DEVELOPMENT OF OZONE-BASED PROCESSES FOR DECONTAMINATION OF FRESH PRODUCE TO ENHANCE SAFETY AND EXTEND SHELFLIFE

DISSERTATION

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ABSTRACT

Although good agricultural practices (GAP) and good handling practices (GHP) may reduce the incidence of pathogens in fresh produce, there is no known defense against contamination due to wildlife or surface water. Additionally, current strategies designed to minimize the risk of contamination during post-harvest operations of fresh leafy greens are not completely effective. Contaminations of fresh fruits with spoilage microorganisms also create economical losses by decreasing products shelflife. Therefore, it is necessary to develop sound mitigation strategies to minimize the health hazards associated with fresh produce.

The main objectives of the current study are to (i) 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 *Escherichia coli* O157:H7 as the pathogen of concern, and (ii) to assess the feasibility of using ozone, carbon dioxide or their combinations, for reducing natural microbiota, and extending the shelf-life of strawberries.

A pilot-scale system has been successfully assembled that allows vacuum cooling and ozone treatment of fresh produce simultaneously. Combinations of vacuum cooling and ozonation (SanVac) successfully inactivated up to 2.4 log *E. coli* O157:H7 CFU/g spinach. Contribution of important treatment variables (ozone concentration,

pressure and treatment time) to process lethality was also investigated using response-surface methodology. Parameters for the optimized SanVac process are of 1.5 g O_3/kg gas-mix (935 ppm, vol/vol), 10 psig holding pressure, and 30 min holding time; these conditions achieve 1.8 log inactivation against *E. coli* O157:H7 with no apparent damage to quality of baby spinach.

A long-term, low-ozone process was also developed to treat fresh produce during transportation or temporary refrigerated storage. This treatment, termed "SanTrans", involves sparging moist gaseous ozone at 16 mg/kg (10 ppm, vol/vol) for up to 3 days. The process inactivated up to 1.4 log *E. coli* CFU/g spinach, and the optimum process resulted in 1.0 log inactivation with minimal effect on product quality. In order to maximize inactivation on *E. coli* O157:H7, contaminated spinach was sequentially subjected to optimized SanVac (at 1.5 g/kg for 30 min and 10 psig) and SanTrans (at 16 mg/kg) processes, using freshly-harvested, unprocessed spinach that was shipped directly from California fields. These sequential treatments inactivated 4.1 to ≥5.0 log *E. coli* O157:H7, depending on the treatment time. Inoculated and vacuum-cooled spinach was analyzed by scanning electron microscopy. It was apparent that vacuum-cooled leaves often contained bacterial cells throughout the intercellular spaces, compared with the non-vacuum cooled leaves.

An ozone-based treatment system was designed and constructed to enhance the storage life of fresh strawberries (*Fragaria* x *ananassa*). The O_3/CO_2 combination treatments showed synergistic effect in delaying mold growth and quality deterioration of the strawberries. When samples were treated with O_3/CO_2 for 4 h and stored at 4°C, the initiation of visual mold appearance was delayed until the 16^{th} day of storage; an 8-day

shelflife extension, compared to untreated samples. These novel technologies are promising alternatives to conventional processes and should enhance the safety and extend the shelflife of fresh fruits and vegetables. Additionally, the new treatments should be relatively easy to integrate into existing fresh produce processes and practices.

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FIELDS OF STUDY

Major Field: Food Science and Technology

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Interest in ozone use in food production and processing has been increasing steadily. This trend is driven by industry's need for potent antimicrobial agents and by the publicity surrounding several successful implementations of this sanitizer. Ozone has been effectively used in the production of bottled drinking water. Additionally, many water treatment plants currently use ozone as a more effective alternative to chlorine. Processors of fresh cut produce who are considering ozone use in their facility are encouraged by the positive experience of few small companies that already integrated ozone into their production lines. Costs of implementing ozone in food processing are not excessively prohibitive and removal of excess sanitizer does not represent a disposal hurdle.

In spite of these successes, many food processors are carefully analyzing the economic benefits and risks associated with ozone implementation. Compared to other sanitizers, the gas has limited solubility in water and thus aqueous applications require efficient gas injection systems and closed treatment vessels. Careful monitoring of ozone dissolution and residues in processing water and the potential for off-gassing may add

technical complexities to processing lines. Corrosiveness of ozone makes it difficult to use with some old equipment (e.g., pumps) in an ozone-upgraded facility. Interestingly, some of the ozone properties that are considered undesirable in a given application make the sanitizer suitable for other applications. The limited solubility of ozone in water can be advantageous, and the gaseous state of the sanitizer has beneficial implementations in food processing. Additionally, some of the drawbacks (e.g., corrosivity) correlate with the potency of ozone as an effective sanitizer.

OZONE CHEMISTRY AND PHYSICS

Ozone is a triatomic molecule (O_3) and a very reactive form of oxygen. It is commonly produced in nature by interactions of molecular oxygen (O_2) with chemicals, electric discharges during lightning, or short ultraviolet (UV) radiation form the sun (Fig. 1.1). These interactions cause rearrangements of atomic oxygen and the formation of the triatomic molecule of ozone. The gas has a characteristic pungent odor that is readily detectable by the human nose at concentrations as low as 0.02 ppm (by volume). Gaseous ozone is colorless at low concentrations and it has a bluish color at high concentrations (Rice et al., 1981).

In the stratosphere, small amounts of ozone (0.05 mg/L) are formed at 15 to 50 km of altitude by photochemical reactions involving the action of solar UV radiation (<240 nm) on molecular oxygen. The troposphere, at approximately 15 km of altitude, contains about 10% of atmospheric ozone. Longer UV radiation (240-320 nm) decomposes ozone to oxygen (Fig. 1.1). Although very low ozone concentrations are naturally present on the earth surface, higher ozone levels can be detected in urban areas

as a result of reactions of atmospheric oxygen with pollutants (i.e., carbon monoxide, hydrocarbons, nitrogen oxides) released by combustion engines and industrial activity (Horváth et al., 1985; Kim et al., 1999; Kim et al., 2003; Wojtowicz, 2004).

Ozone has a molecular mass of 48 and consists of three oxygen atoms arranged in an obtuse angle (116.8°) with a molecular bond length (1.278 Å) slightly larger than that of the a double oxygen bond (1.207 Å) (Mahapatra et al., 2005; Wojtowicz, 2004). Ozone gas can absorb infrared, visible, and UV radiation with maximum and minimum absorption in the UV range at approximately 260 nm and 200 nm, respectively (Horváth et al., 1985). Ozone can be frozen with boiling liquid hydrogen (-252.8 °C at atmospheric pressure), forming blue to violet crystals with a melting point of –192.5 °C under atmospheric pressure (101.3 kPa). Liquid ozone becomes a gas at –111.9 °C (its boiling point) under the conditions just described. The density of gaseous ozone (2.14 g/L) is higher than that of air (1.28 g/L) at 0 °C and one atmosphere (Horváth et al., 1985; Kim et al., 2003).

Ozone is the strongest oxidant currently available for food applications. It has an oxidation potential of 2.07 V, which is higher than that of hypochlorous acid or chlorine (Kim et al., 2003; Rice and Browning, 1980). A list of selected physical properties of ozone is shown in Table 1.1.

Ozone Solubility

Since many of ozone applications in the food industry involve aqueous ozone, solubility of the gas in water will be addressed in the section. Gaseous ozone is more soluble in water than oxygen and nitrogen, but it is less soluble than chlorine and carbon

dioxide. Dissolution of ozone occurs according to Henry's law, which states that at a given temperature, the solubility of the gas is directly proportional to the pressure it exerts above the liquid (Horváth et al., 1985). Water temperature is probably the most important parameter affecting ozone solubility, and the dissolution of the gas increases at lower temperatures. The solubility of ozone in water can vary at atmospheric pressure from 1130 mg/L at 0 °C to 307 mg/L at 60 °C (Fig.1.2) (Bablon et al., 1991; Horváth et al., 1985). For practicality reasons, solubility of ozone in water can be expressed as solubility ratio (S_r) using the formula:

$$S_r = \frac{\frac{mg}{liter\ O_3\ in\ water}}{\frac{mg}{liter\ O_3\ in\ the\ gaseous\ phase}}$$

A negative logarithmic relationship occurs between S_r and water temperature in the range of 0.5 to 43 °C (Bablon et al., 1991).

The design of ozone-water contactors could considerably affect the solubilization rate of the gas for food processing applications. For example, high ozone dissolution can be achieved by injecting the gas into water and generating small bubbles (e.g., 1 to 3 mm diameter), in combination with appropriate gas mixing or turbulence (Katzenelson et al., 1974; Schultz and Bellamy, 2000). On the other hand, organic matter, metals, and other impurities in water can consume ozone or catalyze its decomposition and, therefore, decrease the apparent gas solubilization rate (Khadre et al., 2001). High pH enhances molecular ozone decomposition into hydroxyl radical and also interferes with the ozone solubility rate (Alder and Hill, 1950; Kim et al., 2003).

Solubility of ozone gas in water for food processing can be improved by (i) decreasing water temperature, (ii) increasing water purity, (iii) increasing ozone concentration delivered, (iv) increasing the pressure of the gas above the water, (v) decreasing the size of gas bubbles in the injection systems, (vi) improving gas mixing and distribution, and (vii) extending ozone residence time (Horváth et al., 1985; Khadre et al., 2001).

Ozone Stability

Ozone is more stable in the gaseous than in the aqueous phase (Stumm, 1958). It has been calculated that the theoretical half-life of ozone gas (1.5% wt/wt O_3 in O_2) at 25, 100, and 250 °C is 19.3 years, 5.2 h, and 0.1 s, respectively (Wojtowicz, 2004). Pure mixtures of ozone in oxygen are stable at ambient temperatures in the absence of catalysts and light. However, ozone produced at industrial scale is less stable due to the presence of impurities. It is considered that the half-life of ozone gas at atmospheric pressure, dry conditions, and room temperature is approximately 12 h, and its decomposition to oxygen depends on a series of factors that include temperature, light, organic mater, reactivity with surfaces (e.g., metals), concentration, and pressure (Bablon et al., 1991; Koike et al., 1998; Rice and Browning, 1980; Weavers and Wickramanayake, 2001; Wojtowicz, 2004). The relative stability of ozone gas makes it possible to generate it at one location of a facility and to pipe it some distances, without substantial loss (Rice and Browning, 1980). Explosion of ozone in the gas phase by shock wave may occur at ≥15% wt/wt O_3 in O_2 at 25 °C, and detonation may be enhanced by electrical sparks, heat, or intense light flash. However, in practice, the use of ozone gas at

such concentrations is not common in food processing, and explosions are extremely rare (Guzel-Seydim et al., 2004b; Wojtowicz, 2004).

In the aqueous phase, the half-life of ozone could vary from seconds to hours depending on water quality and temperature (Kim et al., 2003; Weavers and Wickramanayake, 2001). For example, it is generally considered that the half-life of ozone in distilled water at 20 °C is 20 to 30 min (Khadre et al., 2001; Rice and Browning, 1980). Kim (1998) reported that the half-life of ozone in deionized and tap waters at 25 °C was 12 min and 6 min, respectively.

The stability of aqueous ozone is influenced by the presence of ozone-demand material in the water, as well as ozone concentration, temperature, pH, incidence of UV radiation, mechanical stirring, and the presence of metal ions and radical scavengers (Horváth et al., 1985; Kim et al., 2003; Weavers and Wickramanayake, 2001). The pH has a considerable effect on the stability of aqueous ozone. Kim (1998) indicated that the stability of ozone in solution decreased as the pH increased with highest stability at pH 5.0 and undetectable ozone levels at pH 9.0. In food processing, aqueous ozone stability is greatly influenced by the water source, and this factor must be considered while using ozone in processing plants where water contains readily oxidizable organic and inorganic material.

Ozone in aqueous phase continuously decomposes to oxygen in a step-wise mode with the generation of free radical species, which include hydroperoxyl ($HO_2^{\cdot}$), hydroxyl ($^{\cdot}OH$), and superoxide ($^{\cdot}O_2^{-}$) radicals (Bablon et al., 1991; Grimes et al., 1983; Hoigné and Bader, 1975). For example, the decomposition of one mole of aqueous ozone results in the generation of approximately 0.5 mole of $^{\cdot}OH$ (Jans and Hoigné, 1998). The

generated free radicals have a strong oxidizing power, a half-life of microseconds, and are responsible for the high reactivity of ozone (Kim et al., 2003). A schematic representation of the sequence of ozone decomposition reactions to oxygen and free radicals is shown in Fig. 1.3. The relative instability of ozone, in gaseous and aqueous phases, prevents its storage for subsequent use. Therefore, ozone must be generated on-site and at the concentration required for particular food processing applications.

Ozone Reactivity

In the aqueous phase, molecular ozone acts as a dipole with electrophilic and nucleophilic properties and reacts with organic and inorganic compounds (Khadre et al., 2001). As outlined in Fig. 1.3, decomposition of ozone is a chain of reactions that includes initiation, promotion, and inhibition, where various compounds are involved (Jans and Hoigné, 1998; Staehelin and Hoigné, 1985). Free radicals such as superoxide anions ($^{\cdot}O_2^-$) are formed during initiation step with initiator compounds such as hydroxyl (OH^-) and hydroperoxide ions ($HO^{\cdot}_2$), cations (e.g., Fe^{+2}), and organic substances (e.g., glyoxylic acid, formic acid). The UV radiation at 253.7 nm and hydrogen peroxide can also initiate the free radical formation process.

During promotion step, superoxide and hydroperoxide radicals are regenerated from hydroxyl radicals (Fig. 1.3). Organic and inorganic compounds such as formic acids, aryl groups, glyoxylic acid, primary alcohols, humic acids and inorganic phosphate species are common promoters. The reaction rate of superoxide anion radical and ozone is very high; therefore, the radical can also act as a promoter.

The inhibition step involves the consumption of hydroxyl radicals without regeneration of the superoxide anion (Fig. 1.3). Bicarbonate and carbonate ions, alkyl groups, tertiary alcohols, and humic compounds are common inhibitors (Staehelin and Hoigné, 1985). Chain ozone decomposition reactions are also blocked by antioxidants found in food by scavenging the free radicals during decomposition (Kim et al., 2003).

OZONE PRODUCTION

Ozone is usually generated for industrial applications at the point of use and in closed systems by electric discharge methods. Ozone can be produced by various other methods including chemical, photochemical, thermal, chemonuclear, electrolytic, and electrochemical procedures (Horváth et al., 1985). Low concentration of ozone (0.03 ppm) was produced by reaction of oxygen with 185 nm-wavelength radiation in high transmission ultraviolet lamps (Ewell, 1946). UV-based ozone generators are commercially available and these may be suitable for applications that require small quantities of ozone.

Electric discharge systems (corona discharge) are widely used for industrial production of large amounts ozone (Horváth et al., 1985). Production of ozone by corona discharge involves applying a high voltage between two electrodes separated by a dielectric material in the presence of oxygen or dry air (Fig. 1.4A). High-voltage alternating current excites oxygen electrons and induces splitting of oxygen molecules into atoms, which combine with other oxygen molecules to produce ozone. Concentration of ozone produced in this system depends on the voltage, current, frequency, dielectric material, discharge gap, absolute pressure within discharge gap, and the nature of gas

passing through the electrodes (Horváth et al., 1985). Efficiency of ozone production increases at low temperatures, and varies from 1-3% to 2-6% by weight when air and oxygen are used as feed gases, respectively (Weavers and Wickramanayake, 2001). Corona-discharge ozone generators that can produce up to 16% (wt/wt) ozone in oxygen gas mixture are currently available (http://www.ozonia.com).

Andrews and Murphy (2002) indicated that ozone could be generated efficiently by an electrochemical procedure. Electrochemical ozone generation involves electrolysis of water into hydrogen and oxygen atoms (Fig. 1.4B). Hydrogen gas is vented from the gas-water mixture, and oxygen atoms are combined to produce ozone in an oxygen mix. Electrochemical generation is self-pressurized (≤20 psig), and ozone attains a concentration of 12-14% (w/w) in oxygen (Lynntech, 1998).

OZONE MEASUREMENT

Aqueous Phase

There are approximately 20 analytical procedures proposed to measure aqueous ozone using chemical, physical, and physicochemical methods (Horváth et al., 1985; Weavers and Wickramanayake, 2001). Reactivity, instability, volatility, and the effects of interfering substances with ozone should be considered when choosing a method to measure its concentration. Chemical methods are based on the quantification of products resulting from the reaction of ozone with an appropriate reagent. Oxidation of iodine solution by aqueous ozone has been used to analytically determine ozone concentration (Shechter, 1973). However, iodometric methods have variations in sensitivity and accuracy because they do not measure ozone alone, but total oxidants in solution (Alder

and Hill, 1950). Bader and Hoigné (1981) developed the indigo method, a procedure based on the reaction of ozone with sulfonated indigo dye. In this method, ozone reacts with the carbon-carbon double bond of the dye, resulting in its decolorization (Fig. 1.5). Change in color is determined spectrophotometrically (at 600 nm), and the measured absorbance is used to calculate ozone concentration with a minimum detection limit of 0.005 μg/ml. The indigo method gives less interference, due to other oxidant compounds, than iodometric procedures, and it is recommended as a standard to measure residual ozone (APHA, 1995; Gordon et al., 1988). Concentration of ozone in pure aqueous solutions may also be measured by a spectrophotometric method at wave length of 258 nm.

Gaseous Phase

Several methods have been proposed to measure gaseous ozone using iodometry, ultraviolet absorption, and chemiluminescence procedures (Weavers and Wickramanayake, 2001). However, only the UV spectrophotometric method was recommended to measure ozone in gas phase accurately (Gordon et al., 1988). Instruments that measure ozone concentration on the basis of UV absorption are widely used in various applications. In addition, instruments to measure ozone based on calorimetry and amperometric methods are commercially available (Khadre et al., 2001).

ANTIMICROBIAL PROPERTIES OF OZONE

Ozone has strong antimicrobial activity against bacteria, fungi, protozoa, and spores from bacteria and fungi when these microorganisms are present in low ozone demand environments (Khadre et al., 2001). Ozone is also effective against most viruses tested. The mechanisms involved in microbial inactivation by ozone are attributed to its oxidation reactions with cellular components of microorganisms. Oxidation activity of ozone is either directly associated with its molecular form or its decomposition by-products, also known as reactive oxygen species, such as hydroxyl ($^{\cdot}OH$), superoxide anions ($^{\cdot}O_2^-$), and hydroperoxyl ($HO^{\cdot}_2$) radicals (Hunt and Marinas, 1997; Kanofsky and Sima, 1991). Complex oxidation reactions occur against unsaturated lipids in the microbial cell envelope, intracellular enzymes, and genetic material (Khadre et al., 2001; Kim et al., 2003). According to Giese and Christenser (1954), primary target of ozone is the microbial cell surface. Reactions between ozone and double bonds of unsaturated lipids in the cell envelope lead to leakage of cellular constituents and microbial lysis (Scott and Lesher, 1963). Komanapalli and Lau (1996) examined the effect of gaseous ozone at 600 mg/L on *Escherichia coli* K-12 for up to 30 min. The authors found that viability of the bacterium decreased, membrane permeability was compromised, and intracellular proteins were degraded progressively when the ozonation time increased. Cellular damage of ozone treated microorganisms was also observed by electron microscopic analysis (Dave, 1999; Khadre and Yousef, 2001a; Khadre and Yousef, 2001b). It was apparent that ozone causes damage to membranes of Gram-negative bacteria which results in loss of its cellular components. On the other hand, ozone causes

less visible damage to cell wall of Gram-positives and intercellular damage was the main reason for ozone-mediated inactivation of these cells (Kim, 1998).

In addition to the damage to microbial cell envelope, ozone inactivates certain enzymes by oxidizing its sulfhydryl groups (Barron, 1954). According to Dillon et al. (1992), ozone may induce mutagenic effects on *Salmonella enterica* serovar Typhimurium, leading to cell injury or inactivation. Young and Setlow (2004) studied mechanisms of *Bacillus subtilis* spore inactivation and resistance to aqueous ozone. The authors found that DNA damage was not the reason for ozone inactivation of these sporeforming microorganisms. It has been suggested that ozone-mediated inactivation was due to the damage of *B. subtilis* spore inner membrane. Resistance to ozone was attributed to spore coat.

Inactivation of Bacteria

Susceptibility of bacteria to ozone varies among genera and species (Table 1.2). In general, bacterial spores show greater resistance to ozone treatments than vegetative cells. Broadwater et al. (1973) studied the ozone inactivation on vegetative and spore forms of *B. cereus.* The authors reported that 0.12 mg/L ozone decreased the population of *B. cereus* vegetative cells by 2 log units whereas 2.29 mg/L were needed to achieve the same inactivation level for the spores of this bacterium. In another study, Khadre and Yousef (2001b) tested the resistance of spores from eight *Bacillus* spp. to aqueous ozone and the authors reported that *B. stearothermophilus* was the most resistant whereas *B. cereus* was the most sensitive. It has been suggested that *B. stearothermophilus* spores

may be used as an indicator for sanitization efficacy of ozone on various food-contact surfaces.

Contradictory results have been reported on the differences in resistance of Gram-positive versus Gram-negative bacteria to ozone treatments. Kim and Yousef (2000) studied the inactivation kinetics of ozone treated Gram-positive and Gram-negative bacteria in a batch-type reaction system. According to these authors, ozone was very effective in inactivating *E. coli* O157:H7, *Pseudomonas fluorescence*, *Leuconostoc mesenteroides*, and *Listeria monocytogens* and among these microorganisms *E. coli* O157:H7 was the most resistant while *L. monocytogens* was the most sensitive. In other study, Gram-negative bacteria were found to be more sensitive to aqueous ozone than were the Gram-positives among tested microorganisms (Restaino et al., 1995). Similarly, Sobsey (1989) reported that Gram-positives were more resistant to ozone treatments than were the Gram-negatives.

Environment where bacteria are present is also very important for the inactivation efficacy of ozone treatment. When *Salmonella* Enteritidis was treated with 1.5 ppm ozone in distilled water, population of the pathogen was decreased by 6 log units (Kim et al., 1999). Only 1 log reduction in population of *Salmonella* Enteritidis was achieved when the pathogen was inoculated on poultry skin and treated with gaseous ozone at 8% (wt/wt in air) for 15 sec (Ramirez et al., 1994).

Inactivation of *Shigella sonnei* by ozone was assessed when this pathogen was inoculated in water and shredded lettuce (Selma et al., 2007). Treatment of *S. sonnei* with 1.6 and 2.2 ppm aqueous ozone for 1 min decreased the population of the pathogen by 3.7 and 5.6 log units, respectively. Inactivation of this pathogen on shredded lettuce

was only 1.8 log units after treatment with 5 ppm of ozonated water for 5 min. Inactivation of *Yersinia enterocolitica* in water or on potato surface with ozone was evaluated (Selma et al., 2006). Aqueous ozone treatments at 1.4 and 1.9 ppm for 1 min decreased *Y. enterocolitica* count in water (10^8 CFU/ml, initially) by 4.6 and 6.2 log units, respectively. When this pathogen was inoculated on potato surface (~2 x 10^5 CFU/g), ozonated water treatment at 5 ppm for 1 min decreased the population by 1.6 log only.

Sporicidal effect of ozone against *Bacillus* and *Clostridium* was enhanced when the spores were treated in an acidic medium (Foegeding, 1985). The degree of inactivation is also affected by the physiological status of treated bacterium. Cells in their exponential phase were more sensitive to ozone than cells in their stationary phase (da Silva et al., 1998; Kim et al., 2003).

Inactivation of Fungi

Ozone in aqueous and gaseous states is a potent antifungal agent and its fungicidal action varies among species (Table 1.3). Beuchat et al. (1999) investigated the susceptibility of conidia of aflatoxigenic aspergilli to ozone. *Aspergillus flavus* and *A. parasiticus* conidia were treated with 1.74 ppm ozone in phosphate buffer at pH 5.5 and 7.0. The authors reported that the D-values were 1.7 and 1.5 for *A. flavus* and 2.1 and 1.7 for *A. parasiticus* at pH 5.5 and 7.0, respectively.

The antimycotic effect of aqueous ozone against *Candida parapsilosis* was studied by Farooq and Akhlaque (1983). Treatment of *C. parapsilosis* with ozonated water at 0.23 to 0.26 mg/L for 1.67 min decreased the population of this microorganism

by 2 log units. Kawamura et al. (1986) reported that the counts of *C. tropicalis* decreased by 2 log units when the yeast cells were treated with aqueous ozone at 0.02 mg/L for 20 sec or at 1 mg/L for 5 sec.

In another study, ozonated water containing ~0.19 mg/L ozone instantaneously decreased *C. albanicus* and *Zygosaccharomycess bailii* populations by 4.5 log units, but the same concentration decreased *A. niger* spores by <1 log after 5-min treatment (Restaino et al., 1995). The fungicidal action of aqueous ozone, at 0.3-0.5 mg/L, was evaluated by Naitoh and Shiga (1982). According to these authors, the threshold values of fungicidal ozone activity against spores of *Aspergillus, Penicillium,* and *C. paracreus* were 90 to 180, 45 to 60, and 5 to 10 min, respectively. Additionally, the antimicrobial efficacy of ozone increased against all the tested microorganisms with decreasing pH and temperature of treatment water.

Susceptibility of six different yeasts to gaseous ozone at different temperature and humidity conditions was investigated by Naitoh (1993). Cells of *Hansenula anomala, Saccharomycess rosei, Pichia farinose, C. parapsilosis, Kluyveromyces marxianus*, and *Debaryomyces hansenii* var. *hansenii* were treated with gaseous ozone (~5 ppm) for 1 to 5 h at 30 to 60 °C and 25 to 90% relative humidity. Populations of *C. parapsilosis* and *K. marxianus* decreased >1 log with ozone treatment for 5 h at low temperature; however, this treatment did not affect the microbial counts of other tested yeasts. The author reported that the fungicidal effect of ozone was enhanced with increasing treatment temperature, humidity, and time. Mycelial growth of *Botrytis cinerea* was slower when this plant pathogen was inoculated on potato dextrose agar and stored at 2 °C in ozone-enriched (1.5 μg/L) environment (Nadas et al., 2003).

Inactivation of Protozoa

Protozoan parasites such as *Giardia*, *Cryptosporidium*, *Cyclospora* have been implicated in a number of waterborne disease outbreaks worldwide (Clark et al., 2002; Erickson and Ortega, 2006). According to Clark et al. (2002), ozone is more effective chemical disinfectant than chlorine or chlorine dioxide against protozoan parasites in water systems. Selected studies describing inactivation of protozoa by ozone are summarized in Table 1.4. Widmer et al. (2002) investigated the effect of ozone on *G. lamblia* cysts in gerbils using an infectivity assay by scanning electron microscopy, immunoblotting, and flow cytometry techniques. Cysts were treated with ozone at 1.5 mg/L for 0, 30, 60, and 120 sec. The authors reported that ozone exposure for 60 sec or longer effectively inactivated cysts of *G. lamblia* and the treatments caused extensive protein degradation and profound structural modifications to cyst wall.

Differences in resistance of cysts and oocysts of protozoan parasites to ozone treatments have been reported (Erickson and Ortega, 2006). According to Wickramanayake et al. (1984), cysts of *Naegleria gruberi* were more resistant to ozone than were *G. muris* cysts. The author reported that ozone treatment at 0.2 mg/l for 7.5 min inactivated 2 log units whereas only 1.05 min was sufficient to achieve similar inactivation for the cysts of *G. muris*. Population of *C. parvum* oocysts decreased >1 log when the parasite was exposed to 1 mg/L ozone for 5 min (Korich et al., 1990). The researchers also reported that *C. parvum* oocysts were 30 times more resistant to ozone than were *Giardia* cysts when these parasites were treated under the same conditions.

Inactivation of Viruses

Ozone is an effective virucide. This is evident from results of selected studies on inactivation of viruses including bacteriophages and human/animal viruses as summarized in Table 1.5. Ozone at low concentration levels and short contact times are generally sufficient for inactivation of viruses when present in low ozone demand media. When the ozone demand of the medium is high (e.g., in wastewater), long contact time and high ozone concentration are required to inactivate viruses (Kim et al., 1999).

Variation in viruses' susceptibility to ozone has been reported (Khadre et al., 2001). It appears that bacteriophages such as f2, MS2 are the most susceptible viruses to ozone. Resistance of viruses to ozone was greater for hepatitis A than for poliovirus (Herbold et al., 1989). Susceptibility of human rotavirus to ozone was tested (Khadre and Yousef, 2002). Rotavirus suspension at high titer (~10^{11} $TCID_{50}$/ml) was treated with ozone at 5.2 to 25 mg/L for 1 min. These treatments decreased the infectivity of these microorganisms by 2 to 8 log units $TCID_{50}$/ml. Poliovirus type 1 (Mahoney) was treated with 0.25 mg/L of ozone for 5 min and this exposure yielded 2-log inactivation (Harakeh and Butler, 1985). Farooq and Akhlaque (1983) reported 2.5-log reduction of poliovirus type 1 (Mahoney) when the virus suspension was ozonated at 0.23-0.26 mg/L for 1.67 min.

