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Research Paper

Inactivation of *Escherichia coli* O157:H7 and Natural Microbiota on Spinach Leaves Using Gaseous Ozone during Vacuum Cooling and Simulated Transportation

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ABSTRACT

The aim of this study was to integrate an ozone-based sanitization step into existing processing practices for fresh produce and to evaluate the efficacy of this step against *Escherichia coli* O157:H7. Baby spinach inoculated with *E. coli* O157:H7 ($\sim 10^7$ CFU/g) was treated in a pilot-scale system with combinations of vacuum cooling and sanitizing levels of ozone gas (SanVac). The contribution of process variables (ozone concentration, pressure, and treatment time) to lethality was investigated using response-surface methodology. SanVac processes decreased *E. coli* O157:H7 populations by up to 2.4 log CFU/g. An optimized SanVac process that inactivated 1.8 log CFU/g with no apparent damage to the quality of the spinach had the following parameters: O_3 at 1.5 g/kg gas-mix (935 ppm, vol/vol), 10 psig of holding pressure, and 30 min of holding time. In a separate set of experiments, refrigerated spinach was treated with low ozone levels (8 to 16 mg/kg; 5 to 10 ppm, vol/vol) for up to 3 days in a system that simulated sanitization during transportation (SanTrans). The treatment decreased *E. coli* populations by up to 1.4 log CFU/g, and the optimum process resulted in a 1.0-log inactivation with minimal effect on product quality. In a third group of experiments, freshly harvested unprocessed spinach was inoculated with *E. coli* O157:H7 and sequentially subjected to optimized SanVac and SanTrans processes. This double treatment inactivated 4.1 to ≥5.0 log CFU/g, depending on the treatment time. These novel sanitization approaches were effective in considerably reducing the *E. coli* O157:H7 populations on spinach and should be relatively easy to integrate into existing fresh produce processes and practices.

Safety of fresh produce is a critical and urgent concern. Fields are open systems, and product contamination in this environment cannot be completely avoided. Fresh produce is only minimally processed before consumption, and the risk to consumer health from this source is on the rise *(14, 30).* Therefore, it is prudent to develop mitigation strategies to safeguard against unforeseen contamination events.

Chlorine, mostly in the form of sodium or calcium hypochlorite, is commonly used for washing fresh produce. The sanitizer is added to fresh and recycled wash water at 20 to 200 ppm, mainly to maintain the microbial quality of the water and to prevent cross contamination. Unfortunately, this practice results in only modest decreases in the microbial load of fresh produce *(7, 28).* If *Escherichia coli* O157:H7 and similar pathogens become internalized into leafy produce, they can pose an additional challenge to conventional cleaning or sanitization treatments *(6, 27).* Internalization of enteric pathogens such as *Salmonella* or *E. coli* O157:H7 into edible plant parts has been reported for tomatoes *(18),* radish sprouts *(19),* bean sprouts *(37),* and lettuce *(32).*

Ozone is the most reactive sanitizer known, with an oxidation potential of −2.07 V, compared with −1.49 V for hypochlorous acid and −1.36 V for chlorine *(9).* This antimicrobial agent effectively and rapidly inactivates pathogens in low-ozone–demand medium, including microorganisms known to be chlorine resistant *(21).* Previous findings indicated that aqueous ozone can significantly reduce microbial load on fresh-cut produce *(22).* Treatment of fresh-cut lettuce with aqueous ozone inactivated natural microbiota by 1.4 to 4.6 log CFU/g, but the efficacy of the process depended on the method of application and treatment time. Inactivation of *E. coli* O157:H7 on apples depended largely on the method of ozone delivery *(2).* Ozone is a versatile antimicrobial agent that is permitted for use in food in the United States *(23).*

For a given sanitizer, it is better to use it in the gaseous than in the aqueous state. Diffusion of sanitizer molecules is four orders of magnitude faster in gases than in liquids *(8).* Ozone has a longer half-life in the gaseous than the aqueous state *(23).* In previous studies, gaseous ozone was highly effective against pathogenic contaminants on shell eggs when vacuum was applied before sanitization and when sufficient time was provided for the sanitizer to reach embedded microorganisms *(38).* Numerous novel industrial applications of ozone have emerged in recent years. Ozone reactivity, penetrability, and spontaneous decomposition to a nontoxic product (i.e., O_2) make this gas a viable disin-

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fectant for ensuring the safety and quality of water and food products *(17).* However, the reactivity of ozone makes it corrosive to some materials used in food processing, and the toxicity of this gas obligates users to observe certain safety precautions *(23).*

Considering the rapid increase in sales of fresh produce and the frequent disease outbreaks that were recently associated with the consumption of these minimally processed products *(16),* it is vital to explore new approaches to decontamination of these foods. The goal of this study was to enhance the safety of fresh produce in general and baby spinach in particular by integrating ozone-based sanitization steps into existing processing practices while targeting *E. coli* O157:H7 as the pathogen of concern. The objectives of the study were (i) to develop a new ozone-based sanitization process to be used during vacuum cooling of fresh produce, (ii) to optimize treatment conditions, particularly pressure, ozone concentration, and holding time, for maximum process lethality against *E. coli* O157:H7 without damaging product quality, (iii) to develop an effective gaseous ozone treatment that could be used during transportation or temporary storage of fresh produce, and (iv) to assess the antimicrobial efficacy of sequential ozone treatments during vacuum cooling and simulated transportation.

