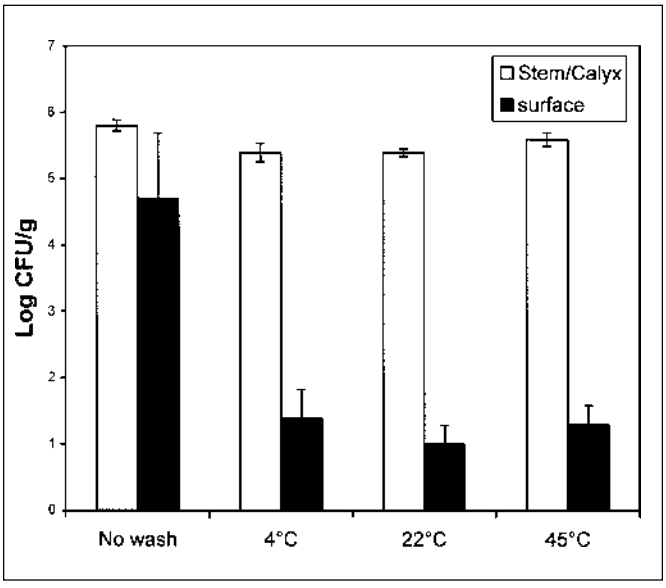

Figure 3—Counts of *E. coli* O157:H7 ($\log_{10}$ CFU/g) on inoculated apples treated with bubbling ozone in water at different temperatures for 3 min; residual ozone at 4 °C: 36 mg/L, 22 °C: 22mg/L, and 45 °C: 18 mg/L. Error bars represent the standard deviation of the mean of 4 apples.

pose cells in these areas to the ozone wash. Spraying the apple's stem-calyx region with water prior to the ozone wash decreased the count of *E. coli* O157: H7 in core samples by 1.5 $\log_{10}$ CFU/g (Figure 4). Control apples, which received a water spray followed by a water rinse showed a reduction of 0.8 $\log_{10}$ CFU/g only. However, the difference in counts between the control (sprayed and water washed) and ozone treated (sprayed and ozone washed) core samples was not significant ($P > 0.1$). The spray followed by ozone wash decreased the population of *E. coli* O157:H7 on the apple surface region 3.6 $\log_{10}$ CFU/g (Figure 4), which is comparable to the decrease observed when apples were treated with bubbling ozone alone (Figure 2). In an earlier study, chicken pieces were inoculated with *Salmonella* Enteritidis and sprayed or dipped in ozonated water. Greater inactivation of *S.* Enteritidis was observed on sprayed than on dipped pieces (Dave 1999).

Combining ozone treatment with apple coring gave different results depending on the sequence of treatments. Counts of *E. coli* O157:H7 on apples that had been ozone-treated and then cored was 3.6 log smaller than that on the whole inoculated apples prior to the treatment. In contrast, counts of *E. coli* O157:H7 on apples that were cored before ozone-treatment was only 1.4 log smaller than that on whole inoculated nontreated apples. Limited inactivation in the latter case can be attributed to (1) internalization of *E. coli* O157:H7 into apple tissues during the coring process, or (2) increase in ozone demand as the result of coring; thus apple tissues may have competed with microorganisms for available ozone.

Population of *E. coli* O157:H7 on whole apples, rinsed in the wetting agent (tetrasodium pyrophosphate) before the ozone wash, decreased 3 to 3.5 $\log_{10}$ CFU/g. The decrease was significantly greater ($P < 0.05$) than that observed on apples washed in water after the wetting agent rinse (Figure 5). Use of surfactants in combination with antimicrobial agents such as hydrogen peroxide and organic acids have been tested earlier (Sapers and others 1999). Organic surfactants have ozone demand, and thus were not used in treatment combinations in this study. Tetrasodium pyrophosphate, a wetting agent with low ozone demand, was used instead. The wetting agent may have enhanced the contact between ozone and bacterial cells that are attached to the hydrophobic surface of the apple, decreased cell attachment on the stem and calyx areas, and thus assisted in exposing entrapped cells to ozone.

Ozone is a superior disinfectant when used against microorganisms suspended in pure water or buffer (Kim 1998; Achen 2000). It is also effective in reducing counts of *E. coli* O157:H7 on the surface region of apples, an area where contamination is most likely to occur when apples drop from the trees. A lesser ozone efficacy was observed on the stem-calyx than the surface region. This may be caused by the attachment of the inoculated bacterium to the rough surfaces of the stem-calyx area or the inaccessibility of the microorganism in this region to the action of the sanitizer. In our experiments, concentration of residual ozone after the apple treatment was high; survival of *E. coli* O157:H7 in the core region in the presence of these high residuals indicates that the bacterium was not exposed to the ozone.