OZONE AND FOOD APPLICATIONS

Food Properties and Ozone Applicability

Reactivity, solubility and disinfection efficacy of ozone are affected by many factors such as temperature, pH, humidity, and presence of ozone-demanding materials in the treated medium. In addition, microbicidal effect of ozone is highly dependent on its accessibility to target microorganisms without interacting with the food components (Fig. 1.6). Microorganisms are generally not found in readily accessible location in food as they are in pure water. Microorganisms that are strongly attached, internalized, or organized as a biofilm on food surfaces or those embedded in the food matrix are not readily inactivated by ozone treatments. Achen and Yousef (2001) reported that washing with aqueous ozone was more effective in inactivating microorganisms on the surface of apples than in decontaminating the calyx and stem areas. The authors also pointed out that the ozone efficacy was reduced when *E. coli* O157:H7 was allowed to attach to the apple surface.

Readily available high ozone demand compounds may compete with microorganisms for ozone. High fat containing foods such as meat require higher ozone concentration than low fat foods such as fruits and vegetables (Kim et al., 2003). Ability of ozone to reduce microbial load in the presence of whipping cream, locust bean gum, soluble starch and sodium caseinate was investigated (Guzel-Seydim et al., 2004a). The authors pointed out that the food components had influenced bactericidal action of ozone against treated microorganisms. Compared to control buffer, starch did not provide any protective effect against microbial inactivation of ozone while moderate or strong protective effects were observed in the presence of other tested components. Similarly,

Restaino et al. (1995) reported that ozone inactivation of microorganisms was not affected by the presence of soluble starch in treatment medium while addition of bovine serum albumin (BSA) reduced the microbial inactivation. Achen (2000) demonstrated that inactivation of bacteria with ozone was dependent on the concentration of BSA added to the medium.

Ozone as an Alternative Sanitizer in Food Processing

Ozone was introduced as a disinfectant in the treatment of drinking water for the first time in 1893 at Oudshourn, Netherlands (Rice et al., 1981). Subsequently, ozone was used for water disinfection in many European countries (Bryant et al., 1992). Ozone may be used in the gaseous or aqueous state in food processing. In general, gaseous ozone is applied for storage applications whereas the aqueous form is used for surface decontamination of foods, equipment or packaging material.

Ozone Treatment System

Ozone can be applied in gaseous or aqueous states for food applications. Sanitization of fresh or fresh-cut vegetables is an example of processes that can make use of both forms of ozone. Fig. 1.7 shows conceptual aqueous and gaseous ozone systems that may be applicable to fresh produce treatments. Essential components for an ozone treatment system for food applications are:

- A gas feed system.
- An ozone generator with electrical power supply.

- An ozone contactor for aqueous ozone applications, or a treatment vessel for gaseous ozone treatments.
- Ozone measurement devices.
- An ozone off-gas destruct system.

High purity oxygen or dry air can be used to generate ozone. Commonly, corona discharge generators are used to produce ozone for food applications, and these generators require high voltage electric supply unit. Gaseous ozone should be dissolved into water for aqueous ozone treatments of foods. Transfer efficiency of ozone from gaseous state to liquid form is an important factor that would affect the process feasibility. There are several ozone dissolution methods for increasing transfer efficiency of this gas into water; these include conventional fine bubble diffusers, turbine mixers, injectors, packed columns, spray chambers, porous plate diffuser contactors, and submerged static radial turbine contactors (Bellamy et al., 1991). Ozone and matrix (e.g., food) to be treated are brought together in treatment vessel. This vessel should be leak proof and equipped with monitoring devices and excess gas destruction unit. The treatment vessel should be designed to permit efficient contact between ozone and the matrix. Automatic control units can be used in conjunction with process flow meters and monitors to maintain target ozone concentration in the process and control ozone generation. There are thermal and catalytic destruction units commercially available to convert excess ozone to oxygen prior to release in the atmosphere. Ozone detectors should be employed in the working environment to routinely monitor the concentration of this gas for employees' safety. Concentration of ozone and time of exposure are critical parameters that determine ozone efficacy during the treatment. For water applications,

efficacy of the treatment is commonly expressed as ozone concentration (mg/L) and contact time (min) that are sufficient to inactivate a given microbial population (e.g., 2 log decrease). The product of multiplication of these two parameters is termed Ct value.

Interaction of ozone with processing equipment and packaging material should be considered for efficacy of the treatment as well as the corrosion stability of the materials used. Ozone's corrosive effect is most pronounced at high concentrations commonly found inside the ozone generator or in the ozone-to-water contacting system. Materials most frequently used in food processing industry are resistant to ozone at moderate concentrations. The materials with high resistance to corrosion by ozone include austenitic (300 series) stainless steel, glass, PTFE (Teflon), Hypalon, and concrete. In addition, plastics commonly used in food industry such as polyvinylchloride (PVC) and polyethylene (PE), are generally resistant at low ozone concentrations. The use of copper alloys and natural rubber should be avoided because of their liability to oxidation and rapid disintegration, respectively. When designing and manufacturing a treatment system, all equipment materials, including the seals, gaskets, and lubricants that come into contact with this sanitizer should be selected from materials of high ozone resistance (Kim et al., 2003).

SELECTED FOOD APPLICATIONS

Potential applications of ozone as an antimicrobial agent in food industry have been extensively studied (Khadre et al., 2001; Kim et al., 1999; Kim et al., 2003). Ozone has been tested on food products such as meat, poultry, fish, fruits and vegetables, and cheese. Other ozone applications tested include the decontamination of food packaging

material, food contact surfaces, and removal of residual pesticides on fruits. Ozone has the advantage of decomposing spontaneously to a nontoxic product, *i.e.*, O_2.

Fruits and vegetables

Fresh fruits and vegetables are susceptible to contamination with pathogenic and spoilage microorganisms, beginning from the pre-harvesting stage through post-processing. Foodborne disease outbreaks linked to minimally processed fruits and vegetables have increased during the past few decades (Sivapalasingam et al., 2004). Microbial contamination of fresh fruits and vegetables not only poses significant risk to public health but also affects the industry financially by decreasing product shelf life.

Ozone has been explored for treating agricultural commodities because it provides more disinfecting power than other sanitizers (e.g., chlorine) and it removes a myriad of contaminants including microorganisms resistant to chlorine treatment (Graham, 1997). Sanitation of fresh produce is one of the most promising applications of ozone. The food industry is strongly interested in using this sanitizer to enhance the shelflife and safety of these perishable products and in exploring new applications of the sanitizer.

Efficacy of ozone against natural microbiota on lettuce was tested (Kim et al., 1999). Mesophilic and psychrotrophic natural contaminants of shredded lettuce were inactivated by 1.4 and 1.8 log units, respectively, when aqueous ozone was applied at 1.3 mM for 3 min. When the ozonation time was increased to 5 min, the counts of these microorganisms were decreased by 3.9 and 4.6 log units, respectively. The authors suggested the bubbling gaseous ozone into wash water is necessary to increase the efficacy of ozone against microorganisms on lettuce. In a more recent study, Beltran et

al. (2005) concluded that sanitization of fresh-cut lettuce using ozonated water was a good alternative to chlorine treatment. Washing shredded lettuce with ozonated water and storing the treated produce under modified atmosphere packaging reduced its microbial populations and extended its shelflife.

Ozone delivery method affects ozone efficacy against microorganisms on fruit surfaces. Achen and Yousef (2001) investigated the inactivation of *E. coli* O157:H7 on apple surfaces by bubbling ozone during washing or by dipping in pre-ozonated water. Counts of *E. coli* O157:H7 decreased by 3.7 and 2.6 log units when the apples ozone-washed by bubbling and dipped, respectively. When the pathogen was inoculated in the stem-calyx region of apples, <1 log inactivation was achieved by the ozone treatments in both delivery methods. The authors suggested that using a surfactant, such as tetrasodium pyrophosphate, to rinse the apples prior to ozone treatment may enhance fruit decontamination.

Gaseous ozone has been studied for inactivation of microorganisms on various fresh fruits and vegetables during storage. Storage of blackberries under 0.1-0.3 ppm gaseous ozone suppressed the fungal growth for 12 days without causing any damage to the tested fruit (Barth et al., 1995). Storage of onions, potatoes, and sugar beets under ozone-enriched atmosphere at 3 mg/L with 6-14 °C and 93-97% humidity reduced the spoilage and microbial population of treated products without affecting their chemical composition or sensory quality (Baranovskaya et al., 1979).

Ozone treatments reduced the fungal decay and extended shelflife of table grapes (Sarig et al., 1996). When the grapes were treated with ozone before or after inoculation with *Rhizopus stolonifer*, significant decrease in decay was achieved. It appeared that the

ozone treatment also induced plant resistance against fungal decay. Depending on treatment conditions, ozone may also cause physiological injury and damage to treated fruits and vegetables (Horváth et al., 1985).

Raw Poultry and Meats

Presence of pathogenic microorganisms such as *Campylobacter* spp., *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7 in raw poultry and meat constitutes significant public health hazards. Pathogenic and spoilage microorganism may contaminate poultry and meat products during slaughtering, handling, processing, and distribution (Zhao et al., 2001). Chemical sanitizers such as chlorine have been tested to decontaminate carcasses and cut meat but the efficacy of these sanitizers was questionable. Therefore, ozone has been tested as an alternative sanitizer in these raw products.

Antimicrobial effects have been demonstrated when ozone was used to treat meat surface (Greer and Jones, 1989; Mitsuda et al., 1990), poultry carcasses (Jindal et al., 1995), poultry-processing chiller water (Diaz et al., 2001), and hatchery equipment (Whistler and Sheldon, 1989). However, variable results were reported when ozone was used to decontaminate beef and beef brisket fat (Kim et al., 2003). Using ozone at low concentration may not be suitable for decontamination of high-ozone demand foods such as meat while ozone at high concentrations may change the chemical compositions and sensory qualities of these types of products (Khadre et al., 2001).

Shell Eggs

Contamination of raw shell eggs with *Salmonella* Enteritidis is a widespread public health problem. Several chemical and physical decontamination methods have been developed to inactivate this pathogen in shell eggs but the microbicidal effectiveness of these treatments was variable (Rodriguez-Romo and Yousef, 2005). Additionally, some of these treatments have deleterious effects on egg quality. Yousef and Rodriguez-Romo (2004) developed methods for inactivation of *Salmonella* Enteritidis in shell eggs using combinations of mild heat and gaseous ozone. These treatments synergistically inactivated this pathogen in shell eggs by more than 6.3 log units without affecting the quality of egg contents.

Gaseous ozone treatment of hatching quail eggs at 10 ppm for 6 h, resulted in more than 3 log inactivation of *Salmonella* (Ito et al., 1999). Koidis et al. (2000) investigated aqueous ozone against *Salmonella* Enteritidis on shell eggs using two concentrations (1.4 and 3.0 ppm) and two temperatures (4 and 22 °C). Ozone treatments at 22 °C with 1.4 ppm inactivated this pathogen by 1 log in 90 sec, whereas treatments at 4 °C using 3.0 ppm resulted in 2 log reduction in the same time period.

Fish and Seafood

Haraguchi et al. (1969) investigated the bactericidal effects of gaseous ozone on the microbiological qualities of fresh jack mackerel (*Trachurus trachurus*) and shimaaji (*Caranx mertensi*). Viable bacterial counts decreased by 2 to 3 log units when the skin of gutted fish was treated with 0.6 ppm ozone in 3% NaCl solution for 30 to 60 min. Ozone treatments extended the shelflife of the fish by 20 to 60%. Shelflife of ozone-washed (1

mg/L for 90 min) shucked vacuum packed mussels was extended 3 days as compared to control counterparts (Manousaridis et al., 2005). Aqueous ozone treatment (4.5 ppm for 10 sec) significantly reduced natural microbiota of both live catfish entering the plant and finished catfish fillets (Sopher et al., 2007).

Dry Food and Food Ingredients

Efficacy of gaseous ozone against microbial contaminants on dry food and food ingredients depends on surface properties of the treated products, ozone concentration, humidity in the environment, temperature, and water activity of the product (Kim et al., 2003). Ozone more effectively detoxified aflatoxins in pistachio kernels than ground pistachios (Akbas and Ozdemir, 2006). Similarly, higher ozone concentration and longer treatment time were needed for cereal flour and ground pepper to achieve comparable microbicidal effect for the whole cereal and pepper (Naitoh et al., 1989; Zagon et al., 1992).

Antimicrobial effect of gaseous ozone against *Bacillus* spp. and *Micrococcus* spp. on cereal grains, peas, beans, and spices was tested (Naitoh et al., 1988). The authors reported that the ozone inactivated these microorganisms by up to 3 log units and that efficacy of the treatment depended on ozone concentration, temperature and relative humidity. Gaseous ozone was found to be an effective anti-fungal fumigant to preserve stored wheat (Wu et al., 2006). Fungal spores associated with wheat were inactivated by 96.9% within a 5-min ozone treatment (0.33 mg/g-wheat × min). The authors also pointed out that the fungicidal effect of ozone was improved by increasing both water activity and temperature of the wheat.

Packaging Material and Food Contact Surfaces

A small number of microbial contaminants are usually present on food packaging materials. Several treatments are currently utilized to achieve sterility of packaging materials including hydrogen peroxide (H_2O_2) alone or in combination with other sanitizers, heat, and UV radiation (Gardner and Shama, 1998; Stefanovic and Dickerson, 1986; Yokoyama, 1990). Sterilization of packaging materials by H_2O_2 is a common practice, yet the process has several disadvantages (Yokoyama, 1990). Unacceptable amount of this sanitizer may remain after the treatment, and this residue may interact with some compounds in the package material (Castle et al., 1995). Alternative sterilization methods have been sought for the food and packaging industries. Ozone in gaseous and aqueous states has been explored for surface disinfection of packages and equipment (Pascual et al., 2007).

Ozone was tested on surfaces of multilaminated aseptic food packaging and stainless steel, against natural bacterial contaminants, bacterial biofilms of *Pseudomonas fluorescence* and dried films of *B. subtilis* spores (Khadre and Yousef, 2001a). Sterility was achieved when un-inoculated multilaminated packaging was treated with 5.9 μg/ml aqueous ozone for 1 min. Population of *P. fluorescence* on multilaminated packaging material was inactivated by up to 8 log units per 12.5 cm^2 surface when the inoculated materials were repeatedly treated with 3.6 μg/ml ozone. Counts of *B. subtilis* spores (10^8/6.3 cm^2) decreased below detection level by washing with 13 μg/ml ozone for the multilaminated packaging material and 8 μg/ml for the stainless steel. The authors found that ozone inactivated *P. fluorescence* biofilms more effectively on stainless steel than on the multilaminated packaging material.

Ozone has been effectively applied to disinfect oak barrels used for aging wine in Australia. Spoilage yeast (i.e., *Brettanomyces* spp.) causes off-taste and other defects in wines and ozone has been proven to control this fungus effectively (Day, 2004).

Pesticides on Agricultural Commodities

Residues of pesticides such as herbicides, insecticides, and fungicides are widely spread in produce and other agricultural commodities. Persistence of these residues and the associated potential adverse health effects raise concerns among consumers and health authorities. Oxidizing agents such as chlorine, chlorine dioxide, peracetic acid, and ozone have been successfully tested to remove several pesticides from apples (Hwang et al., 2002; Ong et al., 1996). The degradation of four pesticides, diazinon, parathion, methyl-parahion, and cypermethrin, by aqueous ozone in water and on vegetable surface (*Brassica rapa*) was investigated (Wu et al., 2007). Ozone treatment at 1.4 mg/L for 30 min was effective in oxidizing 60 to 99% of the four tested pesticides. Ozonated water at 1.4-2.0 mg/L was the most effective in removing up to 60% cypermethrin from the tested vegetable. The authors pointed out that the efficacy of residual pesticides removal by ozone is influenced by its concentration, treatment temperature, chemical properties of pesticides, and properties of produce. Ozone alone or in combination with hydrogen peroxide, UV, or hydrogen peroxide and UV, has been found effective in detoxifying and degrading pesticides in water and wastewater (Ikehata and El-Din, 2005).

COMBINATION TREATMENTS

Combinations of ozone with other technologies have been extensively investigated in order to enhance the treatment's microbicidal efficacy. Ozone-based advanced oxidation processes is a developing technology that utilizes powerful oxidizing intermediates such as hydroxyl radicals, resulting in disinfection efficacy greater than that of ozone alone (Kim et al., 2003). Combination of ozone with hydrogen peroxide, UV, and electron beam facilitates the hydroxyl radicals generation (Sommer et al., 2004). Selected examples of combination treatments will be addressed in this section.

Ozone and Hydrogen Peroxide

Relatively low amounts of aqueous hydrogen peroxide are effective in initiating ozone decomposition and consequently generating hydroxyl radicals (Khadre et al., 2001). Water quality, temperature, pH and the ratio of hydrogen peroxide to ozone are also important factors to be considered in order to reach optimum oxidative performance (Kim et al., 2003). Antimicrobial effectiveness of ozone and hydrogen peroxide combination was evaluated against viruses, spores of *B. subtilis*, and *E. coli* in water (Sommer et al., 2004). Applying initial dosages of 2.5 mg/L ozone and 1.5 mg/L hydrogen peroxide inactivated 6 log units of three tested viruses and *E. coli* after the 4 min treatment. However, only 0.4 log inactivation of *B. subtilis* spore was achieved with the same treatment. Increasing the contact time to 10 min enhanced the sporicidal activity of the combination treatments, causing 1.5 log decrease in spore viability.

Ozone and Chlorine

Chlorine is found as hypochlorous acid or hypochlorite in water under neutral pH. Ozone oxidizes hypochlorites to form the weaker oxidizers, chlorates and chlorides (Bablon et al., 1991). Therefore, possible interactions between chlorine and ozone should be taken into consideration when these sanitizers are applied simultaneously or sequentially. Synergistic action of ozone and chlorine, in sequential applications, against various microorganisms has been reported (Cho et al., 2003; Li et al., 2001; Rennecker et al., 2000). Li et al. (2004) treated *G. lamblia* cysts with combined ozone and free chlorine and investigated the morphological changes of the parasite using transmission electron microscopy (TEM). The TEM results revealed that pretreatment of the cyst wall with the first sanitizer improved the penetrability of the second sanitizer thorough cytoplasm of the trophozoite, demonstrating the synergy of the combination treatment.

Ozone and Other Gases

Sequential or simultaneous application of ozone with other gases such as carbon dioxide, argon, and chlorine dioxide may potentially increase the microbicidal efficacy of the treatment. Vurma et al. (unpublished data) tested the feasibility of reducing natural microbiota and extending shelf life of strawberries using combinations of ozone and carbon dioxide. The researchers found that the combination of these two gasses was more effective in decreasing the natural microbial counts and preserving the quality of the treated strawberries than was either ozone or carbon dioxide treatment alone. Mitsuda et al. (1990) reported the synergistic effect of ozone and carbon dioxide on microbial

inactivation in foods. The synergy was believed to be due to the quenching action of carbon dioxide on the chain decomposition reaction of ozone; this increases the stability and bactericidal effectiveness of the ozone in the treatment environment.

Ozone and Heat

Although rapid decomposition of ozone occurs with elevated temperature, sequential application of ozone and heat could be beneficial (Kim et al., 2003). Rodriguez-Romo et al. (unpublished data) applied mild heat and gaseous ozone in sequence to inactivate *Salmonella* Enteritidis inside shell eggs using a prototype 300-L vessel. Shell eggs, internally contaminated with *Salmonella* Enteritidis (~10^7 CFU/g egg), were heated in water (57-59 °C) for ≤25 min, transferred to the processing vessel, placed under vacuum (10 in-Hg), and subsequently treated with ozone gas (10-12% wt/wt O_3 in O_2; ≤15 lb/in^2-gauge) for ≤40 min. Combined treatment of contaminated eggs significantly decreased internal *Salmonella* population by ≥7 log units, when compared to the un-treated control.

Pre-ozonation of *B. subtilis* spores at sublethal concentration (6 ppm for 1 min) greatly sensitized these microorganisms to subsequent heat treatments (Kim et al., 2003). The D-values observed during heat-treatment of spores were 294, 74.6, and 27 min at 85, 90, and 95 °C, respectively. When the spores were pre-treated with ozone, the D-values decreased to 26.3, 9.3, and 4.0 min for the corresponding heat treatments. Similarly, Novak and Yuan (2004) reported that ozone pre-treatment decreased the resistance of both vegetative and spore forms of *C. perfringens* on beef surfaces to mild heat treatment.

Ozone and Ultraviolet (UV) Radiation

Naitoh (1992) investigated the synergistic antimicrobial action of gaseous ozone and UV against the spores of four *Bacillus* and six *Clostridium* strains. Sporicidal activity was improved when the concentration of ozone or time of exposure to UV increased. Spores for these microorganisms were found to be more sensitive to both ozone and UV treatments when the relative humidity is high. Applying ozone and UV combination reduced the treatment time needed for spore inactivation. Similarly, ozone and UV combination enhanced the inactivation of *Bacillus* spores in water circulation system in the presence of organic compounds such as tryptophan and ribose (Urakami et al., 1997).

Magbanua Jr. et al. (2006) studied the inactivation kinetics of *E. coli* in ozone demand-free media using ozone and UV separately or simultaneously. Combination treatments produced synergy in microbial inactivation; generation of highly reactive hydroxyl radicals via ozone photolysis was believed to contribute to these results. Using UV irradiation followed by gaseous ozone treatment on externally contaminated shell eggs was synergistic against *Salmonella* Enteritidis (Rodriguez-Romo and Yousef, 2005).

Ozone and Pulsed Electric Field (PEF)

Combining selected non-thermal technologies in a sequential or simultaneous mode may have synergistic lethality against microorganisms of concern. Potential synergy between PEF and ozone, when applied in sequence, against *E. coli* 0157:H7, *L. monocytogenes* and *Lactobacillus leichmannii* was investigated (Unal et al., 2001). The authors reported a synergistic antimicrobial action against the tested microorganisms when the cells were pretreated with ozone and followed by PEF treatment. Oshima et al.

(1997) investigated the simultaneous treatment of ozone and PEF against *E. coli* and data from their study showed an additive inactivation effect.

LIMITATIONS, SAFETY CONSIDERATIONS, AND REGULATORY STATUS

Ozone is a powerful and effective sanitizer when applied against microorganisms in low ozone-demand media, e.g., relatively pure water or buffer systems (Kim et al., 2003). However, the oxidizing power of this sanitizer may limit its use in certain food applications. Ozone may cause sensory defects such as discoloration and undesirable odors on some treated products. In addition, food's nutritional components such as vitamins, amino acids, enzymes, essential fatty acids may be altered as a result of oxidation by ozone (Kim et al., 2003). Ozone may also cause physiological tissue damage to treated fruits and vegetables. Adverse effects of ozone depend on the food composition, applied ozone dose, and treatment conditions (Kim et al., 1999). Ozone may lead to the formation of some undesired disinfection by-products (DPB) such as bromate when treated water contains bromide (von Gunten, 2003). Thus, bromide quantity in treatment water should be monitored to prevent the generation of DBP in ozone washed products.

Ozone could be an extremely hazardous gas and its toxicity depends on the concentration and exposure time. The characteristic odor of ozone is detectable at concentrations as low as 0.02 ppm (vol/vol), and higher concentrations of this sanitizer exert acute symptoms in humans. Severe irritation to the upper and lower respiratory tracts is caused by ozone exposure. Symptoms resulting from exposure to high

concentrations of ozone include headaches, dryness of the throat, and pain in the chest. Higher level of exposures may be fatal for humans (Kim et al., 2003).

In the US, current permissible ozone exposure level time-weighted average (PEL-TWA) in the work place environment is 0.1 ppm vol/vol (0.2 mg/m^3), as recommended by the National Institute for Occupational Safety and Hazards (NIOSH) and adopted by the US Occupational Safety and Health Administration (OSHA). According to OSHA, short-term exposure limit (STEL) is 0.3 ppm vol/vol (0.6 mg/m^3) for an exposure less than 15 min and 4 times per day. The immediately dangerous to life or health (IDLH) concentration of ozone in air is 5 ppm (NIOSH, 2005).

Use of ozone in food application involves generation of the gas on site and maintaining the off gas in a closed system until it is destructed. Ozone has relatively short half-life and it decomposes to harmless oxygen. When ozone is generated and used in food applications, precautions and personal safety always must be taken in consideration. Excess ozone should be degassed and separated from the water stream or the treatment vessel and converted to oxygen prior to releasing to the atmosphere. Ambient ozone level should be monitored in the working environment and destruction systems and respirators are needed for the safety of workers in food processing facilities.

The use of ozone in gaseous state was approved by the United States Food and Drug Administration (FDA) in 1975 for the meat aging coolers. Application of ozone for disinfection of bottled water was approved by FDA in 1982. Recently, FDA approved the use of ozone in its gaseous and aqueous phase as an antimicrobial agent in food (Code of Federal Regulations, 2001). There is no labeling requirement for ozone-treated products.

FRESH PRODUCE AND MICROBIOLOGICAL FOOD SAFETY

Fresh fruits and vegetables are major components of a modern healthy diet. Since usually consumed raw, fresh fruits and vegetables are susceptible to contamination with pathogenic and spoilage microorganisms, beginning from the pre-harvesting stage through post-processing. Numerous pathogenic bacteria such as *Salmonella*, *Shigella*, *Escherichia coli* and *Campylobacter* which are commonly found in intestinal tract of animals, may contaminate fresh produce through contact with feces, sewage, irrigation and surface water, as well as during harvesting and post-harvest process handling (Beuchat, 1998). Contamination of fresh produce with viruses such as norovirus, hepatitis A, and other enteric viruses is attributed to an increase in number of foodborne illnesses thus creating serious public health concerns (Fino and Kniel, 2008).

Foodborne outbreaks associated with fresh produce

Foodborne disease outbreaks that are linked to minimally processed fruits and vegetables have increased during the past few decades (Sivapalasingam et al., 2004). Fresh produce caused the second highest number of foodborne disease outbreaks and highest number of disease cases among five major food categories during 1990 to 2005 in the US (Table 1.6.). During this period, a total of reported 713 foodborne disease outbreaks involving 34,049 illnesses were associated with consumption of produce with an average of 48 illnesses per outbreak (CSPINET, 2007).

Various pathogenic microorganisms have been implicated in produce-related outbreaks and the most common bacterial pathogens are *Salmonella* and *E. coli* O157:H7. Jalapeño and serrano peppers were identified as sources of *Salmonella* Saintpaul in a recent US outbreak (CDC, 2008). A total of 1,442 persons had been reported infected with the pathogen. This outbreak caused 286 hospitalizations with the two deaths suspected. A number of additional large fresh produce outbreaks were associated with *Salmonella.* A multi-state salmonellosis outbreak in the US was caused by consumption of raw tomatoes. Cantaloupe and strawberries were also among the fresh produce linked to *Salmonella* outbreaks (Hedberg et al., 1999).

In 2006, an outbreak associated with the contamination of packaged baby spinach with *E. coli* O157:H7 resulted in 205 confirm illnesses and 3 deaths nationwide (FDA, 2007). During same period, two additional outbreaks associated with *E. coli* O157:H7 contaminated leafy greens used in restaurants have been reported. Both outbreaks resulted in a total of 152 illnesses (Doyle and Erickson, 2008). In 2007, processed lettuce contaminated with *E. coli* O157 resulted in a disease outbreak in the Netherlands and Iceland (Friesma et al., 2008). This outbreak resulted in 50 laboratory-confirmed cases.

Microbial contamination of fresh fruits and vegetables not only poses significant risk to public health but also affects the industry financially by decreasing product shelflife. Other pathogens such as *Listeria monocytogenes* and *Clostridium botulinum* can attach to fruits and vegetables (Beuchat and Ryu, 1997). These pathogens have also been isolated from cabbage (Buck et al., 2003). Outbreak has been associated with scallions contaminated with *Shigella flexneri* (Tauxe, 1997). *Campylobacter jejuni* was isolated from various fresh produce such as green onions, peppers, lettuce and spinach (Buck et

al., 2003). Hepatitis A and Norwalk viruses have also been linked to fresh produce outbreaks and lettuce was the source of a Norwalk virus outbreak (Beuchat, 2002).

Enteric bacterial pathogens such as *E. coli* O157:H7 and *Salmonella* among the greatest concerns for fresh produce related outbreaks (Buck et al., 2003) and these two bacteria are considered to be emerging zoonotic pathogens (Sivapalasingam et al., 2005; Tauxe, 1997). This dissertation is focused on *E. coli* O157:H7 as the pathogen of concern, and spinach (*Spinacia oleracea* L.) was selected as a fresh produce model.