MATERIALS AND METHODS

Bacterial strain, culture conditions, and preparation of inoculum. *E. coli* O157:H7 strain B6-914 *(15),* a nonvirulent strain, was used throughout the study. This strain contains genes for green fluorescence protein and for ampicillin and cycloheximide resistance, which enabled enumeration of the bacterium in the presence of the natural microbiota of baby spinach. Stock cultures of *E. coli* O157:H7 were stored at −80°C in Luria-Bertani (LB) broth (Difco, Becton Dickinson, Sparks, MD) containing 40% (vol/vol) glycerol. An isolated *E. coli* O157:H7 colony from incubated plates of LB agar (Difco, Becton Dickinson) was transferred and cultured in LB broth twice before use. Both LB agar and LB broth contained 100 μg/ml concentrations each of ampicillin trihydrate (Fisher Biotech, Subiaco, WA) and cycloheximide (Sigma-Aldrich, St. Louis, MO). Overnight cultures were harvested by centrifugation at 8,000 × *g* for 10 min (Sorval RC-5B, DuPont, Wilmington, DE), washed, and resuspended in 0.1% (wt/vol) buffered peptone water to achieve a final concentration of 10^9 CFU/ml.

Inoculation of spinach. Baby spinach (*Spinacia oleracea* L.) was procured from local grocery stores (Columbus, OH) 1 day before testing. For selected experiments, freshly harvested unwashed spinach that had not been vacuum cooled (Fresh Express Inc., Salinas, CA) was used. Samples of fresh produce (25 g) were spot inoculated with 100 μl of *E. coli* O157:H7 to reach approximately 10^7 CFU/g. Samples were then held in a laminar flow biological hood at room temperature for 2 h to allow inoculum drying and attachment before further treatment. For each experiment, two sets of inoculated samples (two for ozone treatment and two for control) were used.

Enumeration of microorganisms. Treated and untreated baby spinach samples (25 g each) were aseptically placed in polyethylene stomacher bags (PE bags, Fisher Scientific Co., Fair Lawn, NJ) and mixed with 225 ml of peptone water. The bag contents were homogenized for 2 min in a stomacher (model STO-400, Tekmar Inc., Cincinnati, OH). The homogenized samples were serially diluted in peptone water and surface plated onto the LB agar containing antibiotics. Plates were incubated at 35°C for 24 to 48 h, and colonies of *E. coli* O157:H7, which were producing green fluorescence under UV light, were counted. Uninoculated samples, which were prepared and homogenized as described, were plated onto tryptic soy agar (Difco, Becton Dickinson), and plates were incubated at 37°C for 24 to 48 h for determining mesophilic aerobic counts.

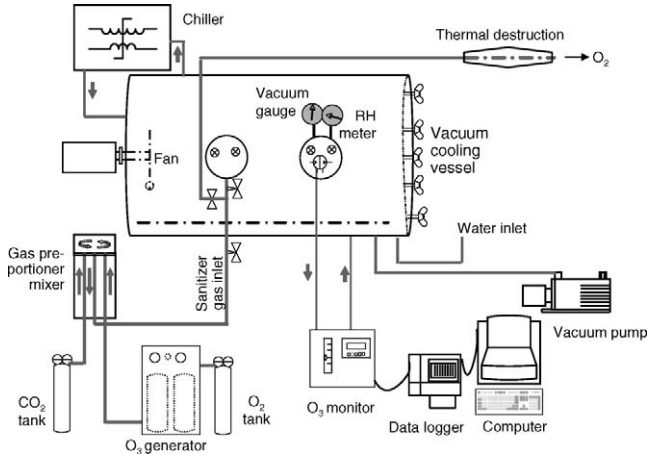

FIGURE 1. *Setup used for treating fresh produce with ozone in tandem with vacuum cooling (SanVac process).*

Ozone generation and measurements. Two ozone generators, Ozat model CSF-7 (Ozonia Inc., Elmwood Park, NJ) and CD-150S (DEL Industries Inc., San Luis Obispo, CA), were used to produce gaseous ozone for these experiments. Model CSF-7 was used in the experiments requiring high ozone concentrations, and model CD-150S was used when low levels of ozone were needed. The concentration of gaseous ozone in ozone treatment chambers was measured using an UV absorption monitor (model LC-L2-2000, IN USA Inc., Norwood, MA). Excess ozone was decomposed into oxygen using a thermal ozone destruct unit (model ODT-006, Ozonia).

Sanitization during vacuum cooling (SanVac), equipment setup. A system was assembled to mimic the industrial vacuum cooling of leafy greens (Fig. 1). In addition to the ozone generator, the system included these main components: (i) a 300-liter stainless steel treatment chamber, (ii) a variable speed control rotary vane pump (model HS 652, Varian Inc., Lexington, MA) to provide the desired level of vacuum during the cooling process, and (iii) a chilling unit (model NESLAB RTE-10, Thermo Electron Corporation, Newington, NH), which circulated a coolant mix (40% propylene glycol; Houghton Chemical Corporation, Allston, MA) through the condensing coil of the treatment vessel. A gas portioning unit (model RK-03218-50, Cole-Parmer Instrument Company, Vernon Hills, IL) was added to the system to deliver a mixture of ozone with other gases, e.g., carbon dioxide. Flow lines for treatment gases were equipped with flow meters to control and regulate the flow rate of each gas. The setup was equipped with a data acquisition system (model 21X micrologger, Campbell Scientific Inc., Logan, UT) to monitor ambient temperature, chamber temperature, vacuum and pressure levels achieved, and concentration of the ozone gas during repressurization and holding steps. A sampling manifold was installed to draw gas samples from two locations in the treatment chamber and to measure the ozone con-

