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Gaseous Ozone Treatment of Baby Spinach within the Existing Production Chain for Inactivation of *Escherichia coli* O157:H7

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ABSTRACT

Sanitization of fresh fruits and vegetables, particularly leafy greens, is limited by penetration of sanitizers to the location of internalized pathogens. It is necessary, therefore, to adapt sanitization operations to practices in the existing produce chain. In this work, we investigated experimentally in a pilot scale, various potential sanitization options using gaseous ozone during and post vacuum cooling on the inactivation of *Escherichia coli* O157:H7 ATCC 43889. It was found that vacuum cooling causes bacterial internalization, making them harder to reach by sanitizer. However, the application of ozone during the vacuum cooling step significantly reduced ($p < 0.05$) population of viable internalized bacteria which otherwise remain unaffected by sodium hypochlorite and UV light surface treatments. The presence of free water inside the vessel available for evaporation during vacuum cooling was found to impact the microbial reduction during combined vacuum cooling and the gaseous ozone treatment. The efficacy of application of high ozone concentration (1.5 g/m^3) short term during the vacuum cooling step in combination with low ozone concentration ($0.032\text{-}0.528 \text{ g/m}^3$) long term sanitization treatments (days) was evaluated. This combination of gaseous treatment was found more effective in microbial reduction compared to a single treatment with a strongly expressed synergistic effect. The post-treatment spinach quality evaluation, however showed an increasing degree of damage as the time of treatment increases even at low ozone concentrations.

INTRODUCTION

The consumption of fresh fruits and vegetables in the U.S. increased by 32% from 1982 to 1997 (Anonymous, 2001). Increased demand and variety of fresh produce, along with growing imports from countries having various standards of sanitation and worker hygiene, have resulted in an increasing number of outbreaks associated with its consumption (Tauxe et al., 1997). According to reported epidemiological data, leafy green vegetables are among the most frequently contaminated food products (Brackett, 2005; deWaal, 2007).

There are multiple ways for pathogens to contaminate fresh produce. Growing on an open field exposes products to animal and bird feces, irrigation water, fertilizer, and other potential sources of pathogens (De Roever, 1998). Irrigation or rainwater usually serves as a transport vehicle. Once on the surface, the pathogens have sufficient time for deep internalization in crevices, pores and stomata. Since it is practically impossible to control contamination of product growing on open fields, the packer/processor may have to rely on postharvest decontamination to protect consumers from foodborne illness (Eschenbach, 2007). To minimize the prevalence of foodborne disease and reduce microbial contaminations in food supplies, an effective strategy for application of sanitizers is essential (Tirpanalan et al., 2011). A number of sanitizers based on organic acids (Mendonca et al., 2004; Ortega et al., 2011; Zhao et al., 2009), chlorine (Pirovani et al., 2001; Singh et al., 2002), biocides (Knowles and Roller, 2001; Singh et al., 2002), ozone (Hunt and Marinas, 1999; Khadre et al., 2001; Singh et al., 2002; Vurma et al., 2009) and their combinations (Zhou et al., 2007) have been proposed over the years. Nevertheless, chlorine based liquid sanitizers (in the range 50 -200 ppm free chlorine) still remain the most widely used for disinfection of fresh produce (Luo et al., 2011; Velázquez et al., 2009). It is commonly accepted that liquid sanitizers have difficulties in reaching bacteria embedded in small crevices

and stomata. Sanitizers in gaseous form are more stable and mobile compared to the aqueous form (Guzel-Seydim et al., 2004; Shynkaryk et al., 2015; Vurma et al., 2009). Several gaseous sanitizers were evaluated in recent years, including ozone (Jin-Gab et al., 1999; Khadre et al., 2001), chlorine dioxide (Sun-Young et al., 2004) and cold plasma (Perni et al., 2008). Among them, ozone was reported to have the highest oxidation potential of -2.07 V (12).