Escherichia coli

Escherichia coli is a Gram-negative, facultative anaerobic, member of the *Enterobacteriaceae*. The bacterium is a common inhabitant of the gastrointestinal tract of animals including cattle, deer, and wild pigs. Most *E. coli* are harmless to humans but a small proportion can cause clinical symptoms in humans and other mammals. Strains of *E. coli* that causes illness are categorized on the basis of their virulence, pathogenic mechanisms, clinical syndromes and antigenic characteristics. The major groups of pathogenic *E. coli* are enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), diffusely adhering (DAEC), enteroaggregative (EAEC) and enterohaemorrhagic (EHEC) (Doyle et al., 1997). Adults usually become sick after 1 to 7 days of exposure with *E. coli* O157:H7 and these individuals show symptoms such as bloody diarrhea. Most healthy adults recover within a week. The pathogen may cause hemolytic-uremic syndrome (HUS) that can lead to death by kidney failure, mostly in children and elderly. The CDC estimated that *E. coli* O157:H7 alone to cause 73,480 illnesses in the United States annually, with approximately 2168 hospitalizations and 61 deaths (Mead et al.,

1999). The most common foods associated this disease are beef, leafy greens, sprouts, and unpasteurized juice, but fresh produce is the leading cause of *E. coli* O157:H7 foodborne disease outbreaks in the US (http://www.ers.usda.gov/AmberWaves/June07/Features/Spinach.htm).

Reasons for increased produce related outbreaks

Better diagnostics and increased outbreak surveillance system could lead to an increased identification of produce-associated outbreaks. Other possible reasons for the increase in produce-related outbreaks are increased consumption of fresh fruits and vegetables as a part of healthy diet, availability of wide variety of fresh produce year around, increased global trade in raw fruits and vegetables, and evolution of food industry from being local farming-trade to global production-processing-trade. Changes in packaging technology that extends the time between harvesting and consumption and the application of post-harvest unit operations such as cutting, slicing and shredding will increase the chance of survival and growth of pathogens by removing the natural protective barriers of the intact plant and for providing nutrients. Large- scale operations in fresh-cut produce industry may easily become contaminated at multiple points, leading to the spread of pathogens into large amount of produce (Beuchat, 1998; Doyle and Erickson, 2008; Tauxe et al., 1997).

Sources of pathogen contamination of fresh produce

Fresh produce are susceptible to contamination with pathogenic and spoilage microorganisms at any point in the production chain. Bacterial contamination sources of fresh produce during pre-harvest and postharvest operations include animals, insects, irrigation water, soil, dirty equipment, cross contamination and human handling (Beuchat and Ryu, 1997). Enteric pathogens such can *E. coli* O157:H7 are extremely capable of surviving on produce. Factors affecting the survival of these pathogens are environment temperature, water/nutrients availability, UV radiation, tissue damage, and the plants natural microbiota (Abdul-Raouf et al., 1993; Aruscavage et al., 2006; Cooley et al., 2006). Value added operations such as cutting, shredding, and washing steps at processing plant may increase the microbial load of produce (Allende et al., 2004), and therefore these operations may lead to the spread of microorganisms, including pathogens.

Leafy Greens Unit Operations

Procedures and unit operations for preparation of minimally processed fresh-cut leafy greens are outlined in Fig. 1.8. Leafy greens are harvested mechanically or by hand and transferred to cooling facility where the produce is cooled immediately by either vacuum cooling/hydro-vacuum cooling or forced-air cooling. Following cooling, fresh produce is transported to processing plant where the fresh-cut value added operations are performed. These operations include cutting the leafy green with mostly automated sharp knives, vigorous washing with water containing chlorine at 200 ppm level for 30 sec,

removing the excess water by drying, packaging with gas flush to create modified atmosphere, and finally transport to retail/ food service operations (Kader, 2002).

Rapid removal of field heat is one of the most important tools for extending shelflife of perishable fresh produce (Brosnan and Sun, 2001; Kader, 2002). Traditionally, vacuum cooling is used to remove field heat from leafy greens within 30 min (Kader, 2002). Rapid evaporation of water from the produce at very low vacuum is the principal of this process. Pressure in the vacuum tunnel is reduced by a powerful vacuum pump, and as the pressure decreases, the boiling temperature of the free water in the product is lowered. When the boiling point of water reaches the initial produce temperature, water starts to evaporate and quickly removes heat from the product. Water vapor generated during evaporation must be removed by cooling coils to avoid its accumulation inside the vessel in order to reach target cooling (Sun and Zheng, 2006). Generally vacuum cooling results in 1% of weight loss for 5-6 °C reduction in a product temperature due to evaporation (Kader, 2002).

Mitigation Strategies to reduce microbial risks associated with fresh produce

Recent research has mainly focused on the prevention of pathogen contamination of fresh produce during pre-harvest, production, and post-harvest operations, in response to current public health concerns about the microbiological safety of produce. Numerous physical, chemical and biological methods are investigated for reducing the potential pathogen load of produce during postharvest processing. Some of the agents tested are chlorine dioxide, bromine, iodine, trisodium phosphate, alkaline compounds, quaternary ammonium compounds, organic acids, hydrogen peroxide, and irradiation. Each of these

agents are partially effective in removing microbial contaminants from fresh produce and their efficiencies depend on the type of produce, characteristics of the produce surface, contact time, applied dosage, and pathogen nature (Beuchat, 1998). Irradiation may reduce the bacterial load of fresh produce appreciably but the process is relatively expensive and is not consumer friendly.

Current sanitization technology of fresh produce relies on chlorine, mostly in the form of sodium or calcium hypochlorite, which is added to fresh and recycled wash water at 20-200 ppm (Sapers, 2006). In spite of its antimicrobial potency against microorganisms in pure cultures, hypochlorites do not adequately decontaminate fresh produce or assure the safety of the retail products (Kim et al., 2003). According to these authors, sanitizers are often neutralized by organic matter in wash water or produce tissues, before they reach the pathogens, particularly those imbedded in surface crevices, internalized or in biofilm within product matrix.

Conventional washing and sanitization technology target pathogenic contaminants on the surface of fresh produce. Unfortunately, these conventional procedures only cause modest decrease in the microbial load of fresh produce (Sapers, 2006). If *E. coli* O157:H7 and similar pathogens become internalized into leafy produce, they pose an additional challenge to these conventional treatments (Bartz, 2006; Sapers, 2001). Internalization of enteric pathogens such as *Salmonella* or *E. coli* O157:H7 into the plants edible parts has been reported for tomatoes (Guo et al., 2002), radish sprouts (Itoh et al., 1998), bean sprouts (Warriner et al., 2003), and lettuce (Solomon et al., 2002). Additionally, spatial and mass transfer considerations hinder the accessibility of sanitizers to locations where microbial contaminants may be hidden. Therefore it is

necessary to develop sound mitigation strategies to minimize health hazards associated with fresh produce.

CONCLUSIONS

Although good agricultural practices (GAP) and good handling practices (GHP) may reduce the incidence of pathogens in fresh produce via sources such as irrigation water, there is no known defense against contamination due to animal, bird or insect intrusion events. Apart from preventing pathogen contamination on fresh fruits and vegetables, there are no mitigation strategies that are commercially available at the moment to minimize the risk of contamination during the post harvest operations of fresh leafy greens. Contaminations of fresh fruits with spoilage microorganisms also create economical problems by decreasing products shelflife. It is necessary, therefore, to devise an efficient sanitization process that uses a potent sanitizer and that ensures its diffusion to hidden and entrapped pathogens.

The food industry is keenly interested in using ozone to enhance the safety and stability of food products and in exploring new applications of this potent sanitizer. This interest was recently matched by a US governmental approval of ozone for the safe use, in gaseous and aqueous phases, as an antimicrobial agent on food (Federal Register. 2001). Ozone has a longer half-life in the gaseous than the aqueous state (Kim et al., 2003). Diffusion within gases is far more rapid than within liquids (Bird et al., 1960). Additionally, gaseous ozone is highly effective against pathogenic contaminants on shellegg, when vacuum is applied prior to sanitization, and when sufficient time is provided for the sanitizer to reach embedded microorganisms (Yousef and Rodriguez-

Romo, 2004). Ozone reactivity, penetrability and spontaneous decomposition to a non-toxic product (i.e., O_2) make this gas a viable disinfectant for ensuring the safety and quality of water and food products (Graham, 1997). Considering the rapid increase in fresh produce sales, and the frequent disease outbreaks that were recently associated with consumption of these minimally processed products (Gorny, 2006), it is of paramount importance to explore ozone-based technologies and processes designed to decontaminate this category of foods.

The main objectives of this dissertation are to (1) enhance the safety of fresh produce by integrating ozone-based sanitization steps into existing processing practices, while targeting *E. coli* O157:H7 as the pathogen of concern, (2) to assess the feasibility of using ozone, carbon dioxide or their combinations, for reducing natural microbiota, and extending the shelf-life of strawberries. In the first part of this dissertation, new ozone-based mitigation strategies and processes are developed in order to inactivate *E. coli* O157:H7 on leafy greens. The second part investigates mainly shelflife extension of fresh strawberries using gaseous ozone. Possibility of using other gases such as CO_2 in combination with ozone in shelflife extension is also explored.

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Property	Value
Melting point	-192.5 ± 0.4 °C
Boiling point	-111.9 ± 0.3 °C
Heat of formation	142.7 kJ/mol
Critical temperature	-12.1 °C
Critical pressure	5532.3 kPa
Density (gaseous phase)[a]	2.14 g/L

[a] Determined at 0 °C and one atmosphere (101.3 kPa).

Table 1.1. Selected physical properties of ozone (Horváth et al., 1985; Wojtowicz, 2004).

Bacteria	Inactivation (log CFU)	Treatment Conditions			References
		Time (min)	Concentration (mg/L)	Medium/Food	
Escherichia coli	4.0	1.67	0.23-0.26	Water	Farooq and Akhlaque (1983)
E. coli O157:H7	~3.7	3	21-25	On Apple surface	Achen and Yousef (2001)
	~0.6	3	21-25	In stem/calyx	
Listeria monocytogenes	0.7 to ~7.0	0.5	0.2 to 1.8	Water (pH at 5.9)	Kim and Yousef (2000)
Shigella sonnei	5.6	1	2.2	In water	Selma et al. (2007)
S. sonnei	1.8	5	5	In shredded lettuce	
Yersinia enterocolitica	4.6	1	1.4	Water	Selma et al. (2006)
	6.2	1	1.9	Water	
	1.6	1	5	Potato surface	
Salmonella Enteritidis	1.0	0.25	8% (wt/wt)	Broiler carcass	Ramirez et al. (1994)
Salmonella Enteritidis	0.6 to ~4.0	0.5	0.5 to 6.5	Water	Dave (1999)
Salmonella Typhimirium	4.3	1.67	0.23-0.26	Water	Farooq and Akhlaque (1983)
Bacillus cereus	>2.0	5	0.12	Water	Broadwater et al. (1973)
B. cereus (spores)	>2.0	5	2.29	Water	
B. cereus	6.1	1	11	Spore suspension	Khadre and Yousef (2001b)
B. stearothermophilus	1.3	1	11	Aqueous ozone mix	
Legionella pneumophila	>4.5	20	0.32	Water	Edelstein et al. (1982)
Fecal streptocci	>2.0	19	2.2	Raw waste water	Joret et al. (1982)

Table 1.2. Inactivation of bacteria by ozone

Fungi	Inactivation (log CFU)	Treatment Conditions			References
		Time (min)	Concentration (mg/L or g)	Medium/Food	
Aspergillus flavus (conidia)	1.0	1.72	1.74	Buffer (pH 7.0)	Beuchat et al. (1999)
A. parasiticus (conidia)	1.0	1.54	1.74	Buffer (pH 5.5)	
	1.0	2.08	1.74	Buffer (pH 7.0)	
	1.0	1.71	1.74	Buffer (pH 5.5)	
A. niger (spores)	<1.0	5.0	0.188	Water	Restaino et al. (1995)
Candida parapsilosis	2.7	1.67	0.23-0.26	Water	Farooq and Akhlaque (1983)
C. tropicalis	2.0	0.30-0.08	0.02-1.0	Water	Kawamura et al. (1986)
C. albanicus	>4.5	Immediate	0.188	Water	Restaino et al. (1995)
Zygosaccharomyces bailii	>4.5	Immediate	0.188	Water	Restaino et al. (1995)

Table 1.3. Inactivation of fungi by ozone

Protozoa	Inactivation (log CFU)	Treatment Conditions			References
		Time (min)	Concentration (mg/L)	Medium/Food	
Giardia lamblia	2.0	1.1	0.7	Water	Wickramanayake et al. (1984)
G. lamblia	>3.0	1.0	1.5	Water	Widmer et al. (2002)
G. lamblia	>3.0	2.0	1.7	Buffer (pH 6.85)	Finch *et al*. (1993)
	>4.0	5.0	1.9	Buffer (pH 6.85)	
G. muris	2.0	2.8	0.5	Water	Wickramanayake et al. (1984)
G. muris	~4.0	5.0	0.6	Buffer (pH 6.70)	Finch et al. (1993)
Cryptosporidium parvum	>1.0	5.0	1.0	Water	Korich et al. (1990)
Naegleria gruberi	2.0	2.1	2.0	Water	Wickramanayake et al. (1984)

Table 1.4. Inactivation of protozoa by ozone

Viruses	Inactivation (log CFU)	Treatment Conditions			References
		Time (min)	Concentration (mg/L)	Medium/Food	
Bacteriophage f2	2.0 to 7.0	0.08	0.09 to 0.8	Water	Kim et al. (1980)
Bacteriophage MS2	2.96	1.0	0.6	Phosphate buffer	Finch and Fairbairn (1991)
Bacteriophage MS2	>3.0	0.17	0.37	Water	Shin and Sobsey (2003)
Norwalk	>3.0	0.17	0.37		
Poliovirus type 1	>3.0	0.17	0.37		
Poliovirus type 1	2.5 to 3.0	1.67	0.23 to 0.26	Water	Farooq and Akhlaque (1983)
Poliovirus type 3	1.63	1.0	0.6	Phosphate buffer	Finch and Fairbairn (1991)
Hepatitis A	~1.0	0.80	0.10	Phosphate buffer	
	2.7	0.02	0.25		Herbold et al. (1989)
	100%	-	0.38		
Hepatitis A	3.9	0.08	0.3 to 0.4	Phosphate buffer	Hall and Sobsey (1993)
Rotavirus human	3.0	6.0	0.1 to 0.3	Phosphate buffer	Vaughn et al. (1987)
Rotavirus SA 11 simian	3.0	6.0-8.0	0.1 to 0.25		
Rotavirus Wa human ATCC	1.to 1.0	1.0	2.1 to 4.2	Water	Khadre et al. (2001)
Rotavirus Wa human Wooster	2.to 5.0	1.0	1.9 to 15.9		
Coxsackie virus A9	>1.7	0.16	0.035	Water	Boyce et al. (1981)
Coxsackie virus B5	2.0	2.0	0.32	Sludge effluent	Harakeh and Butler (1985)
	4.0	2.5	0.40		

Table 1.5. Inactivation of viruses by ozone

Category	Outbreaks	Cases
Eggs	352	11224
Seafood	1053	10415
Beef	506	13873
Produce	713	34049
Poultry	580	17661

Table 1.6. Vehicle categories of number of outbreaks and cases associated with foods during 1990 to 2005 (data from CSPINET, 2007).

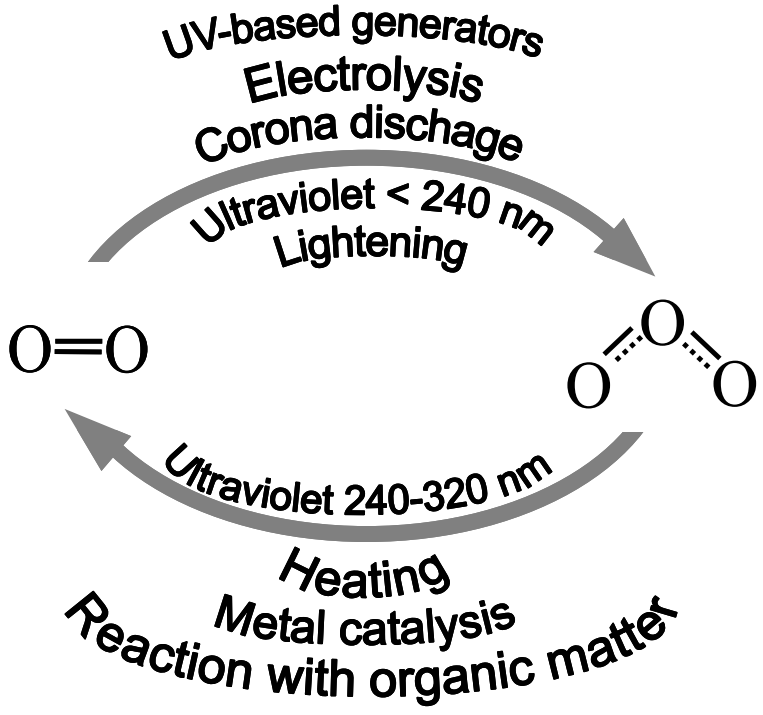

Figure 1.1. Formation and decomposition of ozone in nature or through industrial processes. [O_2: oxygen; O_3: ozone; inner reactions: occur in nature; outer reactions: industrially generated]

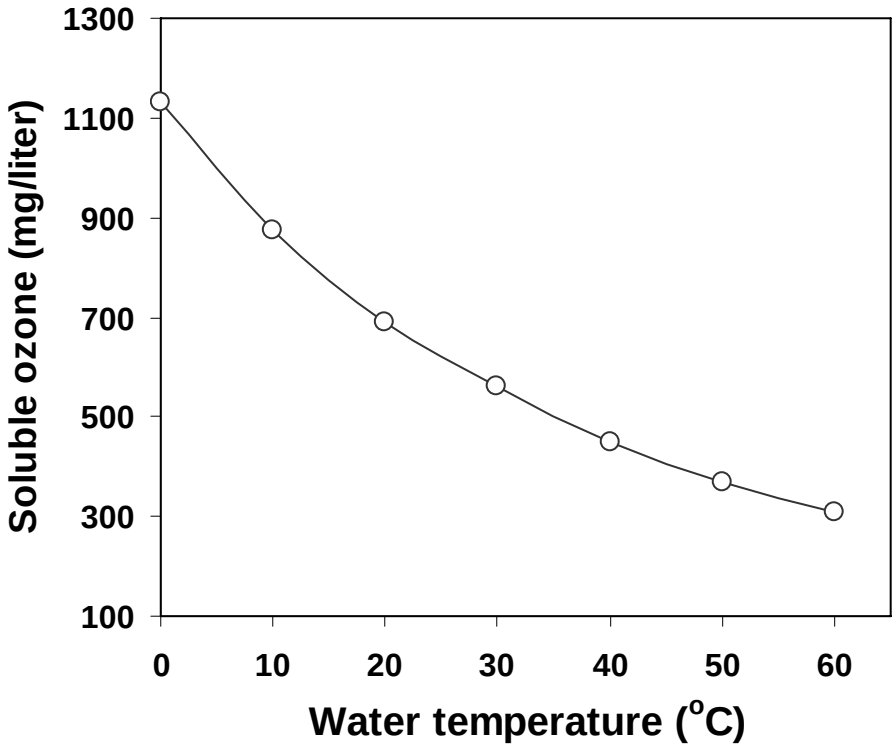

Figure 1.2. Solubility of gaseous ozone in water at atmospheric pressure (101.3 kPa) and different temperatures (Horváth et al., 1985).

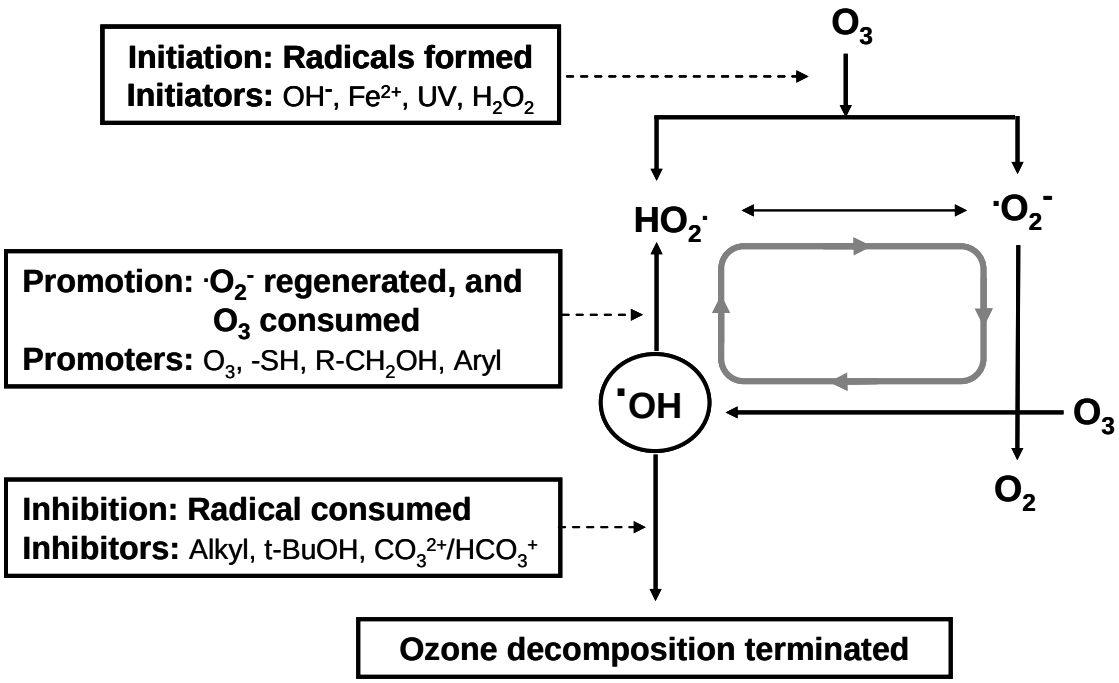

Figure 1.3. Ozone decomposition and free radical formation reactions (Adapted from Khadre et al., 2001). [O_2, oxygen; O_3, ozone; $^{\cdot}OH$, hydroxyl radical; $^{\cdot}O_2^-$, superoxide anion radical; $HO_2^{\cdot}$, hydroperoxide radical]

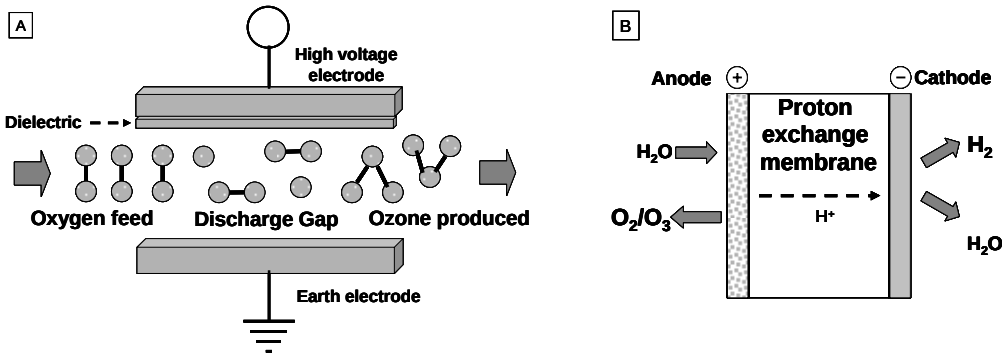

Figure 1.4. Ozone generation by corona discharge method (Adapted from Rice et al., 1981) and electrolysis of water (Lynntech, 1998). [A, corona discharge; B, electrolysis]

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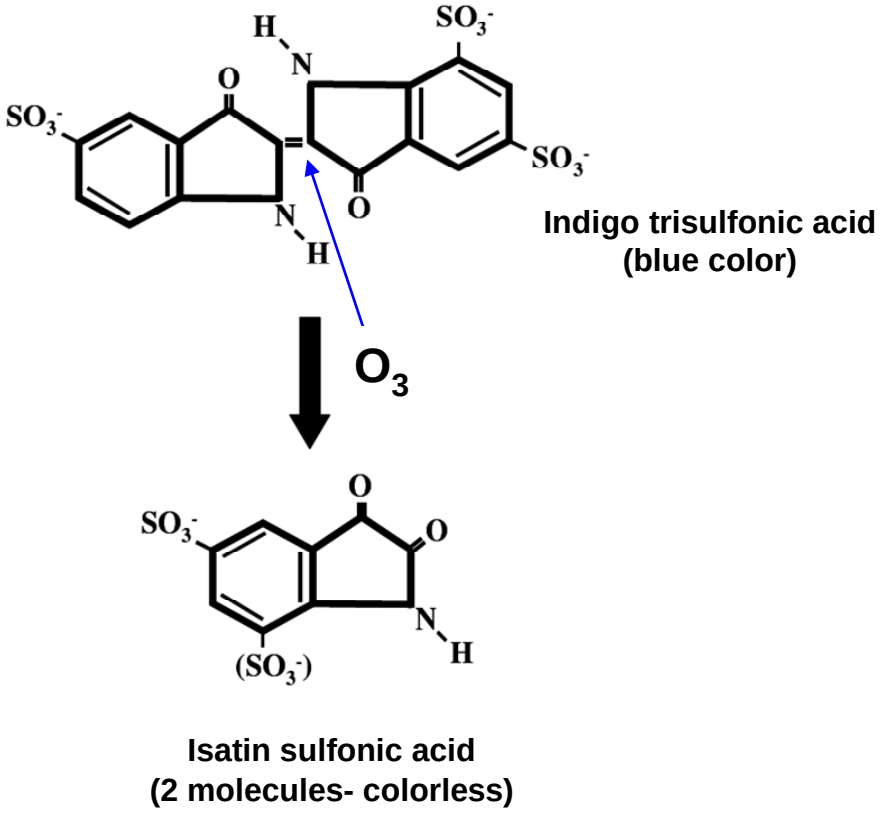

Figure1.5. Reaction between ozone and indigo trisulfonic acid (Modified from Bader and Hoigné, 1981).

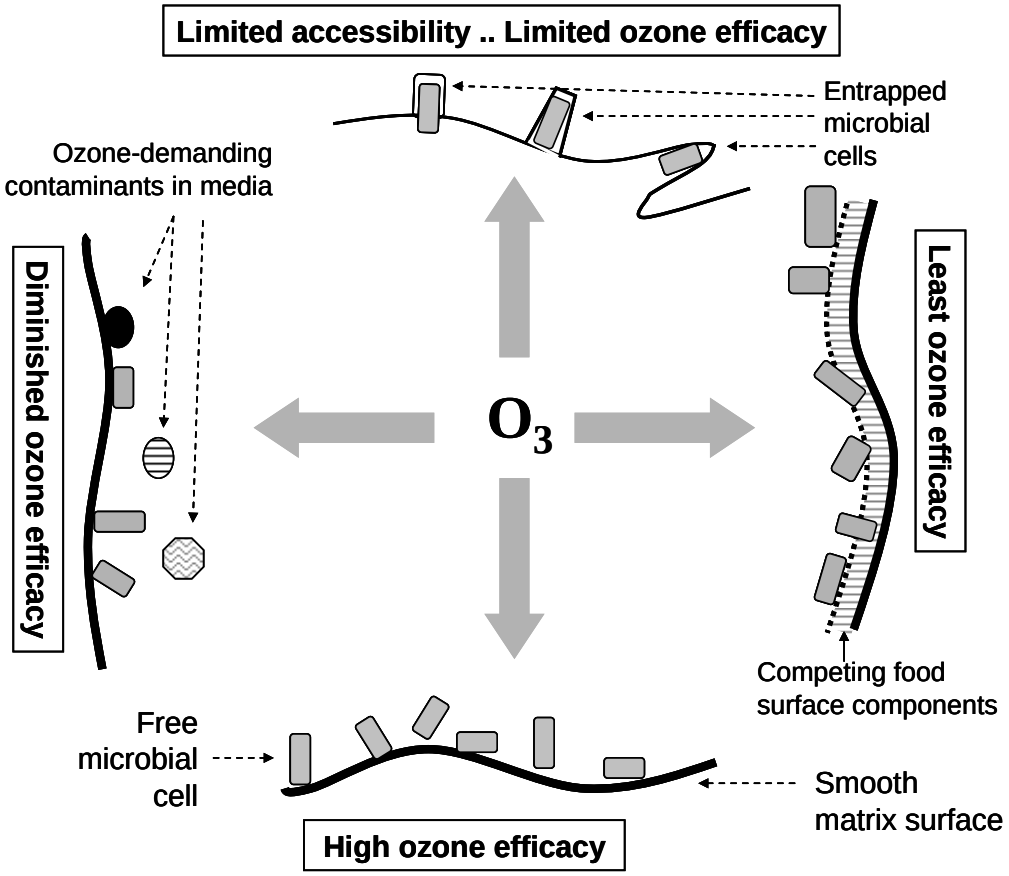

Figure 1.6. Schematic representation of accessibility of ozone to target microorganisms as related to the efficacy of the sanitizer.