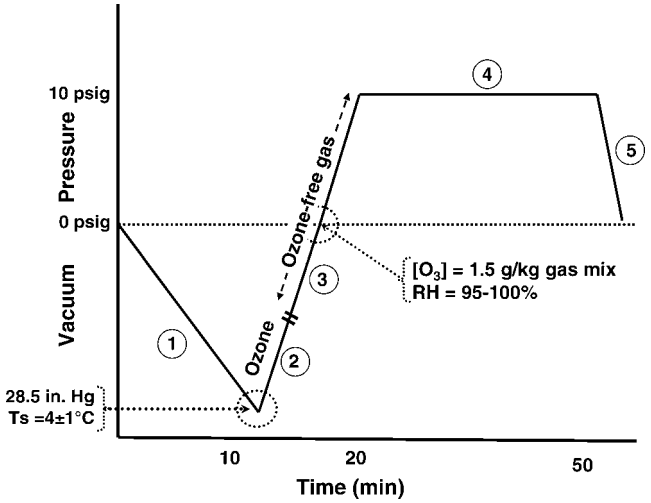

FIGURE 2. *Typical sequence of SanVac treatment, consisting of (1) applying enough vacuum to cool the fresh produce to the desired temperature, (2) repressurizing the vessel with ozone (in oxygen carrier) to reach the targeted concentration of the sanitizer, (3) continuing repressurization with oxygen or other ozone-free gases to reach the target pressure, (4) maintaining the pressure for the specified treatment holding time, and (5) releasing the gases until vessel interior reaches atmospheric pressure.*

centration with an ozone analyzer. The temperature of the condensing coil inside the treatment vessel was maintained at -5 ± 1°C to enhance the vacuum cooling operation. A water inlet line was added to the treatment vessel to adjust the relative humidity (95 to 100%), which was monitored with a thermohygrometer (model OKTAN 03313-70, Cole-Parmer). The system was located in a biosafety level 2 pathogenic plant (Department of Food Science and Technology, The Ohio State University, Columbus). A typical SanVac process sequence is shown schematically in Figure 2. The process included the application of sufficient vacuum to cool the fresh produce to the desired temperature (i.e., 4°C), repressurization of the vessel with ozone (in oxygen milieu) for a specified time and flow rate to reach the targeted concentration of the sanitizer, continued repressurization with oxygen (or other ozone-free gases) to reach the target pressure, maintenance of the pressure for the specified treatment holding time, release of the gases through the thermal ozone-destruct unit, and removal of the product when the vessel interior was at atmospheric pressure and contained a safe level of ozone residue.

Determining *E. coli* O157:H7 lethality at different levels of SanVac treatment variables using response-surface methodology. Samples of inoculated spinach leaves (25 g each) were transferred to the processing vessel and vacuum cooled to reach 4 ± 1°C (~28.5 in. Hg vacuum) within 15 min. Subsequently, the vessel was repressurized with an ozone-oxygen mixture at 55 liters per min flow rate to reach the target ozone concentration (e.g., 1.5 g/kg gaseous atmosphere) and held either under partial vacuum (20 in. Hg vacuum) or above atmospheric pressure (up to 15 psig) for up to 45 min. Surviving *E. coli* O157:H7 populations on spinach were enumerated before and after treatments.

Response-surface methodology was applied to determine the contribution of selected process variables to *E. coli* O157:H7 lethality on spinach. A central composite design was used to develop the response-surface model (JMP IN version 6.0.2; SAS Inc., Cary, NC). Experimental design included the following processing variables: ozone concentration (0.5 to 2.5 g/kg), pressure (0 to 10 psig), and treatment time (15 to 45 min).

Samples of inoculated spinach were vacuum cooled and treated with ozone under the conditions assigned by the central composite design. Each experiment was performed in duplicate, with a total of four samples per experimental condition. Viable *E. coli* O157:H7 populations in spinach were enumerated before and after treatments. Thirty-two experiments were carried out in a random order to develop a response-surface model. The model is represented by the following second order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i<j}^{n} \beta_{ij} X_i X_j + \sum_{j=1}^{n} \beta_{jj} X_j^2 + \varepsilon \qquad (1)$$

where Y is the predicted response, X_i represents the treatment variables, β_0, β_i, β_{ij}, and β_{jj} are the regression coefficients, and ε is the associated random error of the model. In the present study, three variables were tested, hence $n = 3$, and the following equation 2 applies:

$$\begin{aligned}\text{Log reduction } \textit{E. coli} \text{ CFU/g spinach} &= \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \\ &+ \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \\ &+ \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \varepsilon \qquad (2)\end{aligned}$$

where X_1, X_2, and X_3 represent ozone concentration (g/kg), pressure (psig), and treatment time (min), respectively, β_0 is the intercept, β_1, β_2, and β_3 are the linear coefficients, β_{12}, β_{13}, and β_{23} are the interaction coefficients, and β_{11}, β_{22}, and β_{33} are the quadratic coefficients. Statistical analyses included multiple regressions, analyses of variance (ANOVA), and comparisons of bacterial log reduction averages by Tukey's test. The data were analyzed using JMP IN and SAS (version 9.1.3., SAS Inc.). Three-dimensional response-surface figures were generated with commercial software (SigmaPlot version 11.0, Systat Software, Inc., San Jose, CA) to illustrate the main and interactive effects of treatment variables on the inactivation of *E. coli* O157:H7 on spinach. The response-surface model was verified by carrying out six additional experiments with combinations of ozone concentration, pressure, and treatment time.

SanVac process efficacy and survival of *E. coli* O157:H7 during subsequent refrigerated storage. Inoculated spinach (25-g samples) was subjected to the following treatments: (i) vacuum cooling followed by repressurization with ozone, (ii) vacuum cooling followed by repressurization with ozone-CO_2 mixture, (iii) vacuum cooling followed by repressurization with oxygen only (control), and (iv) no treatment (control). Inoculated spinach was vacuum cooled to 4 ± 1°C, repressurized using oxygen, ozone (1.5 g/kg gas mixture), or ozone (1.5 g/kg gas mixture) plus carbon dioxide (50%, vol/vol), and held at 10 psig for 30 min. Surviving *E. coli* O157:H7 cells were enumerated before and after treatments. Samples were refrigerated at ~2°C, and survivors were monitored during storage.

Sanitization during simulated transportation (SanTrans), equipment setup. A separate treatment system was designed to simulate continuous ozonation of fresh produce during 3 days of transportation (Fig. 3). The system consisted of (i) a generator that produces ozone at low concentration (model CD-150S), (ii) a refrigerator retrofitted with ports for ozone inlet and outlet and ozone measurements, and (iii) two glass desiccators modified to serve as treatment vessels, each fitted with a sample holder and three inlet-outlet ports. The system also was equipped with gas flow meters, an ozone monitor, and a thermal ozone destruction unit, as described previously.