Gaseous sanitizers are typically able to penetrate crevices within produce much faster than liquids, since the diffusivity of gases ($\sim 10^{-5} \text{ m}^2/\text{s}$) is four orders of magnitude higher than liquids ($\sim 10^{-9} \text{ m}^2/\text{s}$). However, sanitizers such as ozone or chlorine dioxide also are aggressive oxidizers, and may damage sensitive products such as spinach (Vurma et al., 2009). It has been shown in our previous work (Shynkaryk et al., 2015) that the rates of diffusion and reaction of sanitizer with produce are decisive in determining whether or not a given treatment is effective. For example, a high-speed wash operation is effective for dirt removal, but does not allow enough time for sanitizer diffusion to occur. However, there are various stages of fresh produce processing and handling when an application of sanitizer can be undertaken. In this work, we focus on the possibility of an early postharvest intervention for baby spinach with gaseous ozone treatment during the vacuum cooling and transportation steps. The objectives of this study were to evaluate the effectiveness of gaseous ozone for decontamination of spinach inoculated with *Escherichia coli* O157:H7, and to identify an optimal strategy for application of ozone during existing steps within the produce production chain.

MATERIALS AND METHODS

Operations in the produce chain

First, we identify the sequence of operations commonly associated with the produce chain, in justifying the choice of intervention operations. Figure 1 shows the typical sequence of

operations for leafy vegetables (information partly from Kader 2002, and partly by direct observations). After harvest, leafy greens are typically transported for vacuum cooling, then shipped to packing sites at various locations, where they are cut, washed, centrifuged and packaged. Some potential points of intervention are also detailed in Fig. 1.

The liquid sanitizer spray step provides an opportunity to decrease initial microbial counts in leafy greens by use of a surfactant/organic acid combination. However, this operation is not within the scope of this work, and is addressed in a separate paper. The next treatment; vacuum cooling, involves the application of a vacuum to a load of vegetables, causing evaporation of surface water, and cooling by removal of the latent heat (Karel and Lund, 2003). Thereafter, the vacuum is broken, permitting air or modified atmosphere to enter the chamber. The entire process takes approximately a half-hour. The vacuum cooling operation offers an excellent opportunity for incorporation of gaseous sanitizers (Vurma et al., 2009). Since vacuum cooling is currently a widespread practice, the incorporation of a sanitizer is a modification in an existing process, and could be accomplished at lower cost than a separate stand-alone process. The next step is the transport to the packing facility, which may take up to 96 hours. This is the largest window of time available for sanitizer penetration. By carefully controlled low-concentration dosing of gaseous sanitizer, it should be possible to reach deep internal crevices in produce to inactivate microorganisms that may reside within.

Bacterial strains, culture conditions and preparation of inoculum

A bacterial strain used for experiments was *E. coli* O157:H7 ATCC 43889 obtained from the culture collection of the Department of Food Science and Technology at the Ohio State University. This strain contained genes for green fluorescence protein and for ampicillin resistance, which enabled enumeration of the bacterium in the presence of the natural microbiota

of baby spinach under UV light. In preparation for experiments, a loop of frozen (at – 80°C) culture of *E. coli* was inoculated in LB broth (Difco, Becton-Dickinson, Sparks, MD) and incubated overnight at 37°C. This was followed by another transfer into fresh LB broth for second overnight incubation. Incubated culture was harvested by centrifugation at 8000 rpm for 10 min (IEC Centra MP4R, Needham, MA) and cell pellets were resuspended in 0.1 % wt/vol peptone water (Difco, Becton-Dickinson, Sparks, MD). The concentration of bacterial population was adjusted by spectrophotometric (turbidimetric) analysis (Thermo Spectronic Genesys 5 spectrophotometer, Thermo Fisher Scientific, Waltham, MA), to obtain a population of $\sim 10^9$ CFU/ml in the suspension.