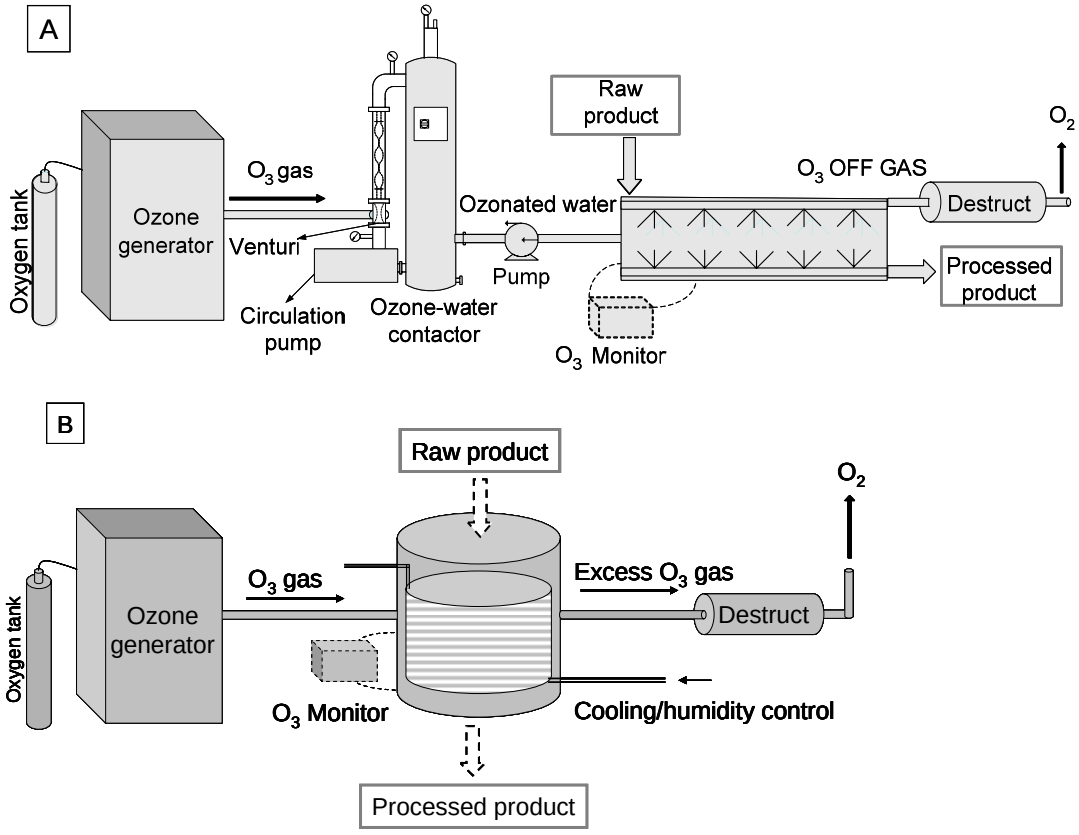

Figure 1.7. Conceptual aqueous and gaseous ozone treatment systems that may be relevant to food applications. [A: aqueous, B, gaseous]

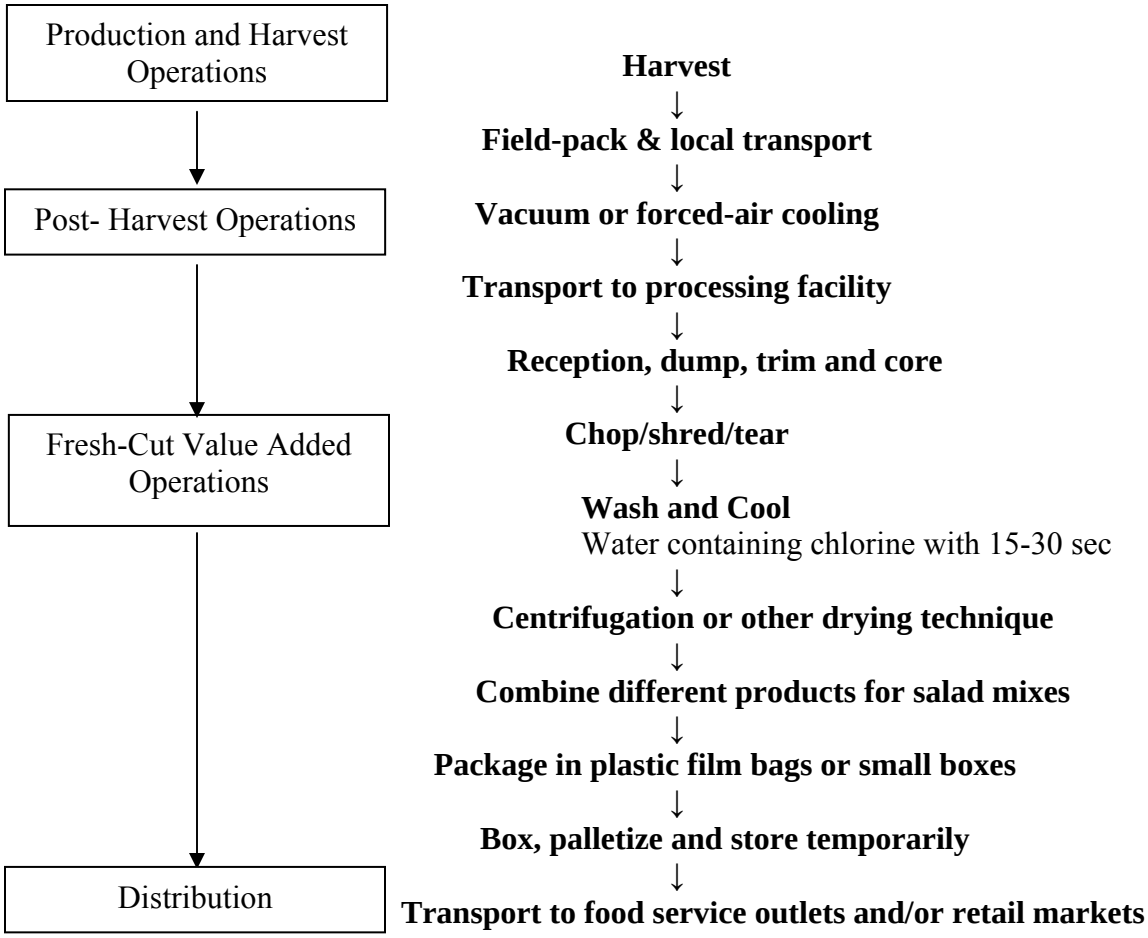

Figure 1.8. Preparation of minimally processed fresh-cut leafy greens (Adapted from Kader, 2002).

CHAPTER 2

DEVELOPMENT OF SANITIZATION TECHNOLOGIES TO INTEGRATE GASEOUS OZONE TREATMENTS INTO FRESH PRODUCE VACUUM COOLING OPERATIONS

ABSTRACT

The aim of this study is to enhance the safety of fresh produce by integrating ozone-based sanitization step into existing practices, while targeting *Escherichia coli* O157:H7 as the pathogen of concern. A process has been developed where fresh produce (baby spinach, *Spinacia oleracea* L) is treated with vacuum to induce cooling and re-pressurized with an ozone-containing gaseous mixture. The process of sanitizing fresh produce during vacuum cooling will be referred to as "SanVac". Inoculated baby spinach (~10^7 CFU *E. coli* O157:H7/g) was treated in a pilot-scale SanVac system. Surviving *E. coli* O157:H7 and natural microbiota in spinach were enumerated before and after treatments. The SanVac processes successfully decreased *E. coli* O157:H7 population on the product by up to 2.4 log. Contribution of important treatment variables (ozone

concentration, pressure and treatment time) to process lethality was also investigated using response-surface methodology. An optimized SanVac process that inactivated 1.8 log of *E. coli* O157:H7 population with no apparent damage to quality of spinach yielded these parameters: 1.5 g O_3/kg gas-mix (935 ppm, vol/vol), 10 psig holding pressure, and 30 min holding time. In conclusion, a novel sanitization approach was effective in reducing considerably the *E. coli* O157:H7 population on spinach and the new treatment should be relatively easy to integrate into existing fresh produce processes.

INTRODUCTION

Fresh produce safety is a critical and urgent concern. Since fields are open systems, contamination cannot, generally be stopped and it is prudent to develop inactivation strategies for all produce to assure consumer safety. The best conceivable practices may be compromised by unforeseen intrusion events. There is an urgent need for methods that will ensure that even internalized and highly resistant microorganisms be inactivated or at minimum, controlled during the passage of produce from farm to fork. Gaseous ozone, when introduced after vacuum cooling, can potentially reach and inactivate internalized and imbedded pathogens.

Fresh produce is only minimally-processed before consumption and the risk to consumer's health form this source is on the rise (Sivapalasingam et al., 2004; Doyle and Erickson, 2008). Chlorine, mostly in the form of sodium or calcium hypochlorite, is commonly used in washing fresh produce. The sanitizer is added to fresh and recycled wash water at 20-200 ppm, mainly to maintain the microbial quality of the water and to

prevent cross contamination. Unfortunately, this practice causes only modest decreases in the microbial load of fresh produce (Beuchat, 1998; Sapers, 2006). If *E. coli* O157:H7 and similar pathogens become internalized into leafy produce, they pose an additional challenge to these conventional cleaning or sanitization treatments (Bartz, 2006; Sapers, 2001). Internalization of enteric pathogens such as *Salmonella* or *E. coli* O157:H7 into the plants edible parts has been reported for tomatoes (Guo et al., 2002), radish sprouts (Itoh et al., 1998), bean sprouts (Warriner et al., 2003), and lettuce (Solomon et al., 2002).

Ozone gas is a versatile sanitizer with excellent antimicrobial properties. The sanitizer effectively and rapidly inactivates pathogens in low-ozone demand media (Khadre et al., 2001). Efficacy of gaseous ozone depends on environment relative humidity, temperature and pressure (Kim et al., 2003). Ozone reactivity, penetrability and spontaneous decomposition to a non-toxic product (i.e., O_2) make this gas a viable disinfectant for ensuring the safety and quality of water and food products (Graham, 1997). It should be cautioned that reactivity of ozone makes it corrosive to some materials used in food processing, and toxicity of the gas obligates users to observe certain safety precautions (Kim et al., 2003).

Fresh produce is preferably chilled immediately after harvesting. Leafy vegetables, such as spinach and lettuce, are chilled through vacuum cooling to remove field heat and to preserve fresh produce during shipping to retail market or processing facility (Brosnan and Sun, 2001; Kader, 2002). Rapid evaporation of water from the produce at very low vacuum is the principle of this process and cooling time is achieved

usually within 30 min for leafy greens (Sun and Zheng, 2006). Water vapor generated during evaporation must be removed by cooling coils to avoid its accumulation inside the vessel (Thompson et al., 2002). In general, vacuum cooling results in 1% of weight loss for each 5-6 °C reduction in a product temperature, due to water evaporation (Cantwell and Suslow, 2002). Vacuum cooling, as currently practiced, may cause internalization of pathogens in produce (Li et al., 2008).

For a given sanitizer, it is advantageous 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 (Bird, 1960). Additionally, ozone has a longer half-life in the gaseous than the aqueous state (Kim et al., 2003). Thus, it is plausible to apply ozone during vacuum cooling in the gaseous state to facilitate the penetration of the sanitizer to points deep within the produce. Based on previous studies, gaseous ozone was highly effective against pathogenic contaminants on shell eggs, when vacuum is applied prior to sanitization, and when sufficient time is provided for the sanitizer to reach embedded microorganisms (Yousef and Rodriguez-Romo, 2004).

Occasional failure of conventional sanitization processes is linked to the inability of sanitizer to reach attached, entrapped, or internalized pathogens in fresh produce. Furthermore, diffusion of ozone into leafy greens is greatly accelerated when the product is subjected to vacuum, followed by introduction of the gaseous sanitizer; this also enables the sanitizer to enter the evacuated interstitial spaces. Therefore, the goal of this study is to enhance the safety of fresh produce in general, and baby spinach in particular, by integrating ozone-based sanitization step into existing processing practices, while targeting *E. coli* O157:H7 as the pathogen of concern. The objectives of the study are to

(i) develop a new ozone-based sanitization process during vacuum cooling of fresh produce, and (ii) optimize treatment conditions, particularly pressure, ozone concentration, and holding time, for maximum process lethality against *E. coli* O157:H7 without damaging product quality.

MATERIALS AND METHODS

Bacterial strain, culture conditions, and preparation of inoculum

Escherichia coli O157:H7 B6-914 (a non-virulent strain), was used throughout the study. This *E. coli* O157:H7 strain contains genes for the green fluorescence protein (GFP) as well as ampicillin and cycloheximide resistance (Fratamico et al., 1997), which enabled enumeration of the bacterium in the presence of the natural microbiota of baby spinach (Fig. 2.1). Similar growth and survival characteristics between the GFP-transformed strains and parent strains of this bacterium have been demonstrated (Fratamico et al., 1997; Tombolini et al., 1997). Stock cultures of *E. coli* O157:H7 were stored at -80°C in Luria-Bertani broth (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) was transferred and cultured in LB broth twice prior to use. Both LB agar and LB broth contained 100 μg/ml of each ampicillin trihydrate (Fisher Biotech, Subiaco, Wash.) and cycloheximide (Sigma-Aldrich, St. Louis, Mo.). Overnight cultures were harvested by centrifugation at 8000 x *g* for 10 min (Sorval RC-5B DuPont,

Wilmington, Del.), washed, and re-suspended in 0.1% (wt/v) buffered peptone water to achieve a final concentration of 10^9 CFU/ml.

Inoculation of spinach (*Spinacia oleracea* L)

Baby spinach was procured from local grocery stores (Columbus, Ohio), a day before testing. For selected experiments, freshly-harvested, unwashed and non-vacuum cooled spinach (Fresh Express Inc., Salinas, Calif.) was used. Samples of fresh produce (25 g) were spot-inoculated with 100 µl *E. coli* O157:H7 to reach aproximately10^7 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 any 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, N.J.) and mixed with 225 ml peptone water. The bag contents were homogenized for 2 min in a stomacher (Model STO-400, Tekmar Inc., Cincinnati, Ohio). The homogenized samples were serially diluted in peptone water and surface-plated onto the antibiotic-containing LB agar. Plates were incubated at 35°C for 24-48 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 just described, also were plated onto

tryptic soy agar (TSA; Difco) and plates were incubated at 37°C for 24-48 h for determining mesophilic aerobic counts.

Ozone generation and measurements

A high capacity Ozat ozone generator (model CSF-7, Ozonia Inc., Elmwood Park, N.J.) used to produce gaseous ozone for these experiments. The concentration of gaseous ozone in ozone-treatment chambers was measured using an UV-absorption monitor (model LC-L2-2000, IN USA Inc., Norwood, Mass.). Excess ozone was decomposed into oxygen using a thermal ozone-destruct unit (model ODT-006, Ozonia Inc.).

Sanitization during vacuum cooling (SanVac)- equipment setup

A system was designed and assembled to mimic the industrial vacuum cooling of leafy greens (Fig. 2.2). In addition to the ozone generator, described earlier, 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, N.H.) which circulated a coolant mix (40% propylene glycol, Houghton Chemical Corporation, Allston, Mass.) through the condensing coil of the treatment vessel. A gas pre-portioning unit (Model RK-03218-50, Cole-Parmer Instrument Company, Vernon Hills, Ill.) was added to the system to deliver a mixture of ozone with other gases, e.g., carbon dioxide (CO_2). Flow lines for treatment gases were equipped with flowmeters 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, Utah) to monitor ambient temperature, chamber temperature, vacuum and pressure levels achieved, and concentration of the ozone gas during re-pressurization and holding steps. A sampling manifold was installed to draw gas samples from two locations in the treatment chamber and measure the ozone concentration using an ozone analyzer. The temperature of the condensing coil inside the treatment vessel was maintained at -5±1^{o}C to enhance the vacuum cooling operation. Additionally, a water inlet line was added to the treatment vessel in order to adjust the relative humidity (95-100%) which was monitored using a thermohygrometer (Model OKTAN 03313-70, Cole-Parmer). The system was located in the biosafety level-2 pathogenic pilot plant, Department of Food Science and Technology, The Ohio State University.

A typical SanVac process sequence is shown schematically in Fig. 2.3. The process included the application of sufficient vacuum to cool the fresh produce to the desired temperature (i.e., 4°C), re-pressurizing the vessel with ozone (in oxygen milieu) for specified time and flow rate to reach the targeted concentration of the sanitizer, continued re-pressurization with oxygen (or other ozone-free gases) to reach the target pressure, maintaining the pressure for the specified treatment holding time, and releasing the gases through the thermal ozone-destruct unit. The product is removed when the vessel interior was at atmospheric pressure and contained a safe level of ozone residue.

Ozone distribution inside the treatment vessel

The fresh produce treatment vessel was equipped with a sampling manifold to measure and compare the distribution of ozone concentration of two sampling points during the vacuum cooling process. The first sampling point was located at the periphery, about 1 cm inside of the treatment vessel (L1), and another point close to the spinach sample (L2) (Fig. 2.4). The fresh produce (1 kg) was vacuum-cooled, re-pressurized with ozone and oxygen mixture, and held at atmospheric pressure and 5 °C for up to 60 min. The sampling ports were used intermittently at 2 min intervals to measure the ozone concentration during holding time. From each sampling port, data were collected in two replicates and the experiment was repeated twice.

Determining the contribution of SanVac treatment variables to *E. coli* O157:H7 lethality using response-surface methodology

Samples of inoculated spinach leaves (25 g each) were transferred to the processing vessel and vacuum-cooled to 4±1^{o}C (~28.5 in. Hg vac.) within 15 min. Subsequently, the vessel was re-pressurized with ozone-oxygen mixture at 55 lpm flow-rate to reach the target ozone concentration (e.g. 1.5 g O_3/kg gaseous atmosphere), and held either under partial vacuum (20 in. Hg vac.), 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, N.C.). 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 at the conditions assigned by the central composite design. Each experiment was performed in duplicates, with a total of 4 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 as described in the result and discussion section. 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 (i.e., Log reduction *E. coli* CFU/g spinach), X_i are the treatment variables, β_0, β_i, β_{ij}, 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 (1) applies:

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

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, β_{11}, β_{22}, and β_{33} are the quadratic coefficients. Raw data of microbial counts (CFU/g spinach) were converted into log units

before analyses. Statistical analyses included multiple regressions, analyses of variance (ANOVA), and comparisons of mean bacterial counts (Log reduction *E. coli* CFU/g spinach) for each treatment by Tukey's multiple comparison test. The data were analyzed using JMP IN and SAS (version 9.1.3.**,** SAS Inc, Cary, N.C.). Three-dimensional response-surface figures were generated by a commercial software (SigmaPlot, version 11.0, Systat Software, Inc., San Jose, Calif.) 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 experimental 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 re-pressurization with ozone, (ii) vacuum-cooling followed by re-pressurization with ozone-CO_2 mixture, (iii) vacuum cooling followed by re-pressurization with oxygen only (control), and (iv) no treatments (control). Inoculated spinach was vacuum-cooled to 4±1^{o}C, subsequently re-pressurized using oxygen, ozone (1.5 g/kg gas mixture), or ozone (1.5 g/kg gas mixture) and carbon dioxide combination (50%, vol/vol), and held at 10 psig for 30 min. Surviving *E. coli* O157:H7 were enumerated before and after treatments. Samples were refrigerated at~ 2°C for up to 7 days and survivors were monitored during storage.

Data Analysis.

All experiments were repeated twice or three times, and each run consisted of duplicate samples. Microbial counts (CFU/g spinach) were converted into log units before analyses and data were analyzed statistically (JMP IN). Mean values for log reduction in bacterial populations were compared by one-way analysis of variance. Tukey's multiple comparisons test was used to analyze mean differences. Values with $P \leq 0.05$ were considered to be 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 AND DISCUSSION

Development of SanVac ozone treatment system

Leafy greens are harvested mechanically or by hand and transferred to a nearby facility where the product is cooled immediately. The vacuum cooling technology has been used extensively for the certain types of horticultural products such as leafy greens (Brosnan and Sun, 2001). Rapid removal of field heat is one of the most important tools for extending shelflife of perishable fresh produce (Brosnan and Sun, 2001; Kader 2002). For leafy greens which have high surface-to-mass ratio (e.g., spinach), vacuum cooling is often accomplished within 30 min (Sun and Zheng, 2006; Thompson et al., 2002).

In the current study, a process has been developed to decontaminate baby spinach during vacuum cooling (SanVac). Baby spinach was treated with vacuum (28.5 in Hg vac.) to induce sufficient cooling (temperature drop of 15±1^{o}C); this was accomplished within 15 min. The cooling time accomplished during SanVac process is comparable to those used in the leafy green processing (Thompson et al., 2002; Kader, 2002). The vessel was then re-pressurized with ozone-oxygen gas mixture. Release of the sanitizing gas was controlled, allowing the measurement of the dose of the sanitizer, product exposure time, temperature, and pressure in the processing environment. To assess treatment uniformity, preliminary experiments were conducted to measure ozone concentration at various locations in the treatment vessel (Fig. 2.4). During 55 min of the holding process ozone concentration decreased from 360 ppm to 280 ppm (Fig 2.5). Both sampling point showed the same ozone concentration during experiments, thus, experimental results confirmed that gaseous mixture is homogenous inside the treatment vessel.

The treatments with ozone, in tandem with vacuum cooling, decreased the population of *E. coli* O157:H7 and natural microbiota by up to 0.5 and 1.7 log CFU/g, respectively, when the samples were held at atmospheric pressure (Tables 2.1 and 2.2). The effect of three levels (60, 80, 95-100 %) of relative humidity (RH) was evaluated on the inactivation of *E. coli* O157:H7 and natural microbiota on spinach during treatments. High humidity in the treatment environment is believed to increase antimicrobial efficacy of gaseous ozone (Kim et al., 2003). Kuprianoff (1953) demonstrated that bactericidal effect of ozone gas was diminished when the RH was 50% or less. It is speculated that the hydration of dry microorganisms makes them susceptible to antimicrobial gases (Kim

et al., 2003). In the current study, no significant ($P \leq 0.05$) inactivation of both *E. coli* O157:H7 or natural microbiota on spinach was observed in response to different RH levels (Tables 2.1 and 2.2). Although treating samples under a high RH environment did not enhance the lethality of ozone, spinach samples treated at 95-100% RH showed similar visual appearances with the untreated samples. Therefore, 95-100% RH level was used in subsequent experiments.

Previous investigators reported that application of gaseous sanitizers under pressure increased penetration of antimicrobial agents deep into food matrix, and consequently enhanced their inactivation efficacy against microorganisms (Padron, 1995; Cox et al., 2000). Therefore, the influence of holding pressure against microbial inactivation during ozonation was investigated. When spinach samples were held under vacuum at 15 in of Hg below atmospheric pressure for 60 min the levels of inactivation observed for *E. coli* O157:H7 and natural microbiota were 0.8 and 1.2 log CFU/g, respectively. Holding samples at 10 psig (i.e., above the atmospheric pressure) increased significantly the levels of inactivation for *E. coli* O157:H7 and natural microbiota to 1.1 and 1.5 log CFU/g, respectively, (Tables 2.1 and 2.2). Noticeably, a higher level of inactivation, 1.8 and 2.0 log CFU/g respectively, for both *E. coli* O157:H7 and natural microbiota were observed when the samples were held for 30 min at high ozone concentration (1.5 g/kg). These experiments clearly showed the benefit of using higher pressure and ozone concentration.

Preliminary screening experiments were carried out to determine the lethal contribution of selected processing variables, namely, ozone concentration, and holding pressure and time. Vacuum cooling of spinach followed by re-pressurization with ozone

mixture decreased the population of *E. coli* O157:H7 by 1.1 to 1.8 log CFU/g, depending on the factors just mentioned (Table 2.3). When spinach samples were treated with 1.5 g O_3/kg gas mixture and held under partial vacuum (20 in. Hg vac.) for 30 min, the levels of *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 *E. coli* O157:H7 CFU/g. However, spinach visual quality was affected and some discoloration of spinach leaves was observed when the ozone concentration was 3.0 g/kg, combined with treatment times greater than 5 min (Table 2.3, experiment no. 3).

Contribution of SanVac treatment variables to process lethality as assessed by response-surface methodology.

Response surface methodology (RSM) is an efficient statistical technique that could be useful to study and predict behavior of microorganisms in foods under multiple treatment conditions (McMeekin et al., 1993; Li et al., 2002). In previous studies, using a carefully selected sequence of vacuum and pressurization, ozone gas penetrated the shell and membranes of whole hen eggs and effectively eliminated embedded *Salmonella* spp. (Yousef and Rodriguez-Romo, 2004). In the current study, RSM was applied to systematically study the contribution of the tested treatment variables to process lethality. The central composite design of three independent variables (ozone concentration, gas pressure, and treatment time) was used to obtain the quadratic predictive model. Measured response (decrease in log CFU/g) at various levels of process variables were fitted by the response-surface model and predicted inactivation levels are shown in Table 2.4. The analysis of variance 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, with significant linear (*P<0.01*) and quadratic (*P<0.01*) effects, and with no significant lack of fit (Tables 2.5 and 2.6). Parameters of estimates (regression coefficients) used in equation 2 are presented in Table 2.6. Each of the three process variables tested in the study significantly (*P<0.01*) influenced 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 to samples treated with ozone at the same concentration and treatment time, but at atmospheric pressure (Table 2.4). Clearly, pressure enhances the lethal effect of ozone against *E. coli* O157:H7 on spinach. Previously, Rodriguez-Romo and Yousef (2005) indicated that using ozone above atmospheric pressure increased lethality of this sanitizer against *Salmonella* Enteritidis on shell eggs.

Predicted responses were plotted against process variables, and Figs. 2.6 and 2.7 show the three-dimensional representations of model data. Although the interaction between ozone concentration and pressure was not significant (*P> 0.05*; Table 2.5), increasing both of the parameters resulted in increasing log reduction (Fig. 2.6). Similarly, Fig. 2.7 illustrates the effects of ozone concentration, treatment time, and their interaction at fixed value of pressure (0 level) on the inactivation of *E. coli* O157:H7 on spinach. Interactions of these two parameters significantly ($P \leq 0.05$) enhanced the inactivation of the bacterium on spinach (Table 2.5).

The response-surface model was verified by carrying out six additional experiments representing various combinations of ozone concentration, holding pressure and treatment time (Table 2.7). Differences between measured and model-predicted

inactivation data were generally negligible, providing evidence that the model was valid. Therefore, the model can be used to predict inactivation of *E. coli* O157:H7 on spinach by these SanVac treatments.

Based on the response-surface model, the most lethal combinations were observed at the maximum levels of the tested treatment variables. These parameters (2.5 g/kg ozone for 45 min at 10 psig) yielded 2.3 log predicted inactivation, however, the visual quality of spinach was negatively affected as compared to control samples. SanVac process resulted in discoloration on some of treated spinach leaves when the holding times were longer than 30 min 1.5 g ozone/kg gas mixture (Fig. 2.8). Therefore, the optimum treatment parameters, producing the greatest lethality with no apparent loss of quality, were selected for subsequent experiments. These conditions include: ozone concentration of 1.5 g/kg, holding pressure of 10 psig, and treatment time of 30 min. The visual quality of treated baby spinach samples was comparable to the untreated samples (Fig.2.8).

In preparation for retail marketing, fresh produce is minimally-processed and packaged in specialized facilities. Conventionally, minimal processing includes cutting, washing and sanitizing with a chlorine solution (Cantwell and Suslow, 2002). Inability of sanitizer 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 precedes conventional minimal processing of produce.

Combining the vacuum cooling with a potent sanitization step (SanVac) not only reduces pathogen population on freshly-harvested produce, it may also minimize or eliminate microbial internalization. Bacterial pathogens such as *E. coli* O157:H7 may become internalized in fresh produce in the field and during post-harvest operations (Solomon et al., 2002; FDA, 1999). Recently, Li et al. (2008) reported that the vacuum cooling process internalizes *E. coli* O157:H7 in lettuce. According to these authors, if produce is contaminated prior to cooling, these contaminants may be forced to internalize during re-pressurization 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. Considering that ozone gas was applied during the re-pressurization step, penetration of the sanitizer into fresh produce is expected; this potentially eliminates the internalized/infiltrated *E. coli* O157:H7. The SanVac process would ideally be applied immediately following the harvest of fresh produce, and before the pathogens have had chance to adapt to refrigeration.

Survival of *E. coli* O157:H7 during refrigerated storage subsequent to SanVac process

During a SanVac process, inoculated spinach samples (~10^7 *E. coli* O157:H7 CFU/g) were vacuum-cooled, subjected to oxygen, ozone (~1.5 g/kg), or ozone (~1.5 g/kg) and carbon dioxide combination (50%, vol/vol), at 10 psig for 30 min, and stored in a refrigerator for 7 days (Fig. 2.9). Surviving *E. coli* O157:H7 populations were monitored during the treatment and refrigerated storage. The ozone treatment alone, or in

combination with carbon dioxide, significantly ($P<0.05$) decreased the *E. coli* O157:H7 population by ~2.0 log CFU/g, compared to untreated or oxygen-treated controls. This 2-log difference remained relatively constant throughout the 7-days of storage. Additionally, populations of *E. coli* O157:H7 on control and treated spinach remained unchanged during refrigerated storage ($P>0.05$).