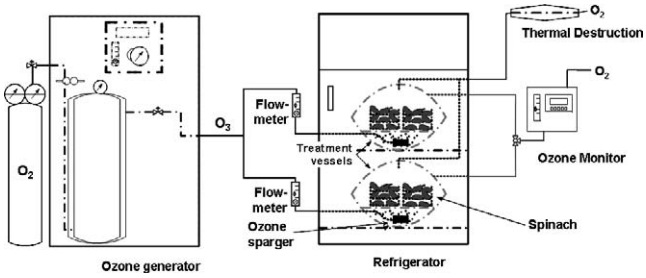

FIGURE 3. *Equipment used for treating fresh produce with low levels of ozone during refrigerated storage, mimicking fresh produce transportation (SanTrans process).*

SanTrans treatment conditions. Spinach samples that were not inoculated (used to test the inactivation of natural microbiota) or were inoculated with *E. coli* O157:H7 were placed in the glass treatment vessel that contained a sterile strainer as a sample holder. Water was added to the bottom of the vessel beneath the sample rack, and ozone was delivered from the ozone generator into the water through a sparger to produce wet ozone gas. This delivery process created bubbling in the water and helped to maintain a high relative humidity (95 to 100%) around the spinach during the 3 days of continuous ozone treatment. Flow rate of the gas mixture inside each of the glass containers was maintained at ~2 liters/min. Ozone concentration was maintained at 8 or 16 mg/kg gaseous atmosphere (5 or 10 ppm, vol/vol) through continuous flushing of the sanitizer into the treatment vessels. A data acquisition system (Campbell Scientific) was used to maintain and monitor the ozone concentration during experiments.

Sequential application of SanVac and SanTrans processes. Freshly harvested unwashed baby spinach that had not been vacuum cooled was used in these experiments. Spinach was inoculated with *E. coli* O157:H7 (~10^7 CFU/g) and subjected to SanVac treatment, which included vacuum cooling followed by ozonation at 1.5 g/kg (i.e., 935 ppm, vol/vol) and pressurization at 10 psig for 30 min. Other inoculated spinach samples were treated with the SanTrans process, which included refrigeration for up to 3 days with continuous sparging with gaseous ozone at 16 mg/kg gas mixture (i.e., 10 ppm, vol/vol). In another treatment, inoculated spinach samples were subjected to SanVac and SanTrans sequentially. Surviving populations of *E. coli* O157:H7 were enumerated before and after treatments and throughout the storage period.

Data analysis. All experiments were repeated twice or three times, and each run consisted of duplicate samples. Microbial counts (CFU per gram of spinach) were converted into log units before analyses, and data were analyzed with the JMP IN program. Mean values for log reduction in bacterial populations were compared using a one-way ANOVA. Tukey's multiple comparisons test was used to analyze mean differences. Values with $P \leq 0.05$ were considered significantly different. Additional statistical analyses were applied to data used for fitting the surface-response model, as indicated earlier. Limited quality assessments, mainly visual observations, of treated spinach were made by the researchers; these observations were not subjected to formal data analysis.

RESULTS

Development of SanVac ozone treatment system. A process has been developed in which fresh produce (baby spinach) was treated with vacuum (28.5 in. Hg vacuum) to induce sufficient cooling (a temperature drop of ~15°C). The cooling was accomplished within 15 min, a rate comparable to those used in the fresh produce industry *(34).* The vessel was repressurized with an ozone-oxygen gas mixture. Release of the sanitizing gas was controlled, allowing the measurement of the dose of the sanitizer and product exposure time. To assess treatment uniformity, preliminary experiments were conducted to measure ozone concentration at various locations in the treatment vessel. The results confirmed that the gaseous mixture was homogeneous inside the treatment vessel.

TABLE 1. *Changes in* Escherichia coli *O157:H7 population on baby spinach when the product was vacuum cooled to 4 ± 1°C and the vessel was repressurized with gaseous ozone and held under pressure (or partial vacuum) for up to 45 min at 95 to 100% relative humidity*[a]

Experiment	Treatment conditions: Ozone concn (g/kg)	Pressure	Time (min)	Decrease in *E. coli* O157:H7 (log CFU/g)[b]
1	3.0	10 psig	0	1.1
2	3.0	10 psig	5	1.5
3	3.0	10 psig	15	1.8
4	2.5	10 psig	15	1.5
5	1.5	10 psig	15	1.4
6	1.5	15 psig	30	1.8
7	1.5	10 psig	45	1.5
8	1.5	20 in. Hg vacuum	30	1.1
9	1.5	10 psig	30	1.8

[a] Initial population on spinach was ~10^7 CFU/g.
[b] Means for two or three independent trials.

Preliminary screening experiments were carried out to determine the lethal contribution of selected processing variables, i.e., ozone concentration and holding pressure and time. Vacuum cooling of spinach followed by repressurization with ozone mixture decreased the population of *E. coli* O157:H7 by 1.1 to 1.8 log CFU/g, depending on these factors (Table 1). When spinach samples were treated with ozone at 1.5 g/kg gas mixture and held under partial vacuum (20 in. Hg vacuum) for 30 min, the *E. coli* O157:H7 inactivation was 1.1 log CFU/g. Holding samples of inoculated spinach at 10 psig with various ozone concentrations inactivated a maximum of 1.8 log CFU/g. However, spinach visual quality was affected when the ozone concentration was 3.0 g/kg, combined with treatment times greater than 5 min (Table 1, experiment 3).