Inoculation of fresh produce leaves and enumeration of microorganisms

Baby spinach used in experiments was grown from the seeds (Burpee Gardens, Warminster, PA) on site in the Biological Sciences Greenhouse at the Ohio State University. Samples of 5 g containing about six leaves were spot inoculated with 20 droplets of 2.5 μ L (50 μ L for one sample) of *E. coli* cell suspension to yield an initial population of $\sim 10^7$ CFU/g. Prior to treatment, samples were held in a biosafety cabinet for ~ 1 hr at room temperature to allow the inoculum to dry. When needed, packaged baby spinach purchased from a local grocery store (Kroger, Columbus, OH) on the same day of the experiment was added to obtain the required bulk weight. For the enumeration of microorganisms treated and untreated (control) samples (~ 5 g) were placed in polyethylene stomacher bags and mixed with 45 ml of peptone water. The bags' contents were homogenized for 2 min in a stomacher STO-400 (Tekmar Inc., Cincinnati, OH). The homogenized samples were serially diluted in peptone water and surface plated onto LB agar (Difco, Becton-Dickinson, Sparks, MD) containing ampicillin 100 μ g/ml, uninoculated samples were plated onto TSA (Difco, Becton-Dickinson, Sparks, MD) for general microbial

load count (data not shown). Inoculated Petri dishes were incubated at 37 °C for 24 hrs. The colonies formed on the plates were enumerated under UV light (Foto/Spectrum 3-3200, Fotodyne inc., New Berlin, WI).

Vacuum cooling and gaseous ozone application

All experiments involving ozone treatment were performed in a pilot scale setup (Fig. 2) permitting application of gaseous ozone to leafy greens under controlled pressure, ozone gas delivery and temperature. The setup was located in a biosafety level 2 pathogenic laboratory (Department of Food Science and Technology, The Ohio State University, Columbus, OH). The main components of the pilot unit comprised a custom 0.3 m³ stainless steel pressure vessel equipped with a condensing coil (Walker stainless equipment company Inc., New Lisbon, WI), NESLAB RTE-10 external chiller (Thermo Electron Corporation, Newington, N.H.), high capacity CSF-7 ozone generator (Ozonias Elmwood Park, NJ) connected to a size K cylinder with extra dry oxygen 99.6% (OSU Stores Department, Columbus, OH), XDS35i scroll vacuum pump (Edwards, Lutín, Czech Republic) and thermal destruct unit (Ozonias Elmwood Park, NJ).

To monitor and record temperature, pressure and ozone gas concentration during the experiment an integrated data acquisition system was built on the basis of programmable logic controller HE-XT102 (Horner APG, Indianapolis, IN). The ozone concentration was measured by continuously sampling the gas from a manifold in a vessel wall and pumping it with a Dia-Vac R101-FP-CA1 pump (Air Dimensions Inc, Deerfield Beach, FL) through a high capacity Mini-Hicon ozone analyzer (IN USA, Inc., Needham, MA). The pressure was measured with a sensor model 912022 (Vacuum Research Corp., Pittsburgh, PA) installed in a vessel wall. Several T-type thermocouples (Omega, Stamford, CT) were introduced inside the vessel via a feed-through panel to measure temperatures of produce and the vessel.

Impact of vacuum and pressurization protocols during vacuum cooling treatment

Herein, we tested the inactivation of *E. coli* O157:H7 after an ozone application using three different protocols: ozone injection and hold under vacuum: “Vacuum, O₃ (Under vac)” ; ozone injection under vacuum with an immediate system pressurization to 69 kPa: “Vac O₃ + 69 kPa”; and ozone application at atmospheric pressure with following pressurization to 69 kPa: “O₃ (Atm) + 69 kPa”. In all cases the time of produce exposure to ozone gas was fixed at 30 min and the vessel load with spinach was 26 g/m³ in order to avoid situations in which ozone demand exceeded its availability. An example of the “Vac O₃ + 69 kPa” treatment is described below.