In conventional apple washing environments, the efficacy of ozone against microbial contaminants may become limited because of the high organic loads in the washing tanks resulting from debris, soils, and fruit saps; these contaminants impose an ozone demand. Decontamination of apples with ozone, however, may become feasible if apples are washed with water first to reduce ozone demand, pretreated with an inorganic wetting agent (such as tetrasodium pyrophos-

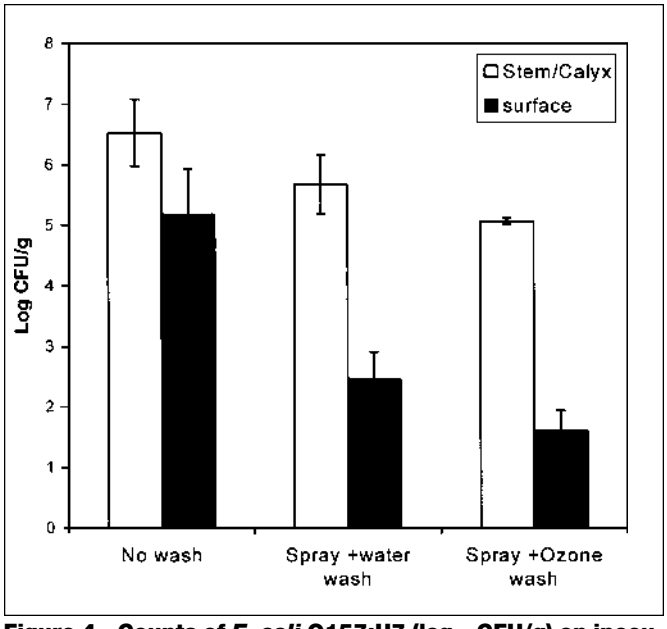

Figure 4—Counts of *E. coli* O157:H7 ($\log_{10}$ CFU/g) on inoculated apples that were spray washed (~20 second water spray on stem and calyx regions) and treated with bubbling ozone in water (23 to 25 mg/L residual ozone) at 22 to 24 °C. Error bars represent the standard deviation of the mean of 6 apples.

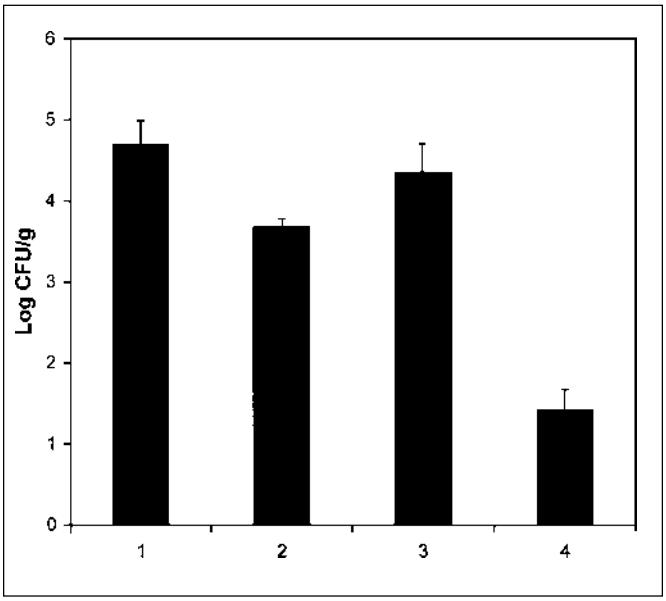

Figure 5—Counts of *Escherichia coli* O157:H7 ($\log_{10}$ CFU/g) on inoculated apples that were rinsed in 0.1% wetting agent (Tetrasodium pyrophosphate) and treated with bubbling ozone in water (23 to 25 mg/L residual ozone) at 22 to 25 °C for 3 min. The bars represent the standard deviation of the mean of 6 apples. 1: No wash; 2: Water wash; 3: Surfactant + water wash; 4: Surfactant + ozone wash

phate), and then treated for 3 min in bubbling ozone water. A similar washing procedure was tested in this study and decreased 3.3 $\log_{10}$ *E. coli* O157:H7/g whole apple. Future research, however, should be directed towards improving ozone delivery methods to increase the accessibility of ozone to the attached cells on all regions of the apple.

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