Contribution of SanVac treatment variables to process lethality as assessed by response-surface methodology. Response-surface methodology was applied to systematically study the contribution of the tested treatment variables (ozone concentration, gas pressure, and treatment time) to process lethality. Measured responses (decrease in population, log CFU per gram) at various levels of the process variables were fitted by the response-surface model, and predicted inactivation levels are shown in Table 2. Predicted responses were plotted against process variables; Figures 4 and 5 provide the three-dimensional representa-

TABLE 2. *Experimental design developed using response-surface methodology to assess the inactivation of* Escherichia coli *O157:H7 populations on spinach in response to changes in ozone concentration, pressure, and treatment time*[a]

Trial	Ozone concn (g/kg)	Pressure (psig)	Time (min)	Measured inactivation (Δ log CFU/g)	Predicted inactivation (Δ log CFU/g)
1	0.5	5	30	0.4	0.6
2	1.5	5	30	1.0	1.1
3	1.5	5	30	1.1	1.1
4	1.5	0	30	0.3	0.8
5	1.5	5	30	1.1	1.1
6	0.5	5	30	0.5	0.6
7	2.5	5	30	1.8	1.5
8	1.5	0	30	0.8	0.8
9	2.5	10	15	1.4	1.5
10	2.5	0	15	0.8	0.7
11	1.5	5	45	1.1	1.1
12	0.5	0	15	0.2	0.2
13	2.5	10	15	1.3	1.5
14	1.5	10	30	1.9	1.6
15	2.5	0	45	1.1	1.2
16	0.5	0	45	0.4	0.1
17	2.5	0	15	0.9	0.7
18	0.5	10	15	0.8	0.7
19	1.5	5	30	1.2	1.1
20	2.5	0	45	1.1	1.2
21	1.5	5	15	0.8	0.7
22	2.5	10	45	2.4	2.3
23	2.5	5	30	1.6	1.5
24	0.5	10	45	0.9	1.0
25	1.5	5	15	0.7	0.7
26	1.5	5	45	1.0	1.1
27	1.5	10	30	1.8	1.6
28	2.5	10	45	2.3	2.3
29	0.5	10	45	1.0	1.0
30	0.5	0	45	0.3	0.1
31	0.5	10	15	0.7	0.7
32	0.5	0	15	0.1	0.2

[a] Initial population on spinach was $\sim 10^7$ CFU/g. During treatments, the product was maintained at 4 ± 1°C and 95 to 100% relative humidity.

tions of model data. The ANOVA for the responses indicated that the model was significant ($P < 0.01$), with a high correlation ($r^2 = 0.926$) between measured and predicted data, significant linear ($P < 0.01$) and quadratic ($P < 0.01$) effects, and no considerable lack of fit (Tables 3 and 4). Parameters of estimates (regression coefficients) used in equation 2 are presented in Table 4. Each of the three process variables tested in the study significantly influenced ($P < 0.01$) the inactivation of *E. coli* O157:H7 on spinach. For example, ozone treatment at 1.5 g/kg for 30 min at 10 psig increased the inactivation of *E. coli* O157:H7 by 1.3 log CFU/g when compared with that for samples treated with ozone at the same concentration and treatment time but at atmospheric pressure (Table 2). Clearly, pressure enhances the lethal effect of ozone against *E. coli* O157:H7 on spinach. Rodriguez-Romo and Yousef *(26)* found that use of ozone above atmospheric pressure increased lethality

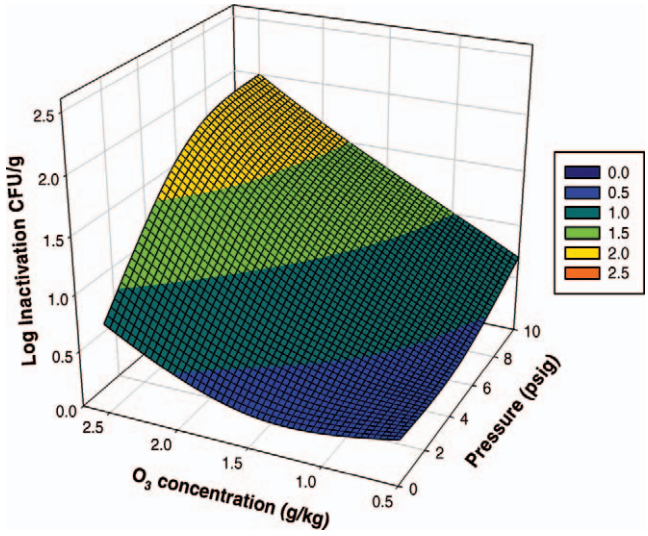

FIGURE 4. *Three-dimensional representation of interaction between ozone concentrations and holding pressures, in relation to inactivation of* Escherichia coli *O157:H7 on baby spinach. Microbial inactivation data were predicted by the response-surface model.*

of this sanitizer against *Salmonella* Enteritidis on shell eggs.

The response-surface model was verified by carrying out six additional experiments representing various combinations of ozone concentration, holding pressure, and treatment time (Table 5). Differences between measured and model-predicted inactivation data were generally negligible, providing evidence that the model is valid. Therefore, the model may be used to predict inactivation of *E. coli* O157:H7 on spinach treated with these SanVac processes.