Prior to vacuum cooling and ozonation the vessel temperature was reduced to about 10°C by circulating the coolant at -5°C through the condensing coil. Then, a tray with inoculated spinach was placed inside the vessel and vacuum applied. When the required pressure (0.7 kPa) was reached, the vacuum pump was stopped and a burst of ozone gas (~10 g/m³) was introduced in the vessel at 3.5 m³/h for 18 seconds to obtain ~1.5 g/m³ of final ozone concentration. Once ozone was applied, the vessel was pressurized to 69 kPa (10 psi) by introducing air from a compressed air line, and held for 30 min. After the treatment was completed, the remaining ozone was exhausted for another 30 min through the thermal destruct unit and the recovered samples were immediately analyzed for microbial load.

Produce loading during vacuum cooling and its impact on ozone decomposition and microbial inactivation

Reaction of ozone with surfaces of fresh produce results in its rapid depletion. Shynkark et al. (2015) evaluated the probability of gaseous ozone reaction with the surface of lettuce leaf and demonstrated that it is a critical factor for the depth of sanitizer penetration into the bulk of

produce. This in particular is a concern with large loads of produce within vacuum cooling chambers, since ozone can be degraded rapidly before it can have its intended effect. To study the impact of produce load on ozone decomposition, the “Vac O₃ + 69 kPa” treatment was applied at at 1.5 g/m³ O₃ and different levels of vessel load with spinach starting at 26600g/m³ (which is the maximum possible load, corresponding to 7980 g spinach in the vessel) 2600 g/m³ (775 g in vessel), 260 g/m³ (78 g in vessel) and 26 g/m³ (7.8 g in vessel). Microbial inactivation was evaluated by placing inoculated samples of 5 g containing about six leaves in the middle of the bulk load of produce. Immediately, after the treatment, inoculated samples were analyzed for microbial population.

Effect of water film and moisture on subsequent ozonation.

To prevent the loss of mass due to water evaporation the produce is often misted with water (Karel and Lund, 2003). Experiments were performed to study the impact of produce misting with water on the ozone effectiveness against *E. coli* O157:H7. Dry and misted (sprayed with water to obtain a continuous film) samples of inoculated baby spinach (26 g/m³) were treated with ozone side by side using two different protocols. In one case samples were subjected to vacuum cooling and ozone was injected under vacuum with immediate system pressurization to 69 kPa “Vac O₃ + 69 kPa”, in the other, ozone was applied at atmospheric pressure followed by pressurization to 69 kPa “O₃ (Atm) + 69 kPa”. Immediately after the treatment, inoculated samples were analyzed for microbial population.

Effect of ozone dosage and duration of application during simulated transport

The produce transport to the packing facility is the largest window in the fresh produce production chain that could be potentially adapted for gaseous sanitizer treatment. The sanitization efficacy of applying low-concentration dosing of ozone for extended periods of time

(up to three days) alone and as a follow-up treatment after the ozonation during the vacuum cooling application “Vac O₃ + 69 kPa” was evaluated. The same experimental setup was used for this study and the vessel was maximally loaded with produce (26000 g/m³). The ozone was flushed through the vessel at 2 l/m, 4 °C and the inlet ozone concentration was maintained at 0.032, 0.106, 0.211 and 0.528 g/m³ O₃. The ozone concentration was monitored with a IN-2000 ozone meter (range 0 - 2.1 g/m³, resolution 0.02 mg/m³, IN USA, Inc., Norwood, MA) and its concentration in the middle of the vessel was periodically sampled with a Dia-Vac R101-FP-CA1 pump (Air Dimensions Inc., Deerfield Beach, FL). Pictures of control leaves were taken every day as well as two samples for microbiological monitoring. Untreated inoculated baby spinach leaves were kept refrigerated (at 4 °C) and plated at the same time as ozone treated samples.