Antimicrobial efficacy of ozone may be enhanced when combined with other gases. It has been previously reported that carbon dioxide may act as a bacteriostatic agent on many microorganisms by inhibiting their decarboxylation enzymes (King and Nagel, 1975; Mitsuda et al., 1990). Mitsuda et al. (1990) reported the synergistic effect of ozone and carbon dioxide on microbial inactivation in foods. Recently, Vurma et al. (unpublished data) tested the feasibility of reducing natural microbiota and extending shelflife of strawberries using combinations of ozone and carbon dioxide. The researchers found that the combination of these two gasses was more effective in decreasing the natural microbial contaminants and preserving the quality of the treated strawberries than was either ozone or carbon dioxide treatment alone. The synergy was believed to be due to the quenching action of carbon dioxide on the chain decomposition reaction of ozone; this increases the stability and bactericidal effectiveness of the ozone in the treatment environment. However, including carbon dioxide in the gaseous mixture during SanVac treatments of baby spinach did not significantly ($P > 0.05$) enhance the lethal effect of ozone against *E. coli* O157:H7 (Fig. 2.9).

In conclusion, a novel sanitization technology (SanVac) was developed for the decontamination of fresh spinach. The SanVac technology relies on using gaseous ozone in tandem with vacuum cooling, and thus dictates only minimal modification of existing

fresh produce processes. Our study shows that an optimized SanVac treatment (1.5 g ozone /kg gas mix for 30 min at 10 psig) is effective in reducing by up to ~2.0 log CFU of both *E. coli* O157:H7 and native surface microbiota present on fresh baby spinach. The results also confirmed that ozone in the gaseous mixture is homogenous inside the treatment vessel during the short processing time. The application of this process serves multiple purposes on produce safety such as early sanitization of produce under conditions of maximum ozone efficacy, and allowing sufficient time for the gaseous sanitizer to reach the entrapped or internalized bacteria. The vacuum cooling operation is already practiced in the field or packinghouses, and therefore, the SanVac process requires relatively little modification for a retrofit. In addition, the SanVac treatments take place immediately following harvest, before the pathogen has had a chance to adapt to refrigeration. The SanVac process is potentially usable for other fresh produce, and possibly against other pathogens.

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Experiment	Treatment Conditions				Inactivation[b] (log CFU)
	Holding Time (min)	RH (%)	Pressure	Concentration (g/kg)	
Low RH	60	60	0 psig	0.41±0.04	0.3
Mid RH	60	80	0 psig	0.41±0.04	0.3
High RH	60	95-100	0 psig	0.41±0.04	0.5
High P	60	95-100	10 psig	0.41±0.04	1.1
High RH Vacuum	60	95-100	15 in. Hg vac.	0.41±0.04	0.8
High RH High P High C	30	95-100	10 psig	1.5 ±0.01	1.8

[a] Initial population was around 10^7 CFU/ g spinach.
[b] Averages for two independent trials.

Table 2.1. Changes in *Escherichia coli* O157:H7[a] population on spinach when the produce is vacuum-cooled to 4±1°C, subsequently re-pressurized using gaseous ozone, and held in the pilot scale vacuum cooler either under vacuum (15 in. Hg vac.), at atmospheric pressure, or above atmospheric pressure (10 psig) for up to 60 min.

Experiment	Treatment Conditions				Inactivation[b] (log CFU)
	Holding Time (min)	RH (%)	Pressure	Concentration (g/kg)	
Low RH	60	60	0 psig	0.41±0.04	1.4
Mid RH	60	80	0 psig	0.41±0.04	1.7
High RH	60	95-100	0 psig	0.41±0.04	1.3
High P High RH	60	95-100	10 psig	0.41±0.04	1.5
Vacuum High RH	60	95-100	15 in. Hg vac.	0.41±0.04	1.2
High P High C	30	95-100	10 psig	1.5 ±0.01	2.1

[a] Initial population was around 10^6 CFU/ g spinach.
[b] Averages for two independent trials.

Table 2.2. Changes in natural microbiota[a] of spinach when the produce is vacuum-cooled to 4±1°C, subsequently re-pressurized using gaseous ozone, and held in the pilot scale vacuum cooler either under vacuum (15 in. Hg vac.), at atmospheric pressure, or above atmospheric pressure (10 psig) for up to 60 min.

Experiment	Treatment Conditions			Decrease in log CFU/g[b]
	Ozone concentration (g /kg)	Pressure	Time (min)	
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 Vac	30	1.1
9	1.5	10 psig	30	1.8

[a] Initial population was ~10^7 CFU/g spinach.
[b] Averages of 2-3 independent trials.

Table 2.3. Changes in *Escherichia coli* O157:H7 population[a] on baby spinach when the product was vacuum-cooled to 4±1°C, and the vessel re-pressurized with gaseous ozone and held under pressure (or partial vacuum) for up to 45 min at 95-100% relative humidity.

Trial	Ozone concentration (g/kg)	Pressure (psig)	Time (min)	Measured inactivation (Δ log CFU/g spinach)	Predicted inactivation (Δ log CFU/g spinach)
1	0.5	5	30	0.4	0.6
2	1.5	5	30	1	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	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	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 was ~10^7 CFU/g spinach.

Table 2.4. Experimental design developed using response-surface methodology to assess the inactivation of *Escherichia coli* O157:H7 population[a] on spinach in response to changes in ozone concentration, pressure and treatment time. During treatments, the product was maintained at 4±1°C and 95-100% RH.

Statistical estimates	Probability
Response-surface model[b]	<.0001
Treatment Variables	
Ozone concentration (g/kg)	<.0001
Pressure (psig)	<.0001
Time (min)	<.0001
Interactions	
Pressure * Time	0.0536
Pressure * Ozone concentration	0.1490
Time * Ozone concentration	0.0306

[a] The SanVac process involved vacuum cooling, re-pressurization with ozone, and holding the product under pressure for a specified time, as described in Table 2.
[b] n=32

Table 2.5. Statistical analyses of results of experiments designed by response-surface model, and significance of model variables used during treatment of *Escherichia coli* O157:H7-contamined spinach with SanVac[a] processes.

Term	Estimate	Standard Error	t Ratio	Prob >|t|
β_o	1.0983	0.0616	17.8400	<.0001*
β_1	0.4700	0.0411	11.4300	<.0001*
β_2	0.4250	0.0411	10.3350	<.0001*
β_3	0.1950	0.0411	4.7420	<.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

* Significant at $P \leq 0.05$

[a] The SanVac process involved vacuum cooling, re-pressurization with ozone, and holding the product under pressure for a specified time, as described in Table 2.

Table 2.6. Parameters of estimates (regression coefficients) of the polynomial model (eq. 2) applied to data obtained during inactivation of *Escherichia coli* O157:H7 on spinach by SanVac[a] processes.

Trial	Ozone concentration (g/kg)	Pressure (psig)	Time (min)	Measured inactivation (Δ log CFU/g spinach)	Predicted inactivation (Δ log CFU/g spinach)
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 was ~10^7 CFU/g spinach.
[b] Product was maintained at 4±1°C and 95-100% RH.

Table 2.7. Verification experiments for response-surface model for inactivation of *Escherichia coli* O157:H7[a] on spinach by SanVac processes at selected treatment parameters[b].

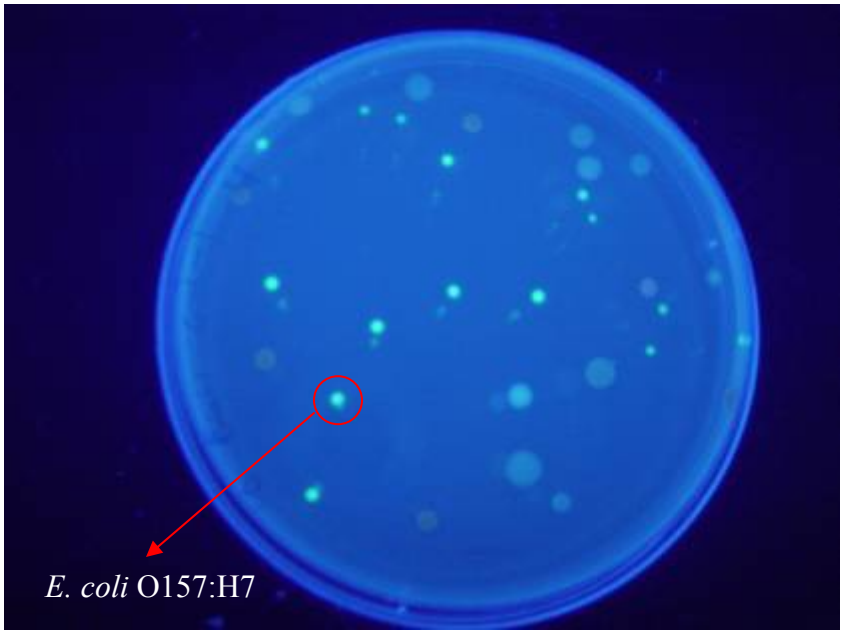


Figure 2. 1. Visualization of *Escherichia coli* O157:H7 strain B6-914 GFP on LB_{ampcyc} agar in the presence of UV light.

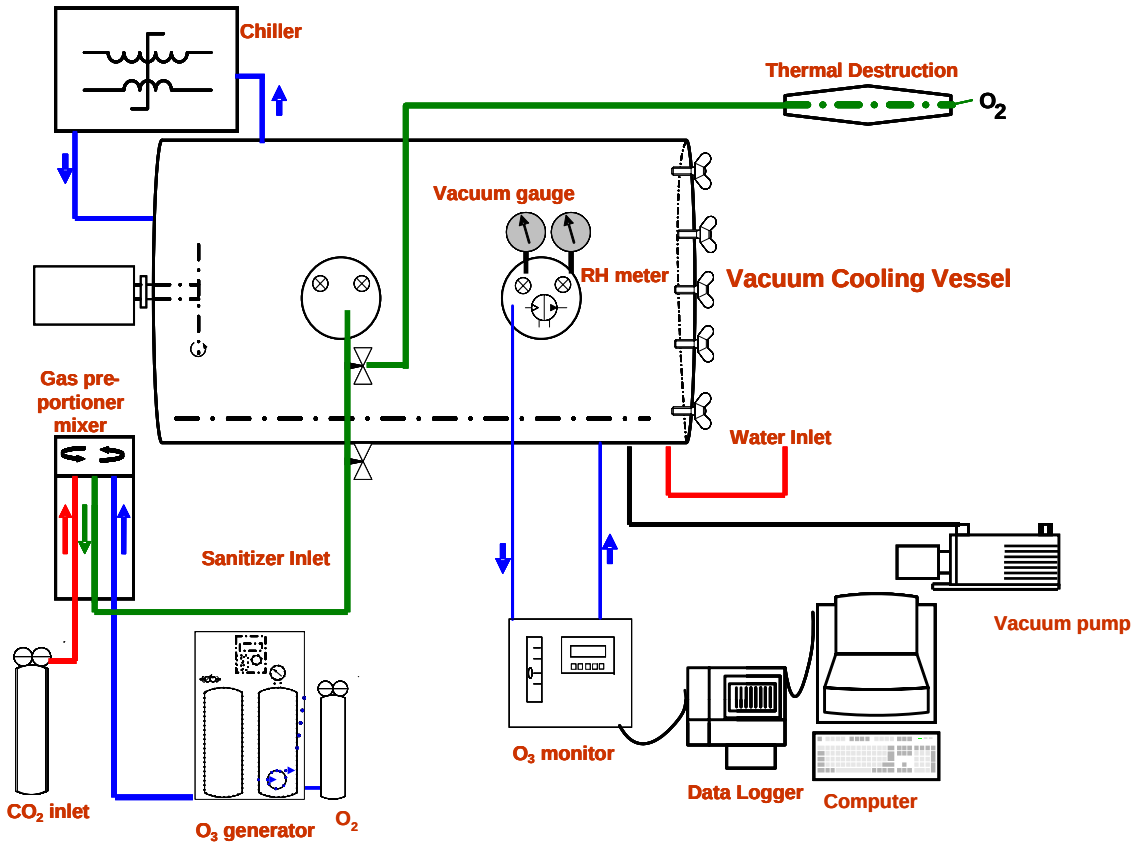


Figure 2.2. Setup used for treating fresh produce with ozone, in tandem with vacuum cooling (SanVac process).

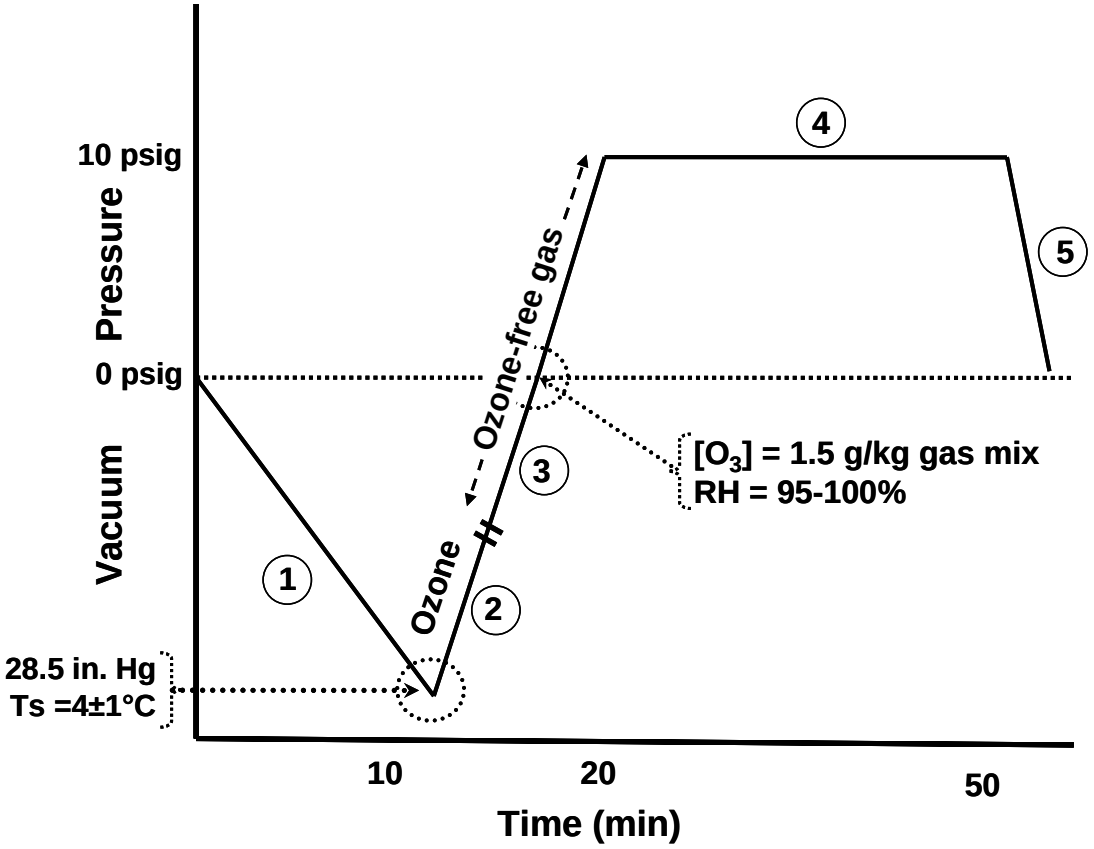
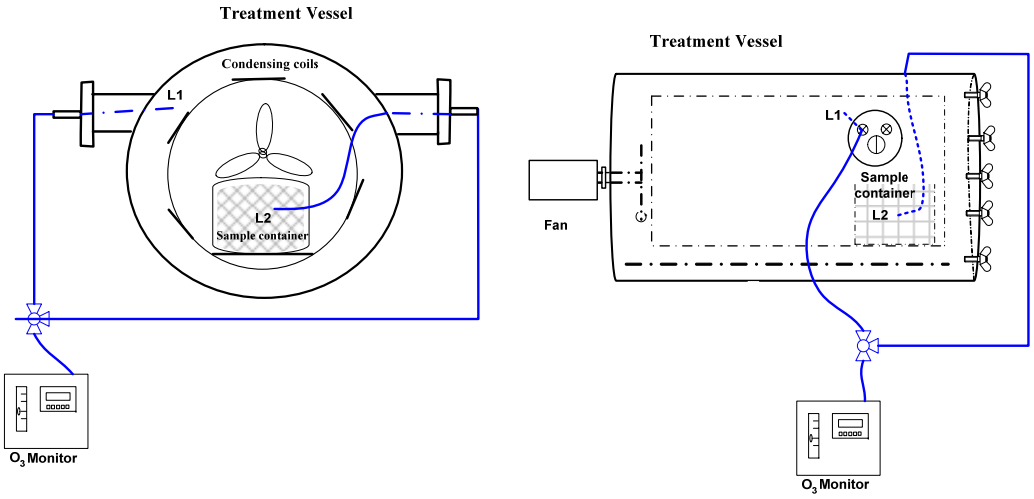


Figure 2.3. An example sequence of SanVac treatment consisting of (1) applying enough vacuum to cool the fresh produce to the desired temperature, (2) re-pressurizing the vessel with ozone (in oxygen carrier) to reach the targeted concentration of the sanitizer, (3) continue re-pressurization with oxygen or other ozone-free gases to reach the target pressure, (4) maintaining the pressure for the specified treatment holding time, (5) releasing the gases until vessel interior reaches atmospheric pressure.



a). Front view

b). Side view

Figure 2.4. Schematic showing the locations of the ozone sampling points (L1 and L2) inside the treatment vessel.

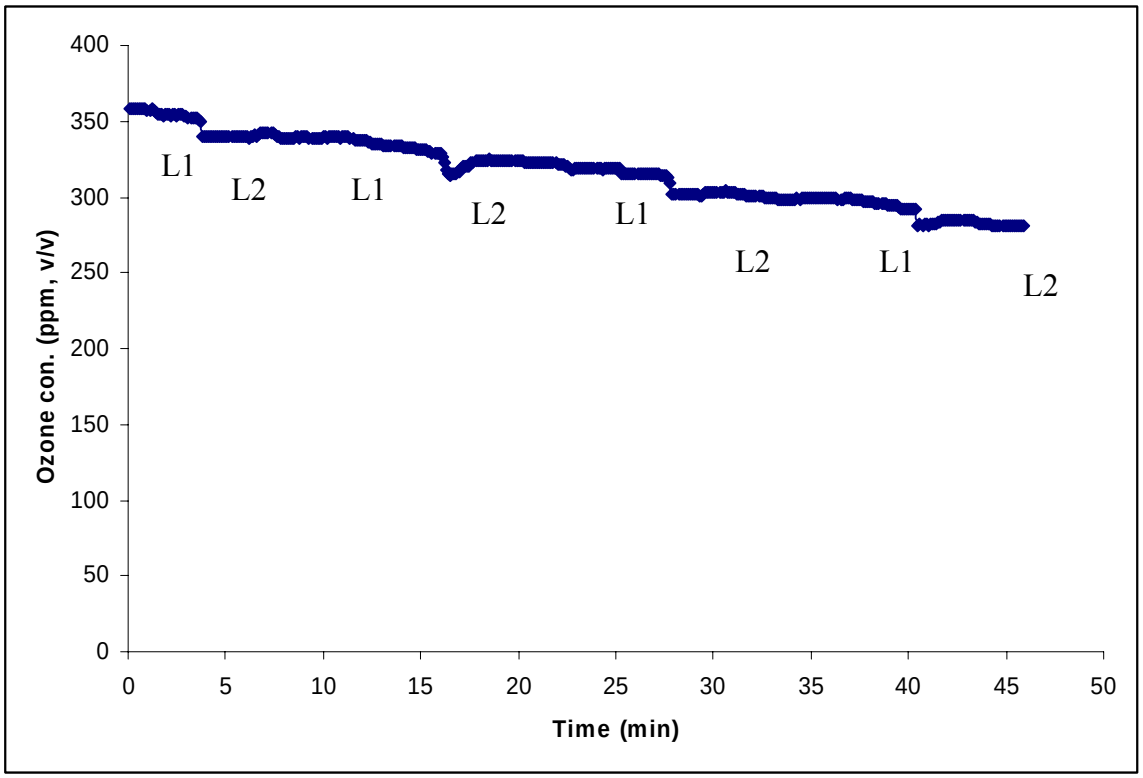


Figure 2.5. Ozone concentration measured at different location inside the treatment vessel. L1 represents a sampling point close to the periphery of the vessel, while L2 represents a sampling point close to the product.

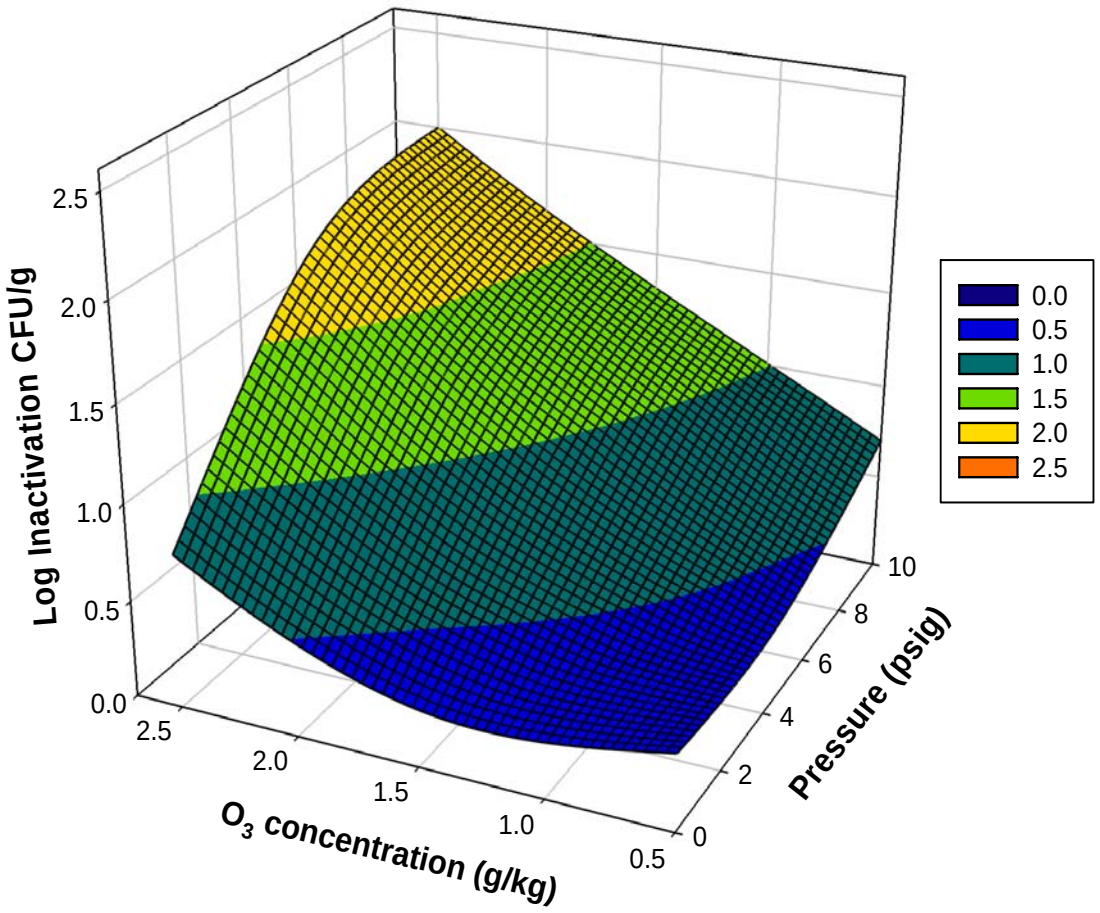


Figure 2.6. Three-dimensional representation of interaction between ozone concentrations and holding pressures, in relevance to inactivation of *Escherichia coli* O157:H7 on baby spinach. Microbial inactivation data shown were predicted by the response-surface model.

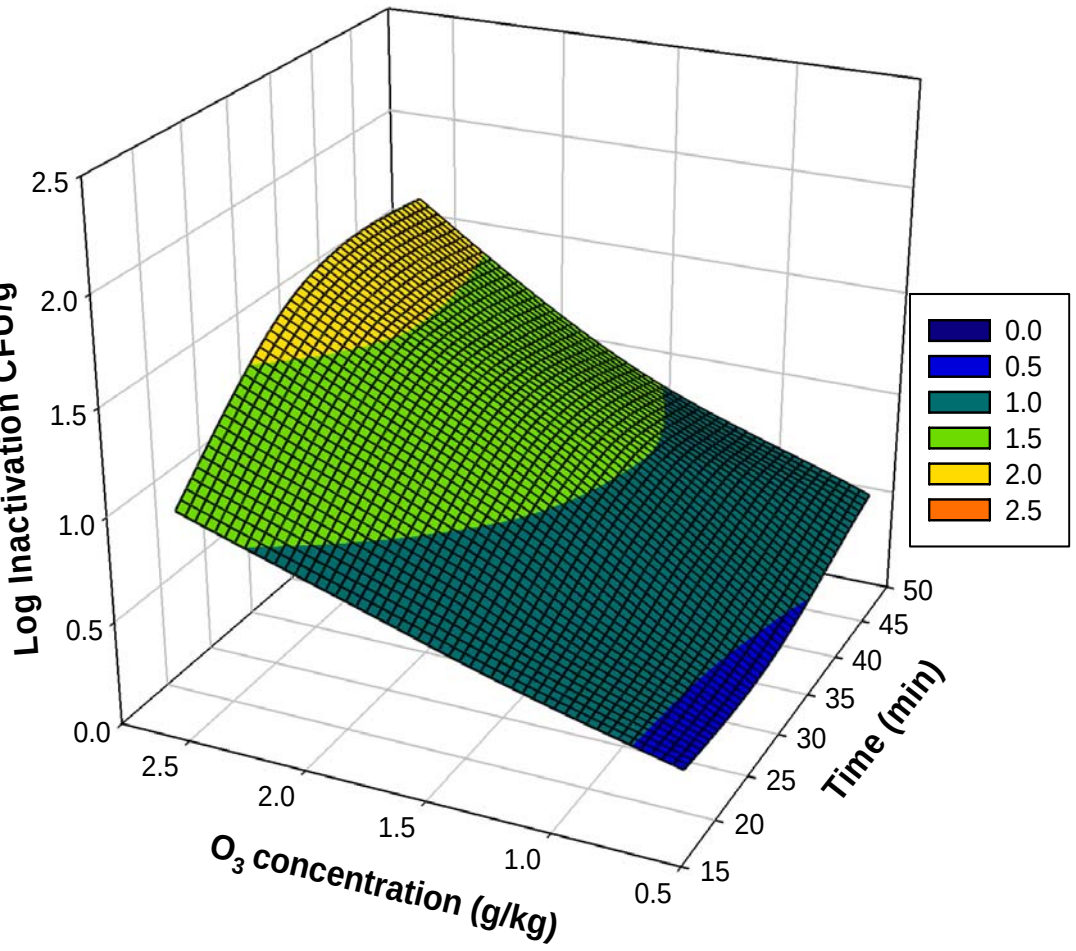


Figure 2.7. Three-dimensional representation of interaction between ozone concentrations and treatment times, in relevance to inactivation of *Escherichia coli* O157:H7 on baby spinach. Microbial inactivation data shown were predicted by the response-surface model.

Control

O_3 treated
1.5 g/kg for 30 min @ 10 psig

O_3 treated
1.5 g/kg for 45 min @ 10 psig

.

Figure 2.8. Baby spinach samples immediately after SanVac treatments.

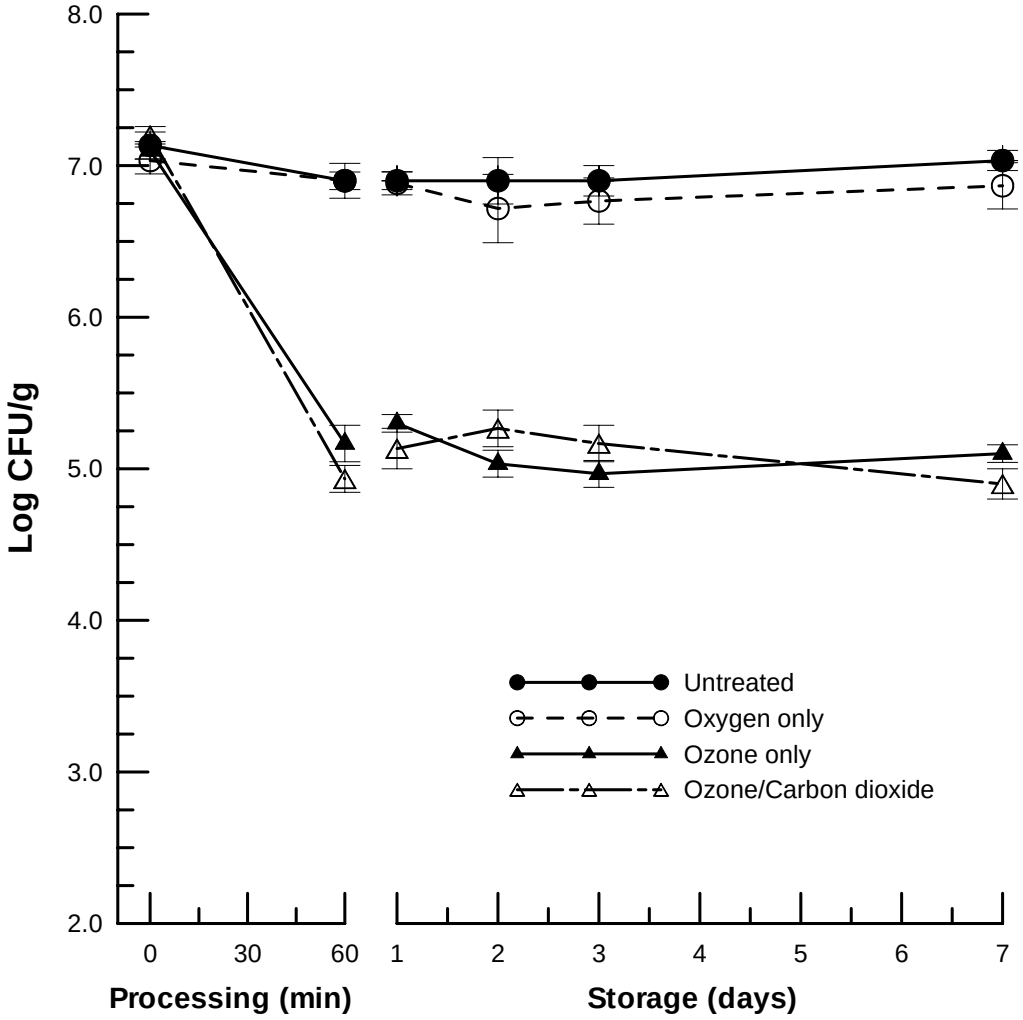


Figure 2.9. Changes in population of *Escherichia coli* O157:H7 on fresh spinach when the inoculated produce was subjected to SanVac process and stored at 2±1°C for 7 days. The SanVac processes includes vacuum-cooling to 4±1°C and treating with O_3 (1.5 g/kg) or O_3+CO_2 (1.5 g O_3/kg kg and 50% CO_2) at 10 psig for 30 min; product was maintained 95-100% RH. Controls included inoculated-untreated or inoculated and oxygen-treated spinach. Data shown are averages of three independent trials. Error bars represent $\pm$ standard error.