Based on the results of the response-surface model, the

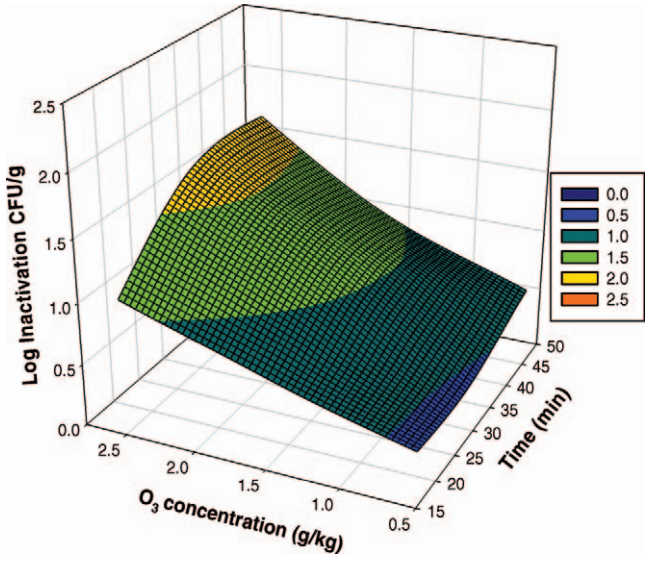

FIGURE 5. *Three-dimensional representation of interaction between ozone concentrations and treatment times, in relation to inactivation of* Escherichia coli *O157:H7 on baby spinach. Microbial inactivation data were predicted by the response-surface model.*

TABLE 3. *Statistical analyses of results of experiments designed by response-surface model, and significance of model variables used during treatment of spinach inoculated with* Escherichia coli *O157:H7 and treated with the SanVac process*[a]

Statistical estimates	Probability
Response-surface model ($n = 32$)	<0.0001
Treatment variables	
Ozone concn (g/kg)	<0.0001
Pressure (psig)	<0.0001
Time (min)	<0.0001
Interactions	
Pressure × time	0.0536
Pressure × ozone concn	0.1490
Time × ozone concn	0.0306

[a] The SanVac process involved vacuum cooling, repressurization with ozone, and holding the product under pressure for a specified time (see Table 2).

greatest lethality was achieved at the maximum levels of the tested treatment variables. These maximum values (2.5 g/kg ozone for 45 min at 10 psig) corresponded to 2.3-log inactivation (predicted), but the visual quality of spinach was negatively affected compared with control samples. Optimum treatment conditions, i.e., those producing the greatest lethality with no apparent loss of product quality, were selected for subsequent experiments. These conditions were ozone concentration of 1.5 g/kg, holding pressure of 10 psig, and treatment time of 30 min.

SanVac process efficacy and subsequent survival of *E. coli* O157:H7 during refrigerated storage. During a SanVac process, inoculated spinach samples (~10^7 CFU/g) were vacuum cooled, subjected to oxygen, ozone (~1.5 g/kg), or ozone (~1.5 g/kg) plus carbon dioxide (50%, vol/vol) at 10 psig for 30 min, and stored in a refrigerator for 7 days (Fig. 6). Surviving *E. coli* O157:H7 populations were monitored during treatment and refrigerated storage. The ozone treatment alone or in combination with carbon dioxide significantly decreased ($P < 0.05$) the *E. coli* O157:H7 population by ~2.0 log CFU/g compared with untreated or oxygen-treated controls. This 2-log difference remained relatively constant throughout the 7 days of storage. Populations of *E. coli* O157:H7 on control and treated spinach remained unchanged during refrigerated storage ($P > 0.05$).

TABLE 4. *Parameters of estimates (regression coefficients) of the polynomial model (equation 2) applied to data obtained during inactivation of* Escherichia coli *O157:H7 on spinach by treatment with the SanVac process*[a]

Term	Estimate	Standard error	*t* ratio	Prob > |*t*|
β_0	1.0983	0.0616	17.8400	<0.0001
β_1	0.4700	0.0411	11.4300	<0.0001
β_2	0.4250	0.0411	10.3350	<0.0001
β_3	0.1950	0.0411	4.7420	<0.0001
β_{12}	0.0688	0.0460	1.4950	0.1490
β_{13}	0.1063	0.0460	2.3110	0.0306
β_{23}	0.0938	0.0460	2.0390	0.0536
β_{11}	−0.0224	0.0801	−0.2800	0.7822
β_{22}	0.1026	0.0801	1.2810	0.2136
β_{33}	−0.1974	0.0801	−2.4650	0.0220

[a] The SanVac process involved vacuum cooling, repressurization with ozone, and holding the product under pressure for a specified time (see Table 2).

TABLE 5. *Verification experiments for response-surface model for inactivation of* Escherichia coli *O157:H7 on spinach by SanVac processes at selected treatment parameters*[a]

Trial	Ozone concn (g/kg)	Pressure (psig)	Time (min)	Measured inactivation (Δ log CFU/g)	Predicted inactivation (Δ log CFU/g)
1	1.5	2	30	0.5	0.9
2	1.5	8	30	1.6	1.4
3	1.5	10	5	0.5	0.6
4	1.5	10	40	1.5	1.7
5	0.4	10	30	0.8	1.0
6	1.6	10	30	1.9	1.7

[a] Initial population on spinach was ~10^7 CFU/g. During treatments, the product was maintained at 4 ± 1°C and 95 to 100% relative humidity.

Inactivation of *E. coli* O157:H7 on spinach during SanTrans treatments. The SanTrans process involved application of low ozone concentrations to spinach held under conditions that mimic transportation. Continuous treatment with a very low ozone concentration of 8 mg/kg (5 ppm,