Spinach injury evaluation by image analysis

Pictures of spinach leaves were taken in raw format using a Sony NEX 5n camera with a Sony 18-55mm f/3.5-5.6 zoom lens (Sony Electronics Inc., New York, NY) under available light (fluorescent) condition. Accurate color rendition was achieved through calibration to known color patches (X-Rite ColorChecker Passport, X-Rite Inc., Grand Rapids, MI) photographed next to the sample. Images were later adjusted to the known color values of the patches using calibration software (ColorChecker Passport, X-Rite Inc., Grand Rapids, MI). The spinach surface color changes were analyzed on selected squares of 10×10 pixels using free online software Image Color Extract (http://www.coolphptools.com/color_extract) and obtained color values were expressed in Lab color space. Where the L* variable represents lightness, a* and b* - color, with a* describing (–) green - (+) red and b* (–) blue - (+) yellow spectrums.

Statistical analysis

There were at least three independent replicates, with two samples per replicate, for each treatment. Average microbial reductions, expressed in logarithmic values, were used in the statistical analyses. Comparisons between several groups were performed by one-way analysis of variance (ANOVA) using Statistica 8.0 (StatSoft Inc., Tulsa, OK, U.S.A.). This was followed by Tukey's post-hoc analysis to analyze mean differences. Differences at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Impact of vacuum and pressurization protocols during vacuum cooling treatment

Figure 3 compares inactivation of *E. coli* O157:H7 after an ozone application using the three different protocols "Vacuum, O₃ (Under vac)"; "Vac O₃ + 69 kPa"; "O₃(Atm) + 69 kPa" described earlier. Baby spinach treated with ozone under vacuum yielded 1.3 log CFU/g reduction compared to control with inoculum dried for one hour ($p < 0.05$). Ozonation after vacuum cooling with subsequent repressurization to 69 kPa achieved 2.1 log CFU/g reduction; however there was no significant difference between microbial reductions after these two treatments. Baby spinach treated with ozone at atmospheric pressure following repressurization to 69 kPa had significantly higher microbial reduction (2.9 log CFU/g) compared with other treatments. The result suggests that applying vacuum cooling can decrease the amount of inactivated bacteria due to possible vacuum internalization.

Erickson (2012) and Li et al. (2008) reported vacuum-induced internalization of bacteria into stomata and crevices on leafy vegetables surfaces which make them harder to reach by sanitizers. To verify this hypothesis, a separate experiment was conducted to determine the population of bacteria internalized during vacuum cooling. For this study inoculated baby

spinach was either treated by vacuum cooling alone (vacuum released at $\sim 4^{\circ}\text{C}$) or with addition of the gaseous ozone treatment at 1.5 g/m^3 with pressurization to 69 kPa and holding for 30 min. These were followed by surface treatment of the leaves with 1.2% sodium hypochlorite (20% bleach, Clorox Inc, Oakland, CA) for 30 seconds. Then samples were washed with sterile deionized water to remove residual hypochlorite and exposed to UV light for 10 min per side. Surface sanitization was tested by gently pressing the surface treated baby spinach leaves on LB agar plates that were later incubated at 37°C for 48 h. The surface sanitization was considered effective when the incubated plates showed no growth (Li et al., 2008). As a control the total cells inoculated on the leaf surfaces were enumerated by plate counts of the homogenized baby spinach leaves without prior surface sanitization. Results are shown in Figure 4.

The initial concentration on the leaves was 7.5 log CFU/g . There was no significant difference in *E. coli* population between untreated samples and samples subjected to vacuum cooling only, without sanitization ($p > 0.05$). The surface sanitization treatment of inoculated and untreated leaf was successful in all cases giving no growth of leaf prints on LB agar plates. No growth was observed from plating inoculated and surface treated samples not exposed to vacuum. To check whether the vacuum cooling step removes microorganisms from the leaf surfaces, we placed empty opened plates containing LB agar in the vessel close to the inoculated spinach samples. After reaching and breaking the vacuum the plates were retrieved and incubated at 37°C for 24 hrs. The experiment was repeated three times. All plates showed no growth. As expected, the vacuum cooling step compromised sanitization and 3.3 log CFU/g of *E.coli* remained unaffected by the surface sanitization treatment. The application of ozone at 1.5 g/m^3 during the vacuum cooling step significantly reduced ($p < 0.05$) the population of viable

internalized bacteria from 3.2 log CFU/g to 2.2 log CFU/g. This suggests that it is more efficient to apply sanitization prior to the vacuum cooling step, if economically feasible.