CHAPTER 3

INACTIVATION OF *ESCHERICHIA COLI* O157:H7 AND NATURAL MICROBIOTA ON SPINACH LEAVES USING GASEOUS OZONE DURING SIMULATED TRANSPORTATION AND STORAGE

ABSTRACT

Conventional sanitization technologies, targeting pathogenic contaminants on the surface of fresh produce, cause only modest microbial inactivation. Reliance on chlorinated water to sanitize produce may have not been a sound mitigation strategy. It is necessary to develop effective inactivation methods to minimize health hazards associated with fresh produce. The goals of this study are (i) to develop a long-term sanitization system that could be used during fresh produce shipment or storage (SanTrans), and (ii) to determine the synergy between the SanVac and SanTrans against *Escherichia coli* O157:H7, when these processes are applied sequentially. Ozone at low concentration was used as a sanitizing agent for extended periods, and efficacy of the

system against microbial contaminants on baby spinach (*Spinacia oleracea* L) was assessed.

Leaves of baby spinach (25 g) were spot inoculated with *E. coli* O157:H7 (~10^7 CFU/g-spinach), air-dried for 2 h, transferred to the treatment vessels located in a refrigerator. The contaminated samples then treated with low levels (8-16 mg/kg gas mix; 5-10 ppm vol/vol) of gaseous ozone for up to 3 day. Additionally, sequential applications of SanVac (application of gaseous ozone during vacuum-cooling at 1.5 g/kg for 30 min and 10 psig) and SanTrans were conducted to maximize inactivation on *E. coli* O157:H7 on baby spinach.

Continuous treatment with a very low ozone concentration at 8 mg/kg level (5 ppm, vol/vol) for 3-day did not change the populations of *E. coli* O157:H7 or natural microbiota on spinach. On the other hand, applying continuous ozone at 16 mg/kg (10 ppm, vol/vol) level demonstrated inactivation of both *E. coli* O157:H7 and natural microbiota by ~1.4 and 1.0 log CFU/g, respectively, compared with the population of *E. coli* O157:H7 on control samples . Inoculated spinach was sequentially subjected to optimized SanVac and SanTrans processes, using freshly-harvested, unprocessed spinach. These sequential combine treatments inactivated 4.1 to ≥5.0 log *E. coli* O157:H7, depending on the treatment time. In conclusion, these novel technologies are promising new alternatives to enhance the safety of fresh produce.

INTRODUCTION

Minimally-processed fresh fruits and vegetables have received a great attention in recent months. Food safety has become a major issue in the agri-food chain, and foodborne disease outbreaks linked to minimally processed fruits and vegetables have increased during the past few decades (Sivapalasingam et al., 2004). Fresh produce caused the second highest number of foodborne disease outbreaks and highest number of disease cases among five major food categories during 1990 to 2005 in the US (CSPINET, 2007).

Fresh produce is susceptible to contamination with pathogenic and spoilage microorganisms at any point in the production chain. Bacterial contamination sources of fresh produce during preharvest and postharvest operations include animals, insects, irrigation water, soil, dirty equipment, cross contamination and human handling (Beuchat and Ryu, 1997). Enteric pathogens such as *E. coli* O157:H7 are extremely capable of surviving on produce. Operations such as cutting, shredding, and washing steps at processing plants may increased the microbial load of produce (Allende et al., 2004), and therefore these operations may lead to the spread of microorganisms including enteric pathogens.

Various sanitation procedures have been explored in recent years to decontaminate fresh fruits and vegetables. Chlorine-based agents, in the form of sodium or calcium hypochlorite, are traditionally used for sanitization purposes by the fresh produce industry at 20-200 ppm level. The antimicrobial activity of this sanitizer depends on several factors such as the availability of the reactivate form of chlorine in wash water,

medium pH, temperature, and the organic load. High organic load in the treatment medium significantly diminishes the antimicrobial efficacy of sanitizers (Parish et al., 2003). Chlorine treatments also may not be sufficient in removing strongly attached, internalized bacteria on produce surfaces. The primary use of this sanitizer is to control microbial load in wash water of fresh produce and to prevent cross contamination (Sapers, 2006). It is necessary, therefore, to devise efficient sanitization process that uses a potent sanitizer in order to enhance the microbiological safety of fresh produce.

Ozone is the most reactive sanitizer known, having an oxidation potential of -2.07 v, compared to -1.49 v for hypochlorous acid, and -1.36 v for chlorine (Bradey and Humiston, 1978). This sanitizer effectively and rapidly inactivates enteric pathogens such as *E. coli* O157:H7, in low ozone-demand media, including pathogens known to be chlorine-resistant (Khadre et al., 2001). Previous investigators demonstrated that ozone in aqueous phase can significantly reduce microbial load on fresh-cut produce (Kim et al., 1999). Treating fresh-cut lettuce with aqueous ozone inactivated 1.4 to 4.6 log CFU of natural microbiota/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 (Achen and Yousef, 2001).

Recent research has mainly focused on prevention of pathogen contamination of fresh produce during production chain. Although good agricultural practices (GAP) and good handling practices (GHP) may reduce the incidence of pathogens in fresh produce via sources such as irrigation water, there is no known defense against contamination due to animal, bird or insect intrusion events. Thus, it is prudent that a mitigation strategy be devised to shield the public from this sporadic health hazard.

Following cooling, fresh produce is transported to fresh market or fresh-cut processing plant where the fresh-cut value added operations are performed. Ample time (3 to 5 days) is available during this period for low-dose sanitizer applications to have lethal effects on pathogenic contaminants. Therefore, the objectives of this study are: (i) to develop an effective gaseous ozone treatment that could be used during fresh produce transportation or temporary storage (the treatment will be refereed as SanTrans), and (ii) assess the antimicrobial efficacy of sequential ozone treatments during vacuum cooling (SanVac) and simulated transportation (SanTrans).

MATERIALS AND METHODS

Bacterial strain, culture conditions, and preparation of inoculum

Escherichia coli O157:H7 B6-914 (does not contain Shiga toxins I or II genes), was used throughout the study. This *E. coli* O157:H7 strain contains genes for green fluorescence protein (GFP) as well as ampicillin and cycloheximide resistance (Fratamico et al., 1997), which enabled enumeration of the bacterium in the presence of the natural microbiota of baby spinach. The growth and survival characteristics of this bacterium are not affected by absence of the Shiga toxin I and II genes (Kudva et al., 1998). Stock cultures of *E. coli* O157:H7 were stored at -80°C in Luria-Bertani broth (LB broth; Difco, Becton Dickinson, Sparks, Md.) containing 40% (vol/vol) glycerol. An isolated *E. coli* O157:H7 colony from LB agar (Difco) was transferred and cultured in LB broth twice prior to use. Both LB agar and LB broth contained 100 μg/ml of each ampicillin

trihydrate (Fisher Biotech, Subiaco, Wash.) and cycloheximide (Sigma-Aldrich, St. Louis, Mo.). Overnight cultures were harvested by centrifugation at 8000 x *g* for 10 min (Sorval RC-5B DuPont, Wilmington, Del.), washed, and re-suspended in 0.1% (wt/v) buffered peptone water to achieve a final concentration of 10^9 CFU/ml.

Inoculation of spinach (*Spinacia oleracea* L)

Spinach samples were procured from local grocery stores (Columbus, Ohio), a day before testing. For SanVac and SanTrans combine experiments, unwashed, un-cooled spinach was used. Samples of fresh produce (25 g) were spot-inoculated with 100 µl *E. coli* O157:H7 to reach aproximately10^7 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 any 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, N.J.) and mixed with 225 ml peptone water. The bag contents were homogenized for 2 min in a stomacher (Model STO-400, Tekmar Inc., Cincinnati, Ohio). The homogenized samples were serially diluted in peptone water and surface-plated onto the antibiotic-containing LB agar. Plates were incubated at 35°C for 24-48 h and colonies of *E. coli* O157:H7, which were producing green fluorescence under UV light, were counted. Samples, which

were prepared and homogenized as just described, also were plated onto Tryptic soy agar (TSA; Difco).

Sanitization during simulated transportation (SanTrans)-equipment setup.

A treatment system allowing continuous ozonation was designed to simulate 3 days transportation. The system consisted of (i) an ozone generator that produces ozone at low concentration (model CD-150S), (ii) a refrigerator retrofitted with ports for ozone inlet and outlet, and for ozone measurements, and (iii) two glass desiccators modified to serve as treatment vessels; each was fitted with a sample holder and three inlet/outlet ports, and (iv) flow meters, a thermal destruction unit, and an ozone monitor were used to conduct these experiments. Details of the experimental setup are provided in Fig. 3.1.

SanTrans treatment conditions

Spinach samples, inoculated with *E. coli* O157:H7 and un-inoculated (for testing the inactivation of natural microbiota), were placed in glass treatment vessel using 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 around the spinach during the three days of continuous ozone treatment. Temperature of the refrigerator was set to 2 ^{o}C and experiments were conducted for three days. Flow rate of the gas mixture inside each of the glass containers was maintained at ~2 lpm. Ozone concentration was maintained at 8 or 16 mg O_3/kg gaseous atmosphere (5 or 10 ppm, vol/vol) through continuous flushing

of the sanitizer into the treatment vessels. Excess ozone and oxygen were discarded through the thermal destruction unit. A data acquisition system (Campbell Scientific Inc.) was used to maintain and monitor the ozone concentration during experiments. These experiments were repeated three times, and each run had two replicates.

Sequential application of SanVac and SanTrans processes

Freshly-harvested, unwashed, and non-vacuum cooled baby spinach was used in these experiments. Spinach was inoculated with *E. coli* O157:H7 (~10^7 CFU/g). Inoculated spinach was subjected to SanVac treatment which included vacuum-cooling followed by ozonation at 1.5 g/kg gas mixture (i.e., 935 ppm, vol/vol) and pressurization at 10 psig for 30 min. Alternatively, spinach was treated with SanTrans process which included refrigeration for up to 3 days with continuous sparing with gaseous ozone at 16 mg O_3/kg gas mixture (i.e., 10 ppm, vol/vol). In another treatment, 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. These experiments were conducted in triplicates.

Ozone generation and measurements

The low ozone capacity generator (model: CD-150S, DEL Industries Inc., San Luis Obispo, Calif.) was used to produce gaseous ozone for SanTrans experiments. The concentration of gaseous ozone in treatment vessels was measured using an UV-absorption monitor (model LC-L2-2000, IN USA Inc., Norwood, Mass.). Excess ozone was decomposed into oxygen using a thermal ozone-destruct unit (model ODT-006, Ozonia Inc.). For the sequential application of SanVac and SanTrans, a high capacity

ozone generator (model Ozat CSF-7, Ozonia Inc., Elmwood Park, N.J.) was used during the SanVac experiments.

Data Analysis.

All experiments were repeated twice or three times, and each run consisted of duplicate samples. Microbial counts (CFU/g spinach) were converted into log units before analyses and data were analyzed statistically (JMP IN version 6.0.2; SAS Inc. Cary, N.C.). Mean values for log reduction in bacterial populations were compared by one-way analysis of variance. Tukey's multiple comparisons test was used to analyze mean differences. Values with $P \leq 0.05$ were considered to be significantly different. Limited quality assessments, mainly visual observations, of treated spinach were made by the researchers; these observations were not subjected to formal data analysis.

RESULTS AND DISCUSSION

Inactivation of *E. coli* O157:H7 and natural microbiota on spinach during SanTrans treatments

The SanTrans processing system was assembled, allowing long term continuous ozonation at low concentration levels during refrigeration and thus simulating shipment of produce from post-harvest to value-added product processing plant. The counts of *E. coli* O157:H7 and natural microbiota on spinach treated with SanTrans using ozone at 8 mg/kg (5 ppm vol/vol) for 3 days were not significantly ($P > 0.05$) different, compared the corresponding counts on untreated controls (Figs. 3.2 and 3.3). In contrast, application of 16 mg/kg (10 ppm vol/vol) ozone for 24 hours significantly ($P \leq 0.05$)

decreased *E. coli* population on spinach by 1.0 log CFU/g compared with the population in control samples (Fig. 3.3). 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. 3.3), and showing a decreasing trend over time. When natural microbiota was monitored, a similar inactivation trend was also observed during a 3-day continuous ozone treatment, but the magnitude of lethality was less than that observed for *E. coli* O157:H7 (Fig. 3.4).

The visual quality of baby spinach samples treated with ozone at low concentration for 1-day was comparable to the untreated counterparts. However, color and texture of samples treated with ozone were slightly affected after 2-days continuous ozonation. At the end of 3-day treatment, some bleaching was observed on ozonated spinach samples (Fig. 3.6). This suggests that while ozone treatment has promise in long-term control of pathogens, it will be necessary to conduct further studies to optimize ozone concentration to maintain quality.

Various pathogenic microorganisms including *E. coli* O157:H7 have been isolated from variety of fresh fruits and vegetables, and some of these pathogens have been implicated in produce-related outbreaks (Beuchat, 1996; Harris et al., 2003). Enteric pathogens such as *E. coli* O157:H7 may well survive and even proliferate on the plant surface during pre-harvest/harvest if the conditions are favorable. The factors affecting pathogen survival on fresh produce include environment and plant temperature, available nutrients and water, tissue damage, and the nature of the plants native microbiota (Abdul-Raouf et al., 1993; Aruscavage et al., 2006; Cooley et al., 2006; Aruscavage et al., 2008). Following harvest, fresh produce are immediately cooled and transported to fresh

market or fresh-cut processing plant where the fresh-cut value added operations are performed. If fresh produce becomes contaminated with *E. coli* O157:H7, the pathogen may adapt to plant environment and possibly survives during refrigerated transportation (Delaquis et al., 2007). In the present study, *E. coli* O157:H7 survived well on baby spinach at refrigerated storage for up to 7 days and the population of this microorganism did not change significantly ($P \leq 0.05$) (Figs. 3.2 and 3.4). Since this pathogen survives well on various fresh produce, its presence may create problems by spreading during subsequent washing and fresh-cut value operations at processing plants and distribution chain. Therefore, application of a low-dose sanitizer such as gaseous ozone during transportation or short term storage may have valuable lethal effects on pathogenic contaminants before the pathogen have had a chance to adapt to the environment and possibly spread during production.

In previous studies, storage of blackberries under very low ozone concentration (0.1-0.3 ppm) suppressed the fungal growth for 12 days without causing any damage to the tested fruit (Barth et al., 1995). Storage of onions, potatoes, and sugar beets under ozone-enriched atmosphere at 3 mg/L with 6-14°C and 93-97% humidity reduced the spoilage and microbial population of treated products without affecting their chemical composition or sensory quality (Baranovskaya et al., 1979). Ozone treatments reduced the fungal decay and extended shelflife of table grapes (Sarig et al., 1996). When the grapes were treated with ozone before or after inoculation with *Rhizopus stolonifer*, significant decrease in decay was achieved.

In the current study, continuous ozone treatment at low concentrations is promising in reducing both *E. coli* O157:H7 and native surface microbiota of baby

spinach by 1-log CFU/g after 1-day treatment without compromising the produce's visual quality. Since the product is intended for shipment to fresh-market or fresh-cut processing facilities thereafter, ample time is available for the sanitizer to have lethal effects on surviving pathogens such as *E. coli* O157:H7.

Sequential Application of SanVac and SanTrans Processes.

Introducing ozone during both vacuum cooling (e.g., the SanVac process) and shipment (e.g., the SanTrans process) may enhance microbial inactivation. Inoculated spinach (~10^7 *E. coli* O157:H7 CFU/g spinach) was ozone-treated (1.5 g/kg, 10 psig for 30 min) while vacuum cooling, then held in a refrigerator under continuous low-ozone flush (16 mg/kg, 10 ppm, vol/vol) for up to 3 days (Fig. 3.7). The SanVac treatment alone significantly ($P \leq 0.05$) decreased the population of *E. coli* O157:H7 by 1.8 log CFU/g immediately after the treatment, and no further reduction was observed during the 3-day refrigerated storage. Application of SanTrans alone for 1 day decreased the population of *E. coli* O157:H7 by 1.0 log, compared to untreated controls. However, when the SanVac treatment was followed by a 1-day or 2-day SanTrans process, populations of *E. coli* O157:H7 on spinach was significantly ($P \leq 0.05$) decreased by 4.1 and 4.6 log CFU/g, respectively. The population of *E. coli* O157:H7 on spinach was undetectable when the contaminated produce was sequentially treated with SanVac and a 3-day SanTrans processes (i.e., ≥5 log inactivation).

Based on the current study, application of gaseous ozone during fresh produce transportation and short-term storage could be an effective sanitization step targeting

pathogenic microorganism such as *E. coli* O157:H7. Using low-level ozonation during refrigerated storage of fresh produce was moderately effective against pathogen populations. Sequential application of two sanitization processes (i.e., ozonation during vacuum cooling as well as during storage or transportation) inactivated *E. coli* O157:H7 population by more than 5.0 log on spinach. The bactericidal efficacy of ozone integrated to an existing produce process, is particularly encouraging, since the additional treatment causes relatively little modification of existing systems.

ACKNOWLEDGMENTS

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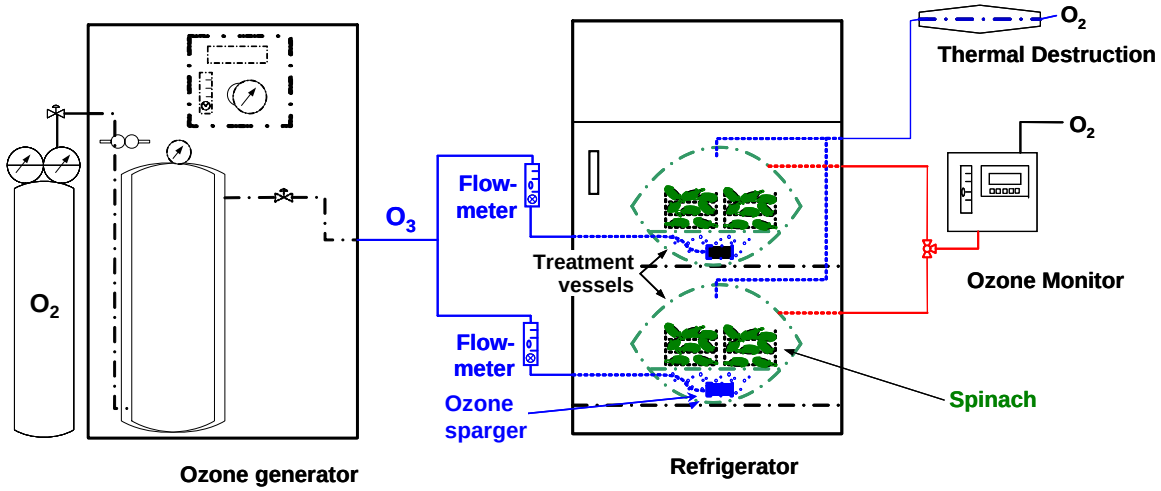

Figure 3.1. Equipment used for treating fresh produce with low levels of ozone during refrigerated storage, mimicking fresh produce transportation (SanTrans process).

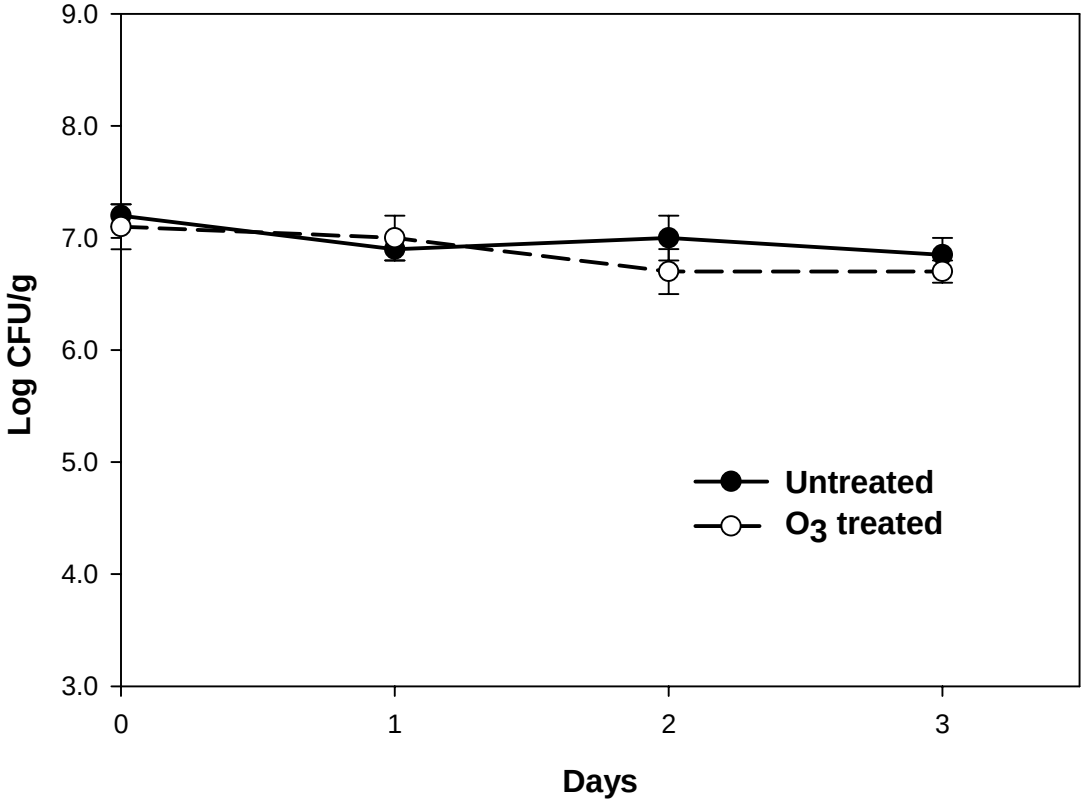

Figure 3.2. Changes in population of *Escherichia coli* O157:H7 on baby spinach when the inoculated produce treated with continuous ozone at 8 mg/kg (5 ppm; vol/vol) in refrigerator (2±1°C) for up to 3 days. Data shown are averages of two independent trials. Error bars represent ± standard error.

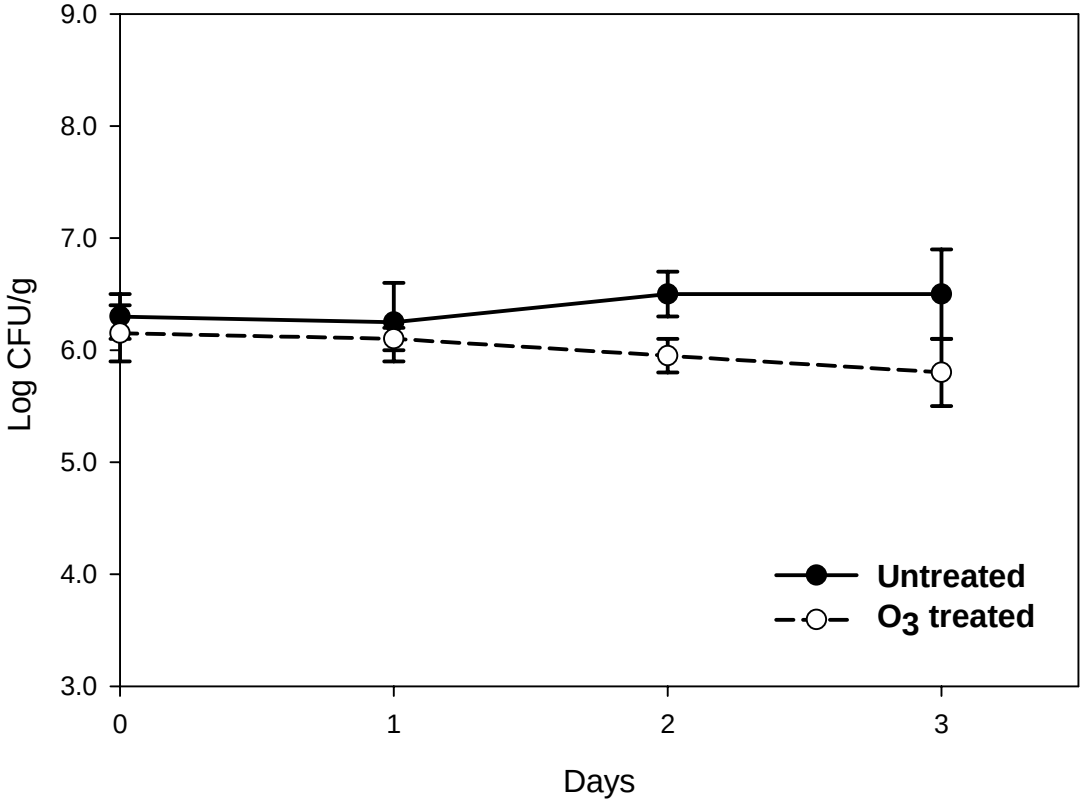

Figure 3.3. Changes in natural mirobiota of baby spinach when the produce treated with continuous ozone at 8 mg/kg (5 ppm; vol/vol) in refrigerator (2±1°C) for up to 3 days. Data shown are averages of two independent trials. Error bars represent ± standard error.

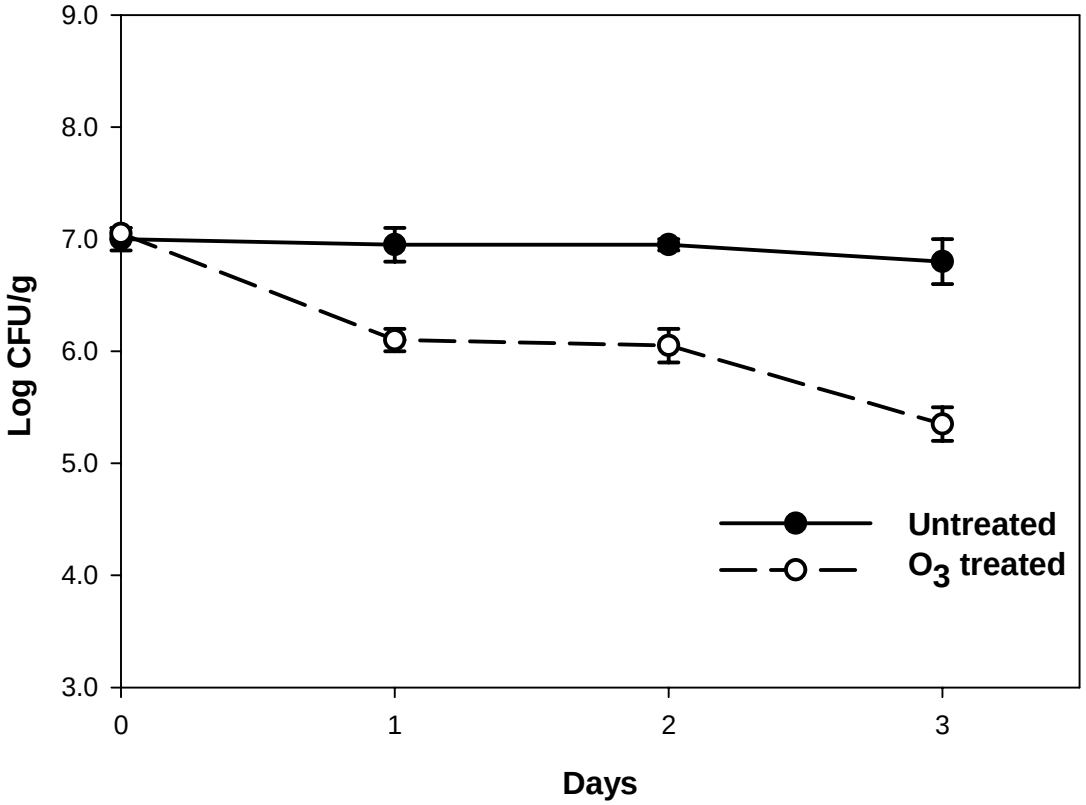

Figure 3.4. Changes in population of *Escherichia coli* O157:H7 on fresh spinach when the inoculated produce treated with continuous ozone at 16 mg/kg (10 ppm; vol/vol) in refrigerator (2±1°C) for up to 3 days (SanTrans process). Data shown are averages of three independent trials. Error bars represent ± standard error.