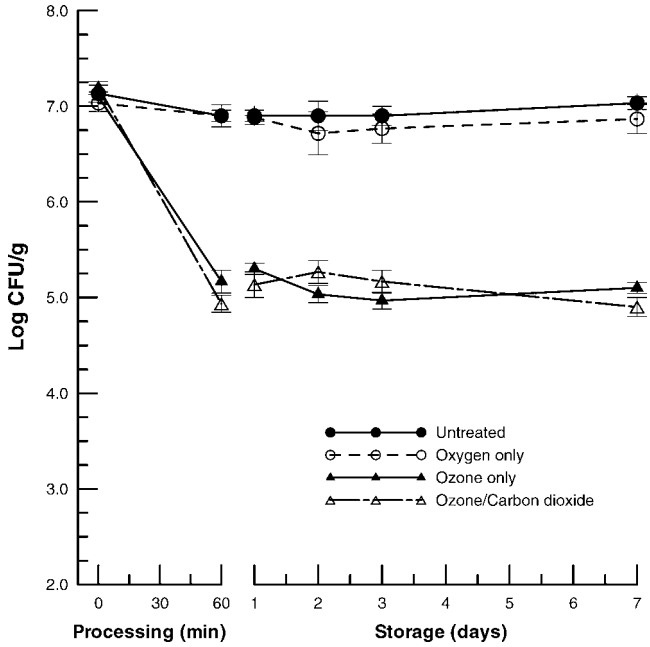

FIGURE 6. *Changes in populations of* Escherichia coli *O157:H7 on fresh spinach when the inoculated produce was subjected to the SanVac process and stored at 2 ± 1°C for 7 days. The SanVac process includes vacuum cooling to 4 ± 1°C and treating with O_3 (1.5 g/kg) or O_3 (1.5 g/kg) plus CO_2 at 10 psig for 30 min. Product was maintained at 95 to 100% relative humidity. Controls included inoculated untreated or inoculated oxygen-treated spinach. Data are means of three independent trials. Error bars represent the standard error.*

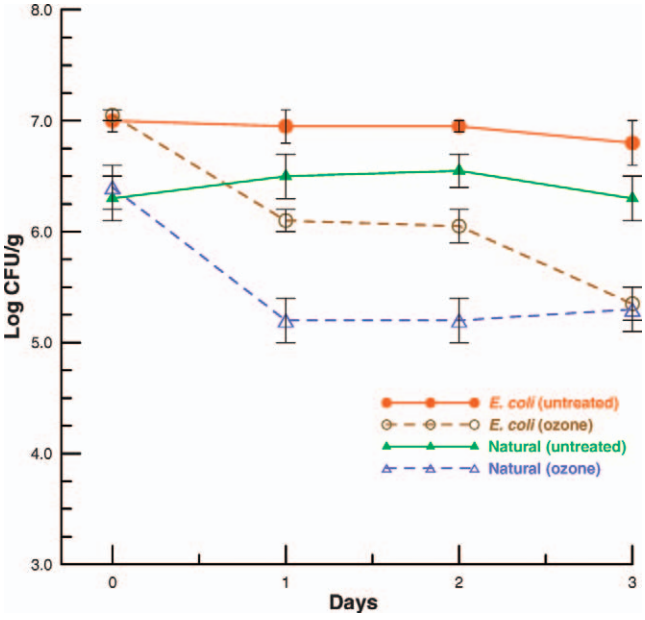

FIGURE 7. *Changes in populations of* Escherichia coli *O157:H7 and natural microbiota on fresh spinach when the inoculated product was treated with continuous ozone at 16 mg/kg (10 ppm) in a refrigerator (2 ± 1°C) for up to 3 days (SanTrans process). Data are means of two independent trials. Error bars represent the standard error.*

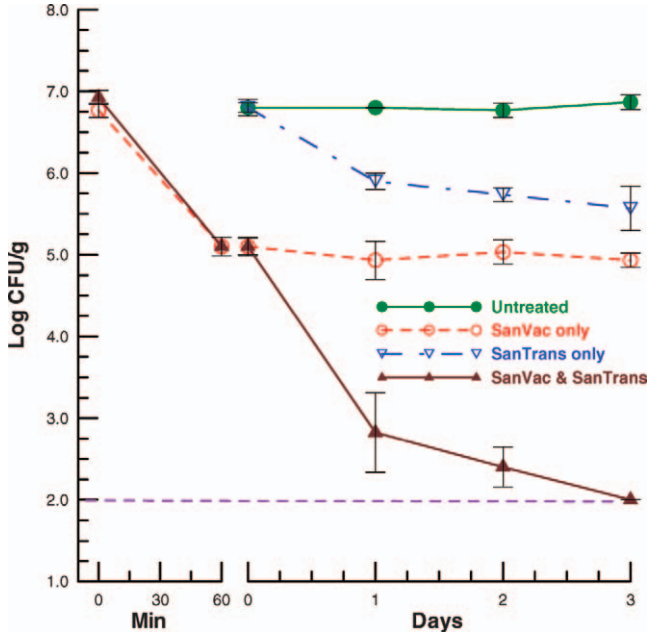

FIGURE 8. *Changes in populations of* Escherichia coli *O157:H7 when inoculated baby spinach was subjected to the SanVac process (for 60 min) and refrigerated (~2°C) for 3 days, the SanTrans process of 3 days of refrigerated storage (~2°C), or SanVac followed by SanTrans. The SanVac process included vacuum cooling to 4 ± 1°C combined with a short-term ozonation (1.5 g/kg at 10 psig for 30 min). The SanTrans process consisted of continuous ozonation at 16 mg/kg (10 ppm, vol/vol) during refrigeration at 2 ± 1°C for 3 days. The horizontal dashed line indicates the detection limit for the* E. coli *O157:H7 enumeration method. The data point at the dashed line was below the detection limit. Data are means of three independent trials. Error bars represent the standard error.*

vol/vol) for 3 days did not change *E. coli* O157:H7 populations or natural microbiota on spinach (data not shown). However, application of 16 mg/kg (10 ppm, vol/vol) ozone for 1 day significantly decreased ($P < 0.05$) *E. coli* population by 1.0 log CFU/g compared with the population on control samples. The difference between *E. coli* O157:H7 counts for ozone-treated and control samples was ~1.4 log CFU/g at the end of a 3-day ozonation process (Fig. 7). When natural microbiota were monitored, inactivation also was observed during the treatment, but the magnitude of lethality after 3 days of ozonation was less than that observed for *E. coli* O157:H7 (Fig. 7). The visual quality of baby spinach samples treated with ozone at this low concentration for 1 day was comparable to that of the untreated spinach.