Produce loading during vacuum cooling and its impact on ozone decomposition and microbial inactivation

Figure 5 depicts the decomposition rate of gaseous ozone during the treatment at different levels of vessel load with spinach. At the low vessel load of 26 g/m³ which represents a ~17:1 mass ratio of produce to ozone gas, the sanitizer concentration was practically unchanged during the whole period of the treatment. It can be also concluded here that ozone decomposition on the vessel's interior walls is insignificant over the studied period of time. Ten- and hundredfold increases in the produce to sanitizer ratio resulted in visible depletion of ozone due to reaction with spinach leaves surfaces. At 1700:1 (2600g/m³) mass of the produce to ozone ratio, only about 50% of the initial ozone concentration remained by the end of the 30 minute cycle. A further tenfold increase in the vessel load with produce resulted in a rapid ozone depletion to below the detection limit within about 5 minutes.

Reductions in microbial counts corresponding to different levels of spinach loads are shown in Figure 6. The obtained reductions in microbial counts correlated with the ozone decomposition rate at different vessel load levels with produce (Figure 4). A maximum inactivation of about 3 log CFU/g was observed when the minimal amount of spinach (26 g/m³) was used in experiments. The efficacy of sanitization decreased and the inactivation data spread widened as the amount of treated spinach was increased. At the maximum vessel load with the produce of 26600g/m³ the microbial reduction varied within 1-2 log CFU/g approaching the

baseline of CFU reduction resulting from the drying of inoculum on the samples during the drying step.

Effect of water film and moisture on subsequent ozonation.

Figure 7 shows results of experiments where dry and misted samples were treated by ozone side by side using two different protocols. As found earlier, the effectiveness of ozonation for dry samples subjected to vacuum cooling may decrease due to bacterial internalization. However, samples of inoculated baby spinach sprayed with water demonstrated the opposite trend. An average inactivation of 2.5 log CFU/g was obtained for ozonation combined with vacuum cooling comparing to 1.9 log CFU/g reduction when the sample was treated at atmospheric pressure and pressurized. Obviously, the formation of a liquid film on produce surfaces represents a barrier for ozone gas and significantly impairs its effectiveness against bacteria. As has been shown by Shynkaryk et al. (2015) the time required for ozone molecules to diffuse through 1mm water film and reach equilibrium concentration on the leaf surface is about 15 min. Ozone decomposition in water is also about 100 times faster than in gas (Khadre et al, 2001; Okafor, 2011). The higher efficiency of ozone application after the vacuum cooling is apparently related with water removal from produce surfaces making them more accessible for ozone.

Another interesting observation was related with the presence of free water inside the vessel and the degree of microbial reduction during combined vacuum cooling and the gaseous ozone treatment application. As it can be seen in Figure 8, the efficiency of ozone is significantly reduced when there is no free moisture available for evaporation during the vacuum cooling i.e. both vessel surface and leaves' surfaces (10 g total weight) are dry. Unger-Bimczok et al. (2008) studied surface decontamination with hydrogen peroxide vapor and observed that higher humidity levels result in a faster inactivation of the test organisms. The authors suggested the

mechanism of the microcondensation when water vapor absorbs gas molecules and forms microscopic deposition of aqueous hydrogen peroxide solution on the surfaces. A similar mechanism is apparently taking place in the present case. When the vacuum in the vessel is broken by injection of the ozone gas, the evaporated water can absorb ozone molecules and condense on produce surfaces.

Effect of ozone dosage and duration of application during simulated transport

Figure 9 shows survival rates of *E. coli* O157:H7 on baby spinach leaves exposed to low dosage of ozone for up to three days simulating the fresh produce transportation step. The initial concentration on the leaves was above 10^7 log CFU/g.