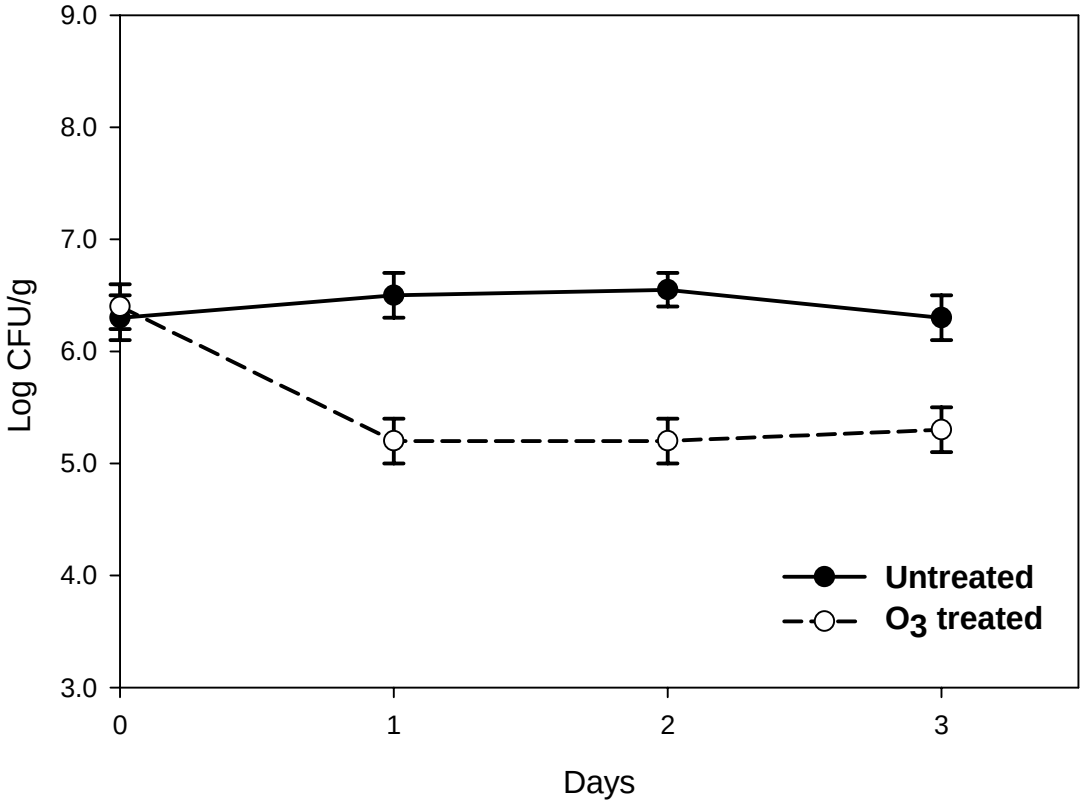

Figure 3.5. Changes in natural microbiota of fresh spinach when the produce treated with continuous ozone at 16 mg/kg (10 ppm; vol/vol) in refrigerator (2±1°C) for up to 3 days (SanTrans process). Data shown are averages of three independent trials. Error bars represent ± standard error.

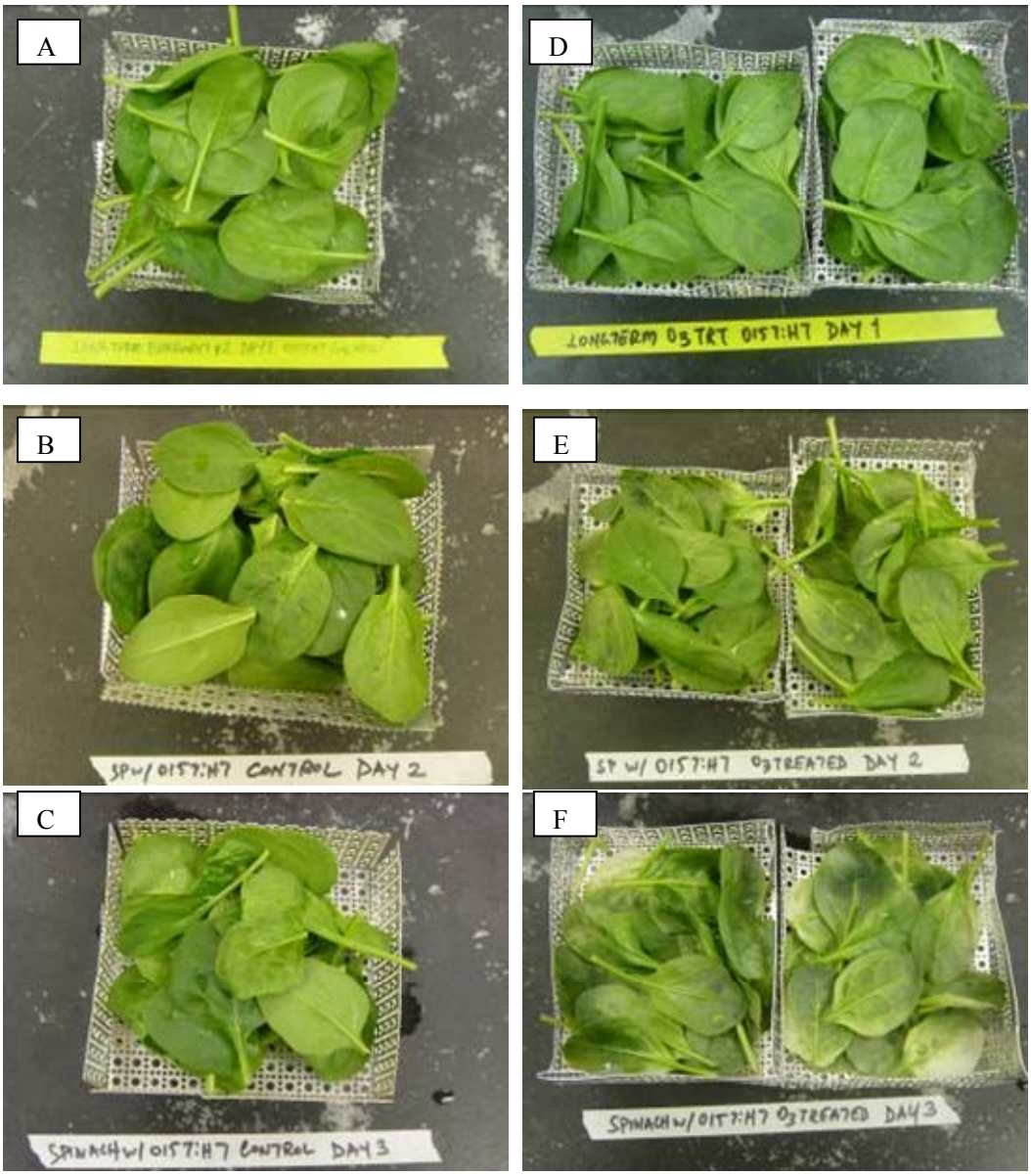

Figure 3.6. Control and ozone treated spinach samples for each sampling point during 3 days: Untreated control samples (A: after 1 day after, B: after 2-days, and C: after 3-days) and treated samples (D: after1 day, E: after 2-days, and F: after 3-days treatment).

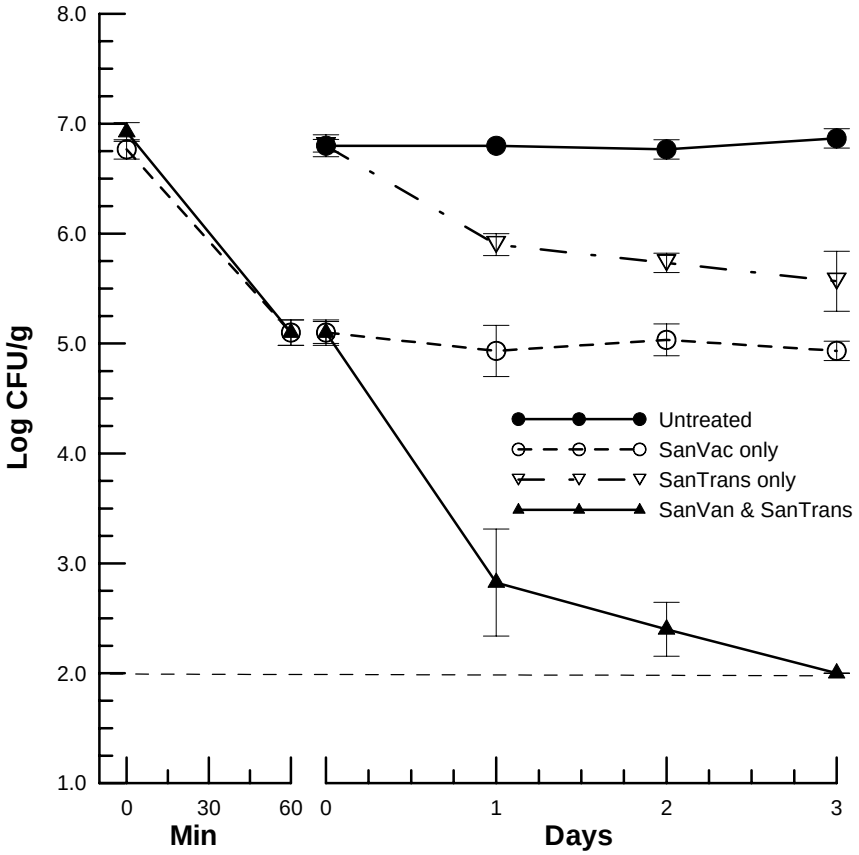

Figure 3.7. Changes in population of *Escherichia coli* O157:H7 when inoculated baby spinach was subjected to SanVac (a 60-min process) followed by 3 days of refrigerated storage (~2°C), SanTrans, or SanVac followed by SanTrans. The SanVac process included vacuum cooling to 4±1°C combined with a short-term ozonation (1.5 g/kg, 10 psig for 30 min). The SanTrans process consisted of continuous ozonation at 16 mg O_3/kg (10 ppm; vol/vol) during refrigeration at 2±1°C for 3 days. The horizontal dashed line indicates the detection limit for *E. coli* enumeration method. The Data point at the dashed line was below the detection limit. Data shown are averages of three independent trials. Error bars represent ± standard error.

CHAPTER 4

INACTIVATION OF NATURAL MICROORGANISMS ON STRAWBERRIES AND EXTENDING THEIR SHELFLIFE USING OZONE AND CARBON DIOXIDE COMBINATION

ABSTRACT

Strawberries (*Fragaria* x *ananassa*) are popular but highly perishable fruits. Fungi are commonly associated with the quality deterioration of strawberries, resulting in significant economic losses. Post-harvest treatments for strawberries are limited, but gaseous sanitizers are potentially useful in improving product shelflife and safety.

The objective of this study was to assess the feasibility of using ozone (O_3), carbon dioxide (CO_2) or their combinations, for reducing natural microbiota, and extending the shelflife of strawberries. A gaseous treatment setup was designed and constructed. The system consisted of an O_3 generator, a 300-liter chamber equipped with O_3 and CO_2 monitors, and various inlet/outlet ports. Strawberries (~1lb), in their original

plastic container, were treated with O_3, CO_2, or their combination, for up to 6 h. In O_3/CO_2 treatments, CO_2 was fed in the chamber for 5 min at 100 scfh flow rate. The concentration of O_3 was maintained at 16 mg/kg gases mixture (10 ppm vol/vol) during each treatment. Control and treated strawberries were evaluated for total mesophilic aerobic, and yeast-mold counts, as well as visual mold growth during storage at 4 and 20°C.

The O_3/CO_2 combination treatments were the most effective in delaying mold growth and quality deterioration of the strawberries. When samples were treated with O_3/CO_2 for 4 h and stored at 4°C, the initiation of visual mold appearance was delayed until the 16^{th} day of storage; an 8-day extension compared to untreated samples. The differences between combination and control treatments in yeast-mold counts were 1.1 log CFU/g immediately after the treatment and approximately 1.8 log CFU/g after 15 days of storage. The overall difference in total mesophilic aerobic counts between treated and untreated samples was approximately 1.2 log CFU/g throughout the storage period.

In conclusion, treating fresh strawberries with O_3/CO_2 combination is a feasible technology for extending the shelflife, and potentially improving the safety, of this perishable fruits.

INTRODUCTION

Strawberries (*Fragaria* x *ananassa*) are extremely delicate fruits with a short shelf life. These perishable fruits are usually harvested by hand and transferred to retail stores without any washing treatment (Knudsen et al., 2001). Strawberries do not receive

any washing since even a small amount of moisture may result in rapid deterioration primarily by fungal growth (Mitcham and Mitchell, 2002) Shelflife of freshly harvested strawberries is typically 1 to 2 weeks, provided that storage temperature is close to 0 °C (Mitcham and Mitchell, 2002). Rapid cooling and refrigerated transportation/storage is one of the most crucial steps to maintain maximum shelflife of strawberries. These fruits are susceptible to contamination with pathogens but they are frequently exposed to contamination with spoilage microorganisms during production, transportation, distribution, handling and processing. High levels of sugar and other nutrients, along with the high water activity, make strawberries ideal for microbial growth. The acid pH of strawberries predominantly creates good environment for fungal spoilage. *Botrytis cinerea* is a fungal plant pathogen, primarily responsible for significant reduction in quality and quantity of strawberries world-wide (Moss, 2008). Recently, Tournas and Katsoudas (2005) reported that 95% (out of total 39 samples) of strawberries were contaminated with spoilage fungi, and 77 % of these fruits were contaminated with *B. cinerea*.

Control strategies for pre- and post-harvest decay of strawberries include physical and chemical methods such as storage at low temperature coupled with modified atmosphere containing usually CO_2, and fungicides applications (Eckert and Ogawa, 1988; Mitcham and Mitchell, 2002). Strawberries usually are air-cooled immediately after harvesting and transport to the retail market with elevated CO_2 atmosphere (Mitcham and Mitchell, 2002). Application of fungicides such as sodium o-phenylphenate, thiabendazole and imazalil, are routinely used on strawberries at preharvest or postharvest stage; thus fungicides are effective in reducing decay during

production (Eckert and Ogawa, 1988; Holmes and Eckert, 1999). However, excessive use of these chemicals may results in the development of resistance of post-harvest pathogens and often decreases overall efficacy of fungicide in fruit production (Eckert et al., 1994; Holmes and Eckert, 1999). Furthermore, there are public concerns and regulatory restrictions regarding the chemical residues on fresh fruits (Smilanick, 1994). Consequently, there is a need for alternative methods that are environment friendly and effective on shelflife extension of fresh fruits.

Ozone, in the gaseous state, is potentially useful in decontamination of surfaces of fruits and vegetables, but detailed investigations of this application are lacking. Carbon dioxide is usually used in modified atmosphere storage at various levels to extend shelflife of minimally processed fruits and vegetables (Mitcham and Mitchell, 2002). Using CO_2 in the environment also decreases respiration rate and ethylene production of the stored produce. Moreover, it has been reported that the ethylene in the environment promotes germination and mycelial growth of plant fungal pathogens such as *B. cinerea* (Kader, 2002). Using elevated level of CO_2 (40-60%) significantly reduced the mycelial growth of fungi (Mokbel and Hashinaga, 2004). Strawberries treated with CO_2 appeared to be firmer in texture compared to air treated controls (Harker et al., 2000). In addition, CO_2 is known to be a good carrier and stabilizer for ozone (Kim et al. 2003). Using CO_2 in the environment with a potent sanitizer such as ozone may be beneficial to decontaminate microorganisms on fresh fruits. Therefore, the objective of this study was to assess the feasibility of using O_3, CO_2 or their combinations, for reducing natural microbiota, and extending the shelf-life of strawberries.

MATERIALS AND METHODS

Fruits

Fresh strawberries were purchased at a local supermarket on the day of testing. The fruits were placed in small carton container (provided by the retailer), refrigerated, and transferred to biological hood about 1 h before testing. In all experiments, unwashed and unhulled strawberries were used. All the strawberries used in the experiments were similar size and weight (20-25 g).

Ozone generation and concentration measurements

Experiments were carried out using Del Ozone generator (model Infinity CD-150S; Del Industries, San Luis Obispo, Calif.). Concentration of gaseous ozone in ozone-treatment chambers was measured using an UV-absorption monitor (model LC-L2-2000, IN USA Inc., Norwood, Mass.). A CO_2 analyzer (Model: 2820, Barcharach, Pa.) was used to monitored CO_2 amount during the treatments. Excess ozone was decomposed into oxygen using a thermal ozone-destruct unit (model ODT-006, Ozonia Inc., Elmwood Park, N.J.).

Gaseous treatment system

A system was assembled to treat strawberries with gaseous ozone (Fig. 4.1). In addition to the ozone generator, the system is comprised of a 300-liter stainless steel treatment chamber, O_3 and CO_2 monitors, O_3 and CO_2 inlet/outlet ports, flow control valves, thermo-hygrometer, and thermal destruction unit. Gas lines were equipped with

flowmeters to control and regulate the flow rate. A sampling manifold was installed to draw gas samples from two locations in the treatment chamber and measure the ozone concentration using the ozone analyzer. For each experiment, ambient and vessel temperatures and concentrations of each gas were monitored. Additionally, a water inlet line was added to the treatment vessel in order to adjust the relative humidity which was monitored using a thermohygrometer (Model OKTAN 03313-70, Cole-Parmer Instrument Company). The system was located in the biosafety level-2 pathogenic pilot plant, Department of Food Science and Technology, The Ohio State University.

Gaseous treatments of strawberries

A preliminary study was conducted to determine important treatment parameters. Strawberries (20 per container), were treated in their original plastic container and total of two containers were used in each treatment. Hot water was added at the bottom of the vessel, beneath the sample rack, to adjust the vessel RH to approximately 95-100%. Once the humidity was adjusted, strawberries were placed in the treatment vessel. Samples were subjected to following treatments for 1, 2, 4, and 6 h: (i) ozone (O_3), (ii) ozone + carbon dioxide (40%, vol/vol) mixture (O_3/CO_2), and (iii) no treatments (control). For ozone treated samples, gaseous ozone was fed into the treatment chamber until the target concentration of 16 mg O_3/kg gaseous atmosphere (10 ppm, vol/vol) was achieved. Ozone concentration was monitored and maintained during each treatment. For samples treated with ozone + carbon dioxide mixture, continuous CO_2 gas was fed through the vessel for 5 min at 100 scfh flow rate to reach the targeted 40% level. Subsequently, gaseous ozone was delivered into the treatment chamber until the target concentration of 16 mg O_3/kg gaseous atmosphere was achieved. Both ozone and CO_2 concentrations were

monitored and maintained during each treatment. For the untreated (controls) and gas-treated samples, humidity, treatment time and temperature were the same. Strawberries were analyzed visually and microbiologically (total mesophilic aerobic count, and yeast and mold count) before and immediately after treatment, and after 1, 2, 3, and 7 days of storage. Treated and control packages were stored at 20°C and ~95-100% RH and the population of natural microbiota was monitored during storage. These experiments were conducted in triplicates.

Based on the most promising preliminary result, 4 h treatment was selected for further studies. In order to determine the effect of storage temperature on the surviving natural microbiota of treated strawberries, two temperatures (4 and 20 °C) were selected. Strawberries (20 per container) were randomly placed in their original containers and treated as follow:

i. Un-treated control samples stored at 20 °C for 7 days
ii. Un-treated control samples stored at 4 °C for 15 days
iii. Combination of O_3 and CO_2 (O_3/CO_2) for 4 h, followed by storage at 20 °C for 7 days
iv. Exposure to CO_2 for 4 h , followed by storage at 20 °C for 7 days
v. Exposure to O_3 for 4 h, followed by storage at 20 °C for 7 days
vi. Combination of O_3 and CO_2 (O_3/CO_2) for 4 h, followed by storage at 4 °C for 15 days

Strawberries were treated with O_3 at 16 mg/kg gaseous atmosphere or O_3 (16 mg/kg gaseous atmosphere) and CO_2 (40%) (O_3/CO_2) combination treatments as described earlier. For strawberries treated with CO_2 alone, the humidity was adjusted

initially as described before, and then the samples were placed in the treatment vessel. Continuous CO_2 gas was fed through the vessel for 5 min at 100 scfh flow rate to reach the 40% level. Subsequently, all the gas valves were closed, and the strawberries were held under the static system during the experimental period. The CO_2 level was monitored and maintained thought the experiments. Humidity, treatment time and temperature were the same for untreated and gas-treated strawberries. Strawberries were analyzed visually and microbiologically (mesophilic aerobic count, and yeast and mold count) as described later. This experiment was conducted in triplicates**.**

Enumeration of microbiota

Treated and untreated strawberry samples were aseptically placed in stomacher bags (PE bags, Fisher Scientific Co., Fair Lawn, N.J.) and weighed. Sterile peptone water (0.1 %, Difco, Becton Dickinson, Sparks, Md.) was added as a diluent into the stomacher bag. The bag was then placed on an orbital shaker (model 361, Fischer Scientific, Pittsburgh, Pa.) and mixed for 2 min at 250 rpm. Bag fluid was serially diluted and plated onto tryptic soy agar (TSA; Difco) for mesophilic aerobic bacteria count and on acidified potato dextrose agar (A-PDA; Difco) for yeast and mold count. Plates were incubated at 37 °C for 24-48 h for mesophilic aerobic counts and at 20°C for 5 days for yeast and mold, and colonies were counted.

Data Analysis

The experiments were conducted in triplicates and data were analyzed statistically (JMP IN version 6.0.2; SAS Inc. Cary, N.C.). Raw data of microbial counts (CFU/g strawberry) were converted into log units before analyses. Mean values for bacterial and fungal counts (Log CFU/g strawberry) were compared by one-way analysis of variance. Tukey's multiple comparison test was used to analyze mean differences. Values with $P \leq 0.05$ were considered to be significantly different.

RESULTS AND DISCUSSION

Gaseous treatments of strawberries for 1, 2, 4 and 6 hours with O_3 and O_3/CO_2 gases

A treatment system has been developed where fresh strawberries were treated, in their original container, in a closed vessel using O_3 or O_3 in combination with CO_2. Fresh strawberries were treated with gases for up to 6 h and held at room temperature and ~95% RH for 7 days. Treatments with O_3/CO_2 combination for more than 4 h significantly ($P \leq 0.05$) decreased yeast and mold count and delayed visual mold growth on strawberries when compared with treatment O_3 alone (Tables 4.1 and 4.2). Treatment of strawberries with O_3 or O_3/CO_2 combination for 4 h decreased the yeast and mold count by 1.2 log CFU/g (Table 4.1). In contrast, the yeast and mold count of control or O_3 treated strawberries did not change significantly. After the 3 days of storage, yeast and mold population on strawberries treated with O_3/CO_2 combination was 1.9 log lower than that of the control, or O_3 alone. The differences between O_3/CO_2 combination and control

treatments in yeast and mold count were 1.0 log CFU/g at the end of the 7-day storage. Similar inactivation pattern was observed when the strawberries were treated with O_3/CO_2 combination for 6 h (Table 4.1). Conversely, the treatment of strawberries with O_3/CO_2 combination for less than 4 h did not show significant ($P > 0.05$) decrease on yeast and mold count, immediately or during the 7 days of storage (Table 4.1). Similar inactivation trend against total mesophilic aerobic count of strawberries was observed when the fruits were treated with O_3 or O_3/CO_2 combinations. Treatment time of 4 h was selected for subsequent experiments.

The antimicrobial activity of gaseous ozone has been tested against microorganisms of various foods. Barth et al. (1995) reported that fungal growth was suppressed on blackberries when the fruits were stored in ozone-enriched environment. Ozone treatments at 3 mg/L, 6-14 °C and 93-97% RH, of onions, potatoes, and sugar beets during storage, reduced the spoilage and microbial population of treated products without affecting their chemical composition or sensory qualities (Baranovskaya et al., 1979). Similarly, ozone treatments reduced the decay caused by various fungi and extended shelflife of table grapes (Sarig et al., 1996). Significant decrease in fungal decay was achieved when the grapes were treated with ozone before or after inoculation with *Rhizopus stolonifer*. Shelflife of various fresh produce, including strawberries, could be doubled if the products were treated with continuous gases ozone at 2-3 ppm level for few hours per day (Ewel, 1940). Since strawberries do not received any washing treatment, gaseous ozone could be an effective choice for the shelflife extension of this category of fruits. It is speculated that the ozone treatment also induced plant resistance

against fungal decay. Ozone may also cause physiological injury and damage to fruits and vegetables depending on treatment conditions (Horváth et al., 1985).

Preliminary experiments, conducted in our laboratory, demonstrated that using low concentration of ozone (i.e., 16 mg/kg in gaseous mix) for long exposure time (up to 24 h) was not effective against the microbial load and this treatment did not extend the shelflife of strawberries (appendix C). An important factor that may have contributed to these results is the oxygen level inside the treatment chamber during the experiments. When strawberries were treated with ozone (e.g., 16 mg/kg gases mixture), the remainder of the atmosphere is made of oxygen which may have activated the growth of aerobic spoilage microorganisms such as fungi. The visual fungal growth was accelerated compare to untreated control samples when the strawberries were treated with oxygen alone, regardless for the treatment time (data not shown). Previous studies revealed that mycelial growth of fungi significantly decreases when CO_2 gas was used in storage at 40-60% levels (Mokbel and Hashinaga, 2004). Garcia-Gimeno et al. (2002) studied the effect of CO_2 enriched atmosphere gas on the growth of *B. cinerea* spores. The authors concluded that CO_2 level in the storage atmosphere should be more than 20% in order to control fungal development. Reports of studies using ozone gas in combination with other gases are limited. Mitsuda et al. (1990) reported the synergistic action of ozone and carbon dioxide combination on microbial inactivation in foods. The synergy was believed to be due to the quenching action of carbon dioxide on the chain decomposition reaction of ozone; this increases the stability and bactericidal effectiveness of the ozone in the treatment environment. Therefore, subsequent experiments were carried out combining O_3 and CO_2, and the contribution of this combination to strawberries visual and

microbiological qualities was investigated during two different storage temperatures (4 and 20 °C).

Strawberries treated with O_3/CO_2 for 4 h and stored at 20°C

The population of yeast and mold on strawberries decreased significantly ($P \leq 0.05$) by ~1.1 log CFU/g when the fruits treated with O_3/CO_2 combination for 4 h compared to controls, immediately after the treatment (Fig 4.2). Treatment with either CO_2 or O_3 for 4 h did not change yeast and mold population. After the 3-day storage, yeast and mold population on strawberries treated with O_3/CO_2 combination was 2.0 log CFU/g lower than that of the control, CO_2 alone, or O_3 alone ($P \leq 0.05$). The differences between combination and control treatments in yeast-mold count were 1.0 log CFU/g at the end of the 7-day storage (Fig. 4.2).

Strawberries that were untreated or treated with CO_2 or O_3 showed visible mold growth on their surfaces after 3 days of storage at 20°C (Fig. 4.5). By the end of the 4^{th} day, nearly all of the control samples were moldy. On the other hand, the visual growth of molds on the strawberries treated with O_3/CO_2 combination was observed only at the end of the 4^{th} day (Fig. 4.6). Mesophilic aerobic count on strawberries treated with O_3/CO_2 combination was ~ 1 log lower than that for the untreated, CO_2–treated or O_3-treated, when the counts were compared after 2 days of storage (Fig. 4.3). At the end of the 3-day storage, control and the treated samples produced similar mesophilic aerobic counts.

Strawberries treated with O_3/CO_2 for 4 h and stored at 4°C

Yeast and mold populations on strawberries treated with O_3/CO_2 combination for 4h decreased by greater than 1.0 log CFU/g immediately after the treatment, compared with populations on control samples (Fig. 4.7). During 15 days of storage at 4°C, yeast and mold count was 1.8 log CFU/g lower on strawberries treated with O_3/CO_2 combination, compared with controls. Visible mold growth was first observed on control strawberries after 8-day storage, and by the 16^{th} day, nearly all of the control samples were moldy. In contrast, strawberries treated with O_3/CO_2 combination showed no visible mold growth throughout the 16-day storage at 4°C (Figs. 4.9 and 4.10). The mesophilic aerobic count of strawberries decreased by ~ 1 log CFU/g after the 4 h O_3/CO_2 treatments (Fig. 4.8). The difference of the counts between treated and control samples were about the 1.0 log CFU/g throughout the storage period.