Sequential application of SanVac and SanTrans processes. Treatments of fresh spinach with ozone during both vacuum cooling (i.e., SanVac process) and simulated shipment (i.e., SanTrans process) were evaluated. Inoculated spinach (~10^7 CFU/g) was treated with ozone (1.5 g/kg at 10 psig for 30 min) while vacuum cooling and then held in a refrigerator under continuous low-ozone flush (16 mg/kg [10 ppm, vol/vol]) for up to 3 days (Fig. 8). The SanVac treatment alone decreased the population of *E. coli* O157:H7 ~1.8 log CFU/g immediately after the treatment, and no further reduction was observed during the 3-day storage period. Application of SanTrans alone for 1 day decreased the population of *E. coli* O157:H7 by 1.0 log CFU/g compared with untreated controls. However, when SanVac treatment was followed by a 1- and 2-day SanTrans process, populations of *E. coli* O157:H7 decreased by 4.1 and 4.6 log CFU/g, respectively. The population of *E. coli* O157:H7 was undetectable (i.e., >5-log reduction) when the spinach was treated with the SanVac process followed by 3 days of the SanTrans process.

DISCUSSION

Leafy greens often are vacuum cooled soon after harvesting to remove field heat and thus extend the shelf life of these perishable products *(10, 20).* For leafy greens that have a high surface-to-mass ratio (e.g., spinach), vacuum cooling often is accomplished within 30 min *(33, 34).* A comparable time scale for cooling of fresh produce was used in this study. In preparation for retail marketing, fresh produce is minimally processed and packaged in specialized facilities. Conventional minimal processing includes cutting and washing with chlorine-treated water *(11).* The inability of the chlorine-treated water to reach attached, entrapped, or internalized pathogens in fresh produce may compromise product safety. Consequently, we tested gaseous ozone in tandem with vacuum cooling as an effective sanitization step that would precede conventional minimal processing of produce. The diffusion of gaseous sanitizers (e.g., ozone) into leafy green produce is expected to be greatly accelerated when the product first is subjected to vacuum and then subjected to the gas treatment. This se-

quence allows the sanitizer to enter the evacuated interstitial spaces.

Combining the vacuum cooling with an ozone-based sanitization process (SanVac) reduced the population of *E. coli* O157:H7 on freshly harvested produce, and the process is potentially useful for minimizing or eliminating microbial internalization. Bacterial pathogens such as *E. coli* O157:H7 may become internalized in fresh produce in the field and during postharvest operations *(31, 35).* Li et al. *(24)* reported that the vacuum cooling process internalizes *E. coli* O157:H7 in lettuce. Thus, if produce is contaminated before cooling, these contaminants may be forced inside the plant tissues during repressurization from vacuum to atmospheric pressure. Therefore, the newly developed SanVac process may minimize the risk of internalization of pathogens in fresh produce tissues during vacuum cooling. Because ozone gas was applied during the repressurization step, penetration of the sanitizer into fresh produce is expected, potentially eliminating any internalized and/or infiltrated *E. coli* O157:H7. The SanVac process would ideally be applied immediately after harvest of fresh produce before the pathogens have had a chance to adapt to refrigeration.

The antimicrobial efficacy of ozone may be enhanced when combined with other gases. Mitsuda et al. *(25)* reported the synergistic effect of ozone and carbon dioxide on microbial inactivation in foods. Vurma *(36)* tested the feasibility of reducing natural microbiota and extending the shelf life of strawberries by using combinations of ozone and carbon dioxide. The researcher found that the combination of these two gases was more effective for decreasing the natural microbial contaminants and preserving the quality of the treated strawberries than was treatment with either ozone or carbon dioxide alone. The synergy was believed to be due to the quenching action of carbon dioxide on the chain decomposition reaction of ozone, thus increasing the stability and bactericidal effect of the ozone in the treatment environment. However, in the present study inclusion of carbon dioxide in the gaseous mixture during SanVac treatments of baby spinach did not significantly enhance ($P > 0.05$) the lethal effect of ozone against *E. coli* O157:H7 (Fig. 6).

Enteric pathogens such as *E. coli* O157:H7 may survive or even proliferate on plant surfaces during preharvest and harvesting. Factors that favor this survival and/or growth include environment and plant temperature, availability of nutrients and water, tissue damage, and the native microbiota of the plants *(1, 3, 12).* After harvest, fresh produce is immediately cooled in preparation for transportation to retailers or to processing facilities where value-added operations (e.g., cutting) are performed. If fresh produce becomes contaminated with *E. coli* O157:H7, the pathogen may adapt to the processing plant environment and possibly survive during refrigerated transportation *(13).* In the present study, *E. coli* O157:H7 survived on baby spinach for up to 7 days of refrigerated storage, with minimum changes in populations (Fig. 6).

The results of the current study indicate that application of gaseous ozone during transportation and short-term storage of fresh produce may be an effective sanitization step against pathogenic microorganism such as *E. coli* O157:H7. Use of low-level ozonation during refrigerated storage of fresh produce was moderately effective against bacterial populations. Sequential application of two sanitization processes (i.e., ozonation during vacuum cooling and during storage or transportation) decreased *E. coli* O157:H7 populations by more than 5.0 log CFU/g. In previous studies, storage of blackberries under very low ozone concentrations (0.1 to 0.3 ppm) suppressed fungal growth for 12 days without causing any damage to the tested fruit *(5).* Storage of onions, potatoes, and sugar beets under an atmosphere enriched with ozone at 3 mg/liter, 6 to 14°C, and 93 to 97% humidity reduced the spoilage and microbial population of treated products without affecting their chemical composition or sensory qualities *(4).* Ozone treatments reduced the fungal decay and extended the shelf life of table grapes *(29).* When the grapes were treated with ozone before or after inoculation with *Rhizopus stolonifer,* a significant decrease in decay was achieved.

A novel sanitization technology was developed for the decontamination of fresh spinach. This new approach relies on use of gaseous ozone in tandem with vacuum cooling immediately after harvest and possibly during transportation of fresh produce. Adaptation of the proposed technology should result in only limited modification of existing fresh produce processes. The new ozone-based sanitization technologies also may be useful for other fresh produce products and against other pathogens.

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