Keeping inoculated samples in a refrigerator (at 4 °C) as well as continuous treatment with oxygen inside the vessel for three days did not reduce *E. coli* O157:H7 populations on spinach. However, continuous application of 0.032 and 0.106 g/m³ of ozone at atmospheric pressure, which resulted in ~0.21 mg/m³ and ~ 0.42 mg/m³ of ozone inside the treatment vessel respectively, significantly decreased ($p < 0.05$) *E. coli* population by 0.9 log CFU/g and 1.1 log CFU/g compared with the population on control samples. The treatment with 0.211 g/m³ (~ 4.23 mg/m³ inside the vessel) resulted in 1 log CFU/g microbial reduction at the end of the first day, 1.9 log CFU/g and 3.7 log CFU/g *E. coli* O157:H7 reduction at the end of the second and third days respectively ($p < 0.05$). Ozonation with the inlet concentration of sanitizer at 0.53 g/m³ (~10.6 mg/m³ inside the vessel) achieved 1.6 log CFU/g of *E. coli* O157:H7 reduction at the end of the first day and 4.5 log CFU/g at the end of the second day, however there was no significant difference between microbial reductions after the second and third days.

Figure 10 show pictures of baby spinach leaves exposed to low ozone concentration treatment (0.106 g/m³ and 0.211 g/m³ inlet) taken before and after one, two and three days of

treatment. The open squares show area where color measurements were taken and the enlarged squares are filled with measured color and contain Lab color space data. For the ozone treatment at 0.106 g/m³ a slight changes in leaf color (with L* increase from 46 to 51, and b* shift by 10 units toward yellow) was observed even after the third day. A loss of leaf turgor, however, was present due to water loss, despite the vast availability of condensate inside the vessel. An increase of the ozone inlet concentration to 0.211 g/m³ resulted in a formation of light green spots already after the first day of treatment and more pronounced leaf bleaching as the time of exposure increased from 1 to 3 days. The bleaching translated into L* channel increase from 51 to 83 from day 0 to day 3 and slight shift in a spectrum a* toward the red color with spectrum b* remaining practically unchanged.

Figure 11 depicts results of combination treatment with gaseous ozone when inoculated spinach was first vacuum cooled, sanitized at 1.5 g/m³ ozone with pressurization to 69 kPa for 30 min and then subjected to 0.106 g/m³ of ozone at 2 l/min for up to three days as discussed above. The combination gaseous treatment was more effective in microbial reduction compared to a single high short term or low long term ozone treatment with a strongly expressed synergistic effect. The difference between *E. coli* O157:H7 counts for samples treated with 0.106 g/m³ ozone at inlet and treated with combinational gaseous treatment control samples was 0.5 log CFU/g (p < 0.05) at the end of the 1st day of ozonation treatment. For the second and third days, the difference in microbial counts increased to 1.3 log CFU/g and 3.8 log CFU/g respectively (p < 0.05).

Pictures of intact spinach and spinach after different stages of treatment with gaseous ozone are shown in figure 12. Samples photographed immediately after an application of ozone gas at 1.5 g/m³ under vacuum followed by pressurization to 69 kPa for 30 min did not show any visible

changes in color or leaf appearance. The pictures of the same samples taken after one, two and three days of treatment with low ozone concentration demonstrated an increasing degree of damage as the time of treatment increased. Pictures taken after two and three days also showed significant increase in lightness (L^*) and variations in spectrums a^* and b^* .

It should be noted that our studies involved baby spinach, which is by far the most sensitive to ozone treatment. It is expected that such treatments may be applied to other fruits, vegetables or leafy greens with less quality damage.

CONCLUSIONS

The effectiveness of gaseous ozone application varies depending on the mass ratio of produce to the ozone gas. The microbial count reduction significantly drops when ozone demand significantly exceeds its availability. Vacuum cooling removes liquid from the produce surfaces and increases efficiency of ozone gas against *E. coli* O157:H7. At the same time the sanitizer application after vacuum cooling is less effective due to vacuum internalization of bacteria into produce tissue. On the other hand an application of ozone during the vacuum cooling step significantly reduces population of viable internalized bacteria which otherwise remain unaffected by surface sanitization by sodium hypochlorite and UV light. A mechanism of microcondensation is apparently taking place when the vacuum in the vessel is broken by injection of ozone gas, and the evaporated water absorbs ozone molecules and condenses on produce surfaces. Short time high ozone concentration treatment followed by long term and low ozone concentration sanitization was found to have a synergistic effect on the inactivation of *E. coli* O157:H7. Spinach quality, however, is significantly affected after an extended produce exposure even to low ozone concentrations.

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FIGURE CAPTIONS

- Figure 1. Current postharvest operations for leafy vegetables, showing approximate timelines. Proposed interventions are shown by dotted arrow callouts.
- Figure 2. Pilot scale experimental setup for vacuum cooling and ozone gas treatment.
- Figure 3. Effect of pressure on inactivation of *E. coli* O157:H7. Different letters on bars represents significant difference ($p < 0.05$). Error bars indicates $\pm$ standard deviation of the mean after three replicates.
- Figure 4. Effect of vacuum cooling on internalization of *E. coli* O157:H7. Different letters on bars represents significant difference ($p < 0.05$). Error bars indicates $\pm$ standard deviation of the mean after three replicates.
- Figure 5. The decomposition rate of gaseous ozone at single burst injection at 1.5 g/m^3 , 30 min holding time and different levels of vessel load with spinach. Symbols are the experimental data; dashed lines are drawn for the guidance of the eye.
- Figure 6. Reductions in microbial count for different levels of spinach loads after 30 min treatment and single burst injection of ozone (1.5 g/m^3). Symbols are the experimental data; dashed lines represent the logarithmic fit to the experimental data.
- Figure 7. Effect of the presence of water on the leaves on the inactivation of *E. coli* O157:H7 with ozone. Different letters on bars represents significant difference ($p < 0.05$). Error bars indicates $\pm$ standard deviation of the mean after three replicates.
- Figure 8. Effect of the presence of water on the leaves on the inactivation of *E. coli* O157:H7 with ozone. Different letters on bars represents significant difference ($p < 0.05$). Error bars indicates $\pm$ standard deviation of the mean after three replicates.
- Figure 9. Survival rates of *E. coli* O157:H7 on baby spinach leaves exposed to low dosage of ozone (0.032, 0.106, 0.211, 0.528 g/m^3) and oxygen at atmospheric pressure for up to three days at maximum vessel load with spinach of 26600 g/m^3 and untreated leaves kept in a refrigerator at 4°C .
- Figure 10. Pictures of baby spinach leaves exposed to low dosage of ozone ($0.106, 0.211 \text{ g/m}^3$) at atmospheric pressure for up to three days at maximum vessel load with spinach of 26600 g/m^3 . Open squares shows areas where color measurements were taken and enlarged squares are filled with measured color and shows Lab color data.
- Figure 11. Survival rates of *E. coli* O157:H7 on baby spinach leaves exposed to low dosage of ozone (0.106 g/m^3 , 2 l/m) for up to three days at the maximum vessel load with spinach of 26600 g/m^3 and untreated leaves kept in a refrigerator at 4°C . The circle shows the point at which samples were pretreated by ozone gas at 1.5 g/m^3 applied under vacuum with pressurization to 69 kPa for 30 min.

Figure 12. Pictures of baby spinach pretreated by ozone gas at 1.5 g/m^3 applied under vacuum with pressurization to 69 kPa for 30 min which followed by exposure to low dosage of ozone (0.106 g/m^3 , 2 l/m) for up to three days at the maximum vessel load with spinach of 26600 g/m^3 .

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