Prevention of postharvest loses of perishable fruits such as strawberries due to microbial decay have been a great challenge. To prevent fruit decay, these fruits usually held under controlled atmosphere, coupled with low temperatures. However, postharvest loses still take place during agri-food chain. There is an urgent need to develop effective, environment friendly, alternative technologies for preventing postharvest loses. In the current study, attempts were made to extend the shelflife of strawberries using low O_3 concentrations with or without CO_2 in the gas mixture. The O_3/CO_2 combination was the most effective in keeping the quality of the strawberries. The combination delayed the initiation of mold growth for 2 days throughout 7-day storage at 20°C. This positive effect of the two-gas combination was appreciable when the fruits were treated for 4 h and stored at 4°C. Initiation of visual mold appearance was delayed until 16^{th} day of

storage, compared to the 8th day for untreated control. Therefore, treatments of strawberries with O_3/CO_2 combination may be an effective means of extending shelflife and controlling post harvest losses of these perishable fruits.

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Treatment	Treatment time (h)	Yeast and mold count on strawberries (CFU/g)				
		Storage (days)				
		0	1	2	3	7
O_3	0[a]	4.6 ± 0.2	4.8 ± 0.2	5.2 ± 0.2	5.9 ± 0.1	6.6 ± 0.2
	1	4.4 ± 0.3	4.7 ± 0.2	5.0 ± 0.1	6.0 ± 0.3	6.5 ± 0.1
	2	4.5 ± 0.1	4.9 ± 0.3	5.1 ± 0.2	5.8 ± 0.2	6.4 ± 0.1
	4	4.8 ± 0.3	5.0 ± 0.0	5.3 ± 0.2	6.1 ± 0.3	6.6 ± 0.4
	6	4.5 ± 0.3	4.8 ± 0.0	5.1 ± 0.2	5.9 ± 0.2	6.6 ± 0.2
$O_3\backslash CO_2$	0	4.5 ± 0.2	5.0 ± 0.2	5.3 ± 0.3	6.1 ± 0.1	6.5 ± 0.2
	1	4.2 ± 0.3	4.9 ± 0.1	5.0 ± 0.1	5.7 ± 0.2	6.2± 0.2
	2	4.3 ± 0.2	5.0 ± 0.1	4.8 ± 0.1	5.5 ± 0.1	6.3 ± 0.1
	4	3.5 ± 0.1	3.7 ± 0.2	3.9 ± 0.0	4.0 ± 0.1	5.6 ± 0.1
	6	3.4 ± 0.2	3.6 ± 0.2	4.0 ± 0.0	3.9 ± 0.1	5.4 ± 0.2

[a] Treatment time at 0 (h) represents yeast and mold count before treatment

Table 4.1. Changes in yeast and mold count (log CFU/g strawberry) on strawberries when the fruits were treated with O_3 or O_3/CO_2 combinations for up to 6 h and stored at 20 °C. Holding control strawberries at 20 °C for up to 6 h did not significantly change the yeast and mold count. Data shown are averages of three independent trials, each with replicate samples (n=6).

Treatment	Treatment time (h)	Mesophilic aerobic count on strawberries (CFU/g)				
		Storage (days)				
		0	1	2	3	7
O_3	0[a]	5.1 ± 0.2	4.8 ± 0.3	4.5 ± 0.2	4.1 ± 0.3	3.9 ± 0.2
	1	4.9 ± 0.2	4.6 ± 0.4	4.2 ± 0.4	4.0 ± 0.2	3.7 ± 0.1
	2	4.9 ± 0.1	4.8 ± 0.2	4.4 ± 0.1	4.1 ± 0.2	3.9 ± 0.3
	4	4.8 ± 0.2	4.8 ± 0.2	4.1 ± 0.3	3.6 ± 0.2	3.5 ± 0.3
	6	4.7 ± 0.4	5.0 ± 0.2	4.3 ± 0.1	3.6 ± 0.2	3.2 ± 0.4
$O_3\backslash CO_2$	0	4.8 ± 0.1	4.8 ± 0.3	4.6 ± 0.3	4.0 ± 0.3	3.7 ± 0.1
	1	4.9 ± 0.1	4.7 ± 0.3	4.4 ± 0.1	3.9 ± 0.1	3.8 ± 0.2
	2	5.0 ± 0.1	4.6 ± 0.3	4.0 ± 0.1	4.0 ± 0.1	3.8 ± 0.2
	4	4.5 ± 0.1	4.1 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.2 ± 0.2
	6	4.3 ± 0.3	3.9 ± 0.2	3.8 ± 0.1	3.5 ± 0.1	3.3 ± 0.1

[a] Treatment time at 0 (h) represents yeast and mold count before treatment

Table 4.2. Changes in mesophilic aerobic count (log CFU/g strawberry) on strawberries when the fruits were treated with O_3 or O_3/CO_2 combinations for up to 6 h and stored at 20°C. Holding control strawberries at 20 °C for up to 6 h did not significantly change the yeast and mold count. Data shown are averages of three independent trials, each with replicate samples (n=6).

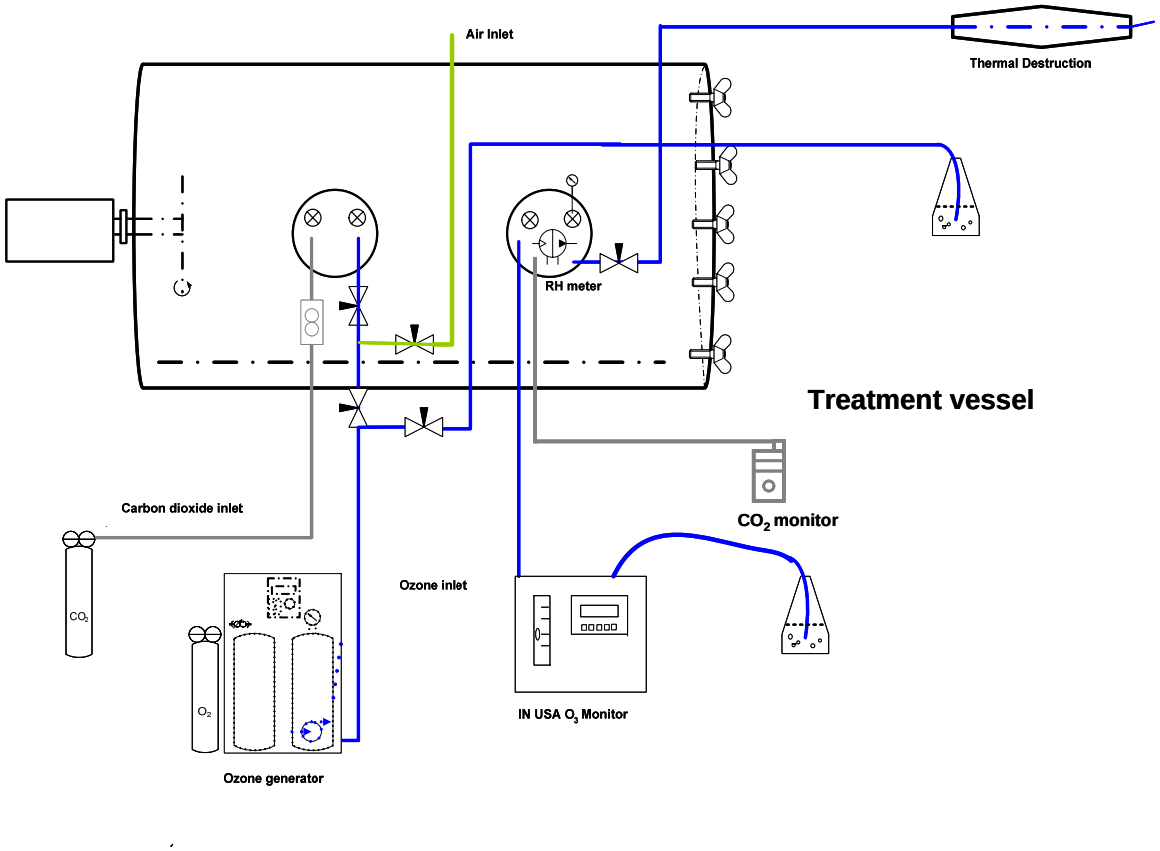

Figure 4.1 Schematics of the gaseous treatment system for fresh strawberries.

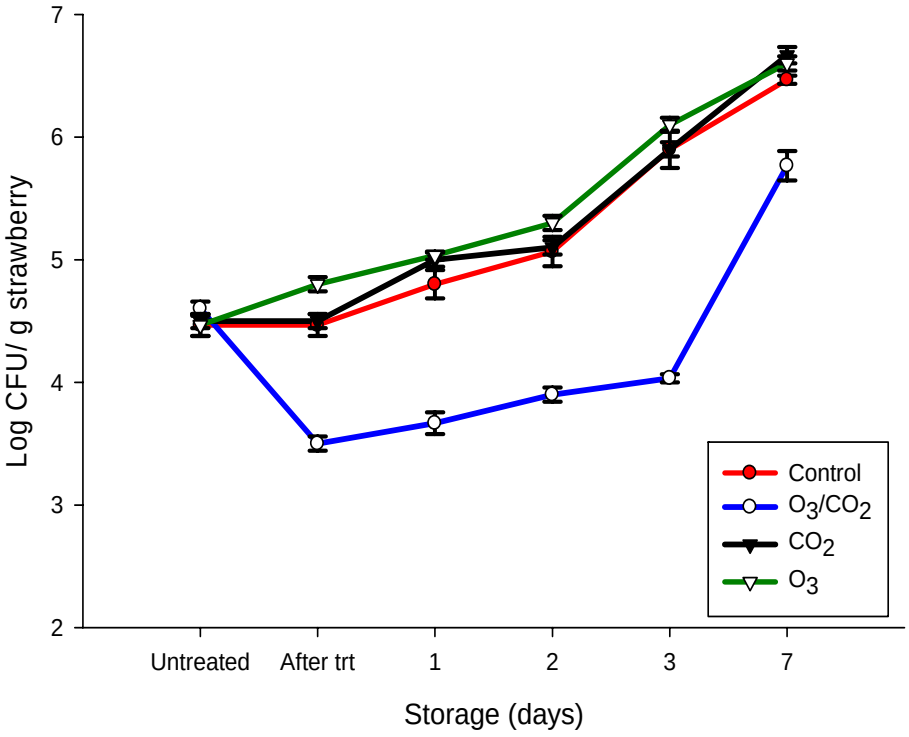

Figure 4.2. Changes in yeast and mold count of strawberries treated with O_3, CO_2 or their combinations for 4 h when the fruits were stored at 20 °C. Data shown are averages of three independent trials, each with replicate samples (n=6). Error bars represent $\pm$ standard error.

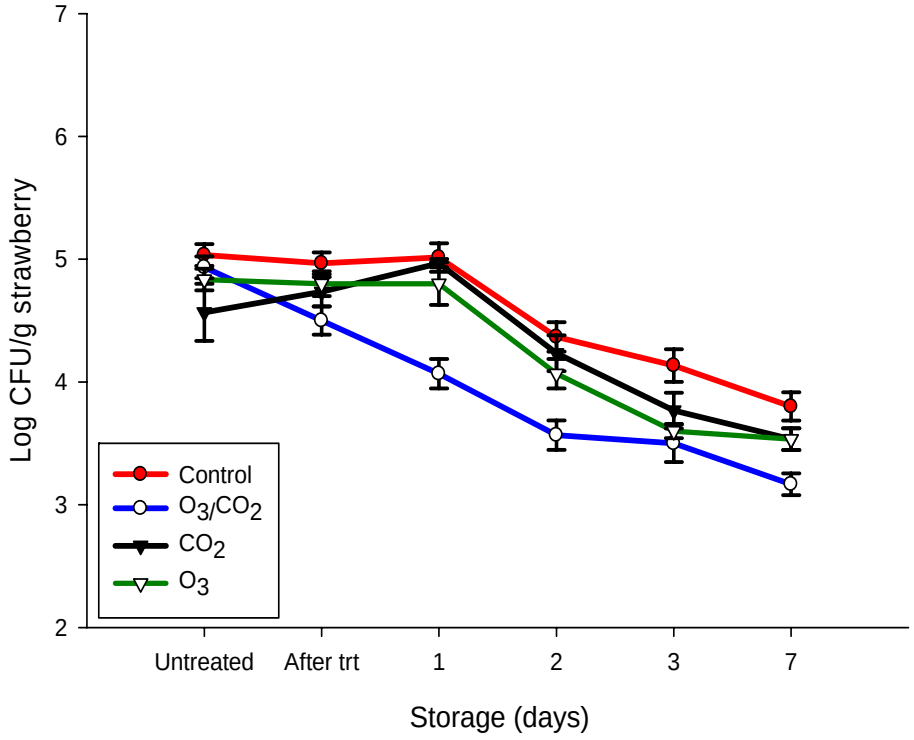

Figure 4.3. Changes in mesophilic aerobic count of strawberries treated with O_3, CO_2 or their combinations for 4 h when the fruits were stored at 20°C. Data shown are averages of three independent trials, each with replicate samples (n=6). Error bars represent $\pm$ standard error.

Before O_3/CO_2 treatment

O_3/CO_2 treatment

After O_3/CO_2 treatment

Untreated

Figure 4.4. Strawberries before, during and immediately after treatment with O_3/CO_2 for 4 h.

Untreated

CO_2 only

O_3 only

O_3/CO_2 combination

Figure 4.5. Strawberries that were untreated or treated with gases (for 4 h) after 3 days storage at 20 °C.

Untreated

CO_2 only

O_3 only

O_3/CO_2 combination

Figure 4.6. Strawberries that were untreated or treated with gases (for 4 h) after 4 days storage at 20 °C.

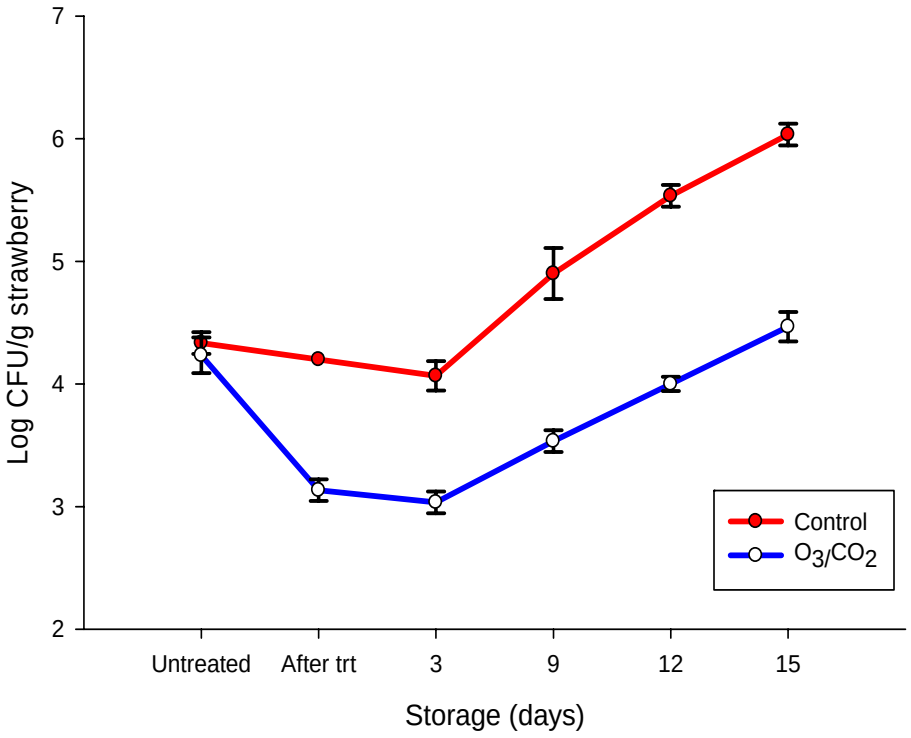

Figure 4.7. Changes in yeast and mold count of strawberries treated with or without O_3/CO_2 combination for 4 h when the fruits were stored at 4°C. Data shown are averages of three independent trials, each with replicate samples (n=6). Error bars represent $\pm$ standard error.

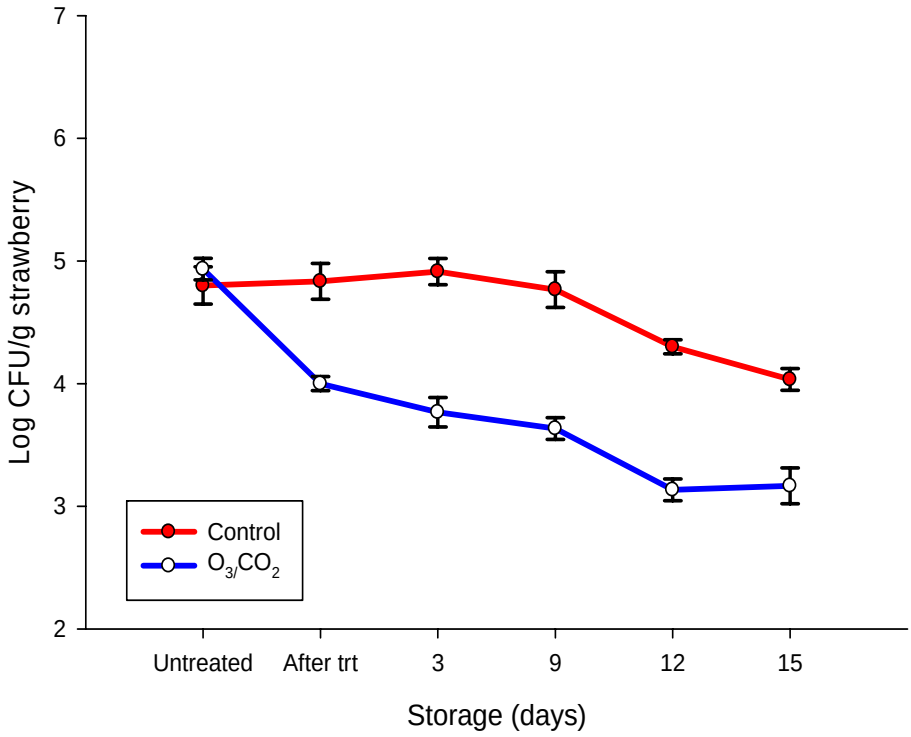

Figure 4.8. Changes in mesophilic aerobic count of strawberries treated with or without O_3/CO_2 combination for 4 h when the fruits were stored at 4°C. Data shown are averages of three independent trials, each with replicate samples (n=6). Error bars represent $\pm$ standard error.

Before O_3/CO_2 treatment

Untreated

After O_3/CO_2 treatment

Figure 4.9. Strawberries before, during and after treatment with O_3/CO_2 for 4 h.

Untreated at day 8

O_3/CO_2 treated at day 8

Untreated at day 16

O_3/CO_2 treated at day 16

Figure 4.10. Strawberries that were untreated or treated with gases (for 4 h) after 16 days storage at 4 °C.

APPENDIX A

OZONE CONCENTRATION CONVERSION FROM VOLUME/VOLUME TO MASS/MASS

Ozone concentration during some of our experimentation was measured in ppm (vol/vol). This section explains how measured concentration on volume/volume basis (ppm) was converted to mass/mass basis. Concentration of ozone measured in ppm is defined as milliliter of pure O_3 presence in one cubic meter of O_2-O_3 at atmospheric pressure and standard room temperature.

Therefore, $$\text{ppm (vol/vol)} = \frac{ml\ of\ O_3}{m^3\ of\ O_2}$$

To convert from vol/vol to mass/mass, density of O_3 gas and O_2 should be considered. Gas mix could be as simple as O_2+O_3 or it could be more complex, e.g. O_3 in air (N_2, O_2, CO_2, and water vapor in addition to O_3). For simplicity, the gas mix considered here is O_2+O_3. In the current study, the gas mix was mostly O_2 since very small O_3 concentrations were used. It is known that 22.4 L of O_3 at STP contain 48 g of O_3. Hence,

volume of O_3 at standard room temperature and pressure can be calculated using the following formula:

$$\frac{V_1}{T_1} = \frac{V_2}{T_2}$$

Where V_1 = 22.4 L, T_1 = 273.15 K, V_2 = ? and T_2 = (273.15 + 25) K

$$V_2 = \frac{V_1 \times T_2}{T_1} = \frac{22.4 \times 298.15}{273.15} = 24.45\,L$$

For conversion of 1 ppm (vol/vol) O_3 in O_2 into mg O_3/m^3 O_2;

$$\frac{ml\ of\ O_3}{m^3\ of\ O_2} = \frac{\frac{48}{24.45}}{m^3\ of\ O_2} = 1.96 \text{ mg } O_3/m^3 \text{ gas mix (or 2.14 mg/m}^3 \text{ at STP)}$$

Since 32 g O_2 gas occupy 24.45 L at room temperature (25°C) and pressure (1 atm),

Therefore, O_2 density = $\frac{32}{24.45} = 1.31\ g/L = 1.31$ kg O_2/m^3

Hence, 1 $\frac{g\ of\ O_3}{m^3\ of\ O_2} = \frac{1\ g\ of\ O_3}{1.31\ kg\ of\ O_2} = 0.76 \frac{g\ of\ O_3}{kg\ of\ O_2}$ (or $0.70 \frac{g\ of\ O_3}{kg\ of\ O_2}\ at\ STP$)

APPENDIX B

MICROSCOPIC DEMONSTRATION OF INTERNALIZATION/INFILTRATION OF *ESCHERICHIA COLI* O157:H7 INTO SPINACH BY VACUUM COOLING

Bacteria can become internalized in fresh produce in the field and during post-harvest operations. Solomon et al. (2002) reported that *E. coli* O157:H7, when inoculated in manure and added to planting soil, internalized and survived on lettuce plants grown in that soil. According to the researchers, this pathogen was observed using confocal scanning laser microscopy (CLSM) inside of the lettuce tissue at depths as low as 45 μm (Solomon et al., 2002).

Li et al. (2008) recently reported that the vacuum cooling process internalizes *E. coli* O157:H7 in lettuce. According to these authors, under vacuum conditions, balance between lettuce interior and surrounding atmosphere is disrupted. Water in the vacuoles and empty spaces will evaporate into the environment as a nature of vacuum cooling, and forced to open stomata found on produce surface. If produce contaminated prior to cooling, during re-pressurization from vacuum to atmospheric pressure, these contaminants may be forced to internalize by the resulting powerful suction.

Bacterial internalization into produce surfaces can be visualized by electron microscopy. Therefore SEM was used to demonstrate the possible internalization/infiltration of *E. coli* O157:H7 into spinach by vacuum cooling process.

EXPERIMENTAL APPROACH

Scanning electron microscopy (SEM) Procedure

Baby spinach samples (25 g) were spot-inoculated with 100 µl *E. coli* O157:H7 strain B6-914 to reach aproximately10^7 CFU/g. Samples were then held in laminar flow biological hood at room temperature for 2 h for allowing drying and attachment before any further treatment. The inoculated and dried baby spinach samples were then treated as follow:

- Untreated control
- Vacuum cooling and combined with O_2 treatment
- Vacuum cooling combined with O_3 at 0.41 g/kg for 5 min

Control and untreated spinach samples were immersed in a 2.5% glutaraldehyde–0.1 M phosphate buffer saline (PBS, pH 7.4) for more than 2 h and then washed in the PBS solution to remove the glutaraldehyde. Samples were then dehydrated by exchange in a graded series of ethanol solutions (50, 70, 80, 95 and 100% for 2x 10 min each), frozen in liquid nitrogen, and fractured manually with cold fine-tipped tweezers. Frozen fragments were thawed into ethanol (100%) and critical point dried using liquid carbon dioxide. Dry fragments were mounted onto specimen stubs with fracture faces oriented

upward and sputter coated with a thin layer of gold. Intercellular spaces near the fracture faces were examined with a Quanta 400 FEG SEM operated at an accelerating voltage of 5 kV in the high-vacuum–secondary electron imaging mode of operation. Experiments were repeated two times. Results of selected SEM micrographs as shown on Fig. A1.

In conclusion, the SEM micrographs revealed bacterial cells throughout the intercellular spaces in baby spinach after inoculated leaves were vacuum cooled.

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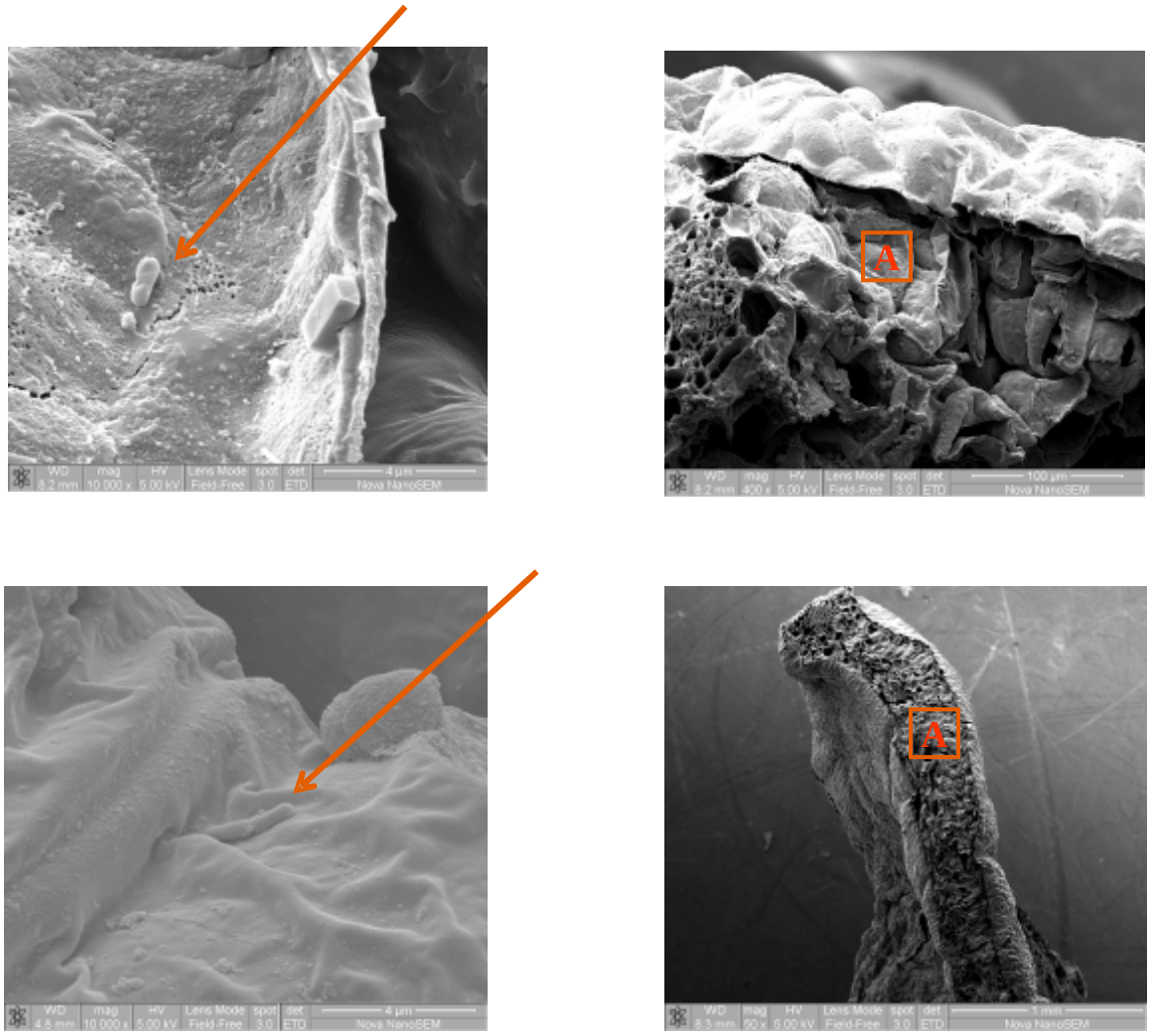

Figure B.1. Scanning electron micrographs of baby spinach leaf pieces that were inoculated with *Escherichia coli* O157:H7 and vacuum cooled. In the left images, orange arrows indicate bacterial cells in intercellular spaces, and the letters A indicate the area of magnification (shown on the right images).

APPENDIX C

GASEOUS OZONE TREATMENTS OF FRESH FRUITS

Overall objective of the study was to develop a gaseous ozone treatment system to inactivate pathogenic and spoilage microorganisms on fruits and extend their shelflife. The study was consisted of three parts: 1) high ozone concentration-short treatment time, 2) low ozone concentration-long treatment time, and 3) very low ozone concentration-long treatment time.

Strawberries or raspberries were inoculated with *Listeria monocytogenes* OSY-328 or *Salmonella enterica* subsp. *enterica* serovar Muenchen ATCC 8388 (*S.* Muenchen) and inactivation was measured in response to various ozone concentrations and treatment time. Additionally, inactivation of natural microbial contaminants on fruits was also measured with the ozone treatments.

1. INACTIVATION OF PATHOGENIC AND NATURAL MICROORGANISMS ON FRUITS WITH HIGH OZONE CONCENTRATION AND SHORT TREATMENT TIME

Experiments were focused on assessing the effect of high ozone concentration with short treatment times on inactivation of pathogenic and natural microorganisms and shelflife of perishable fruits.

MATERIALS AND METHODS

Fruits

Fresh and unblemished strawberries and raspberries were purchased at a local grocery store the day before the experiment and were stored in the refrigerator. All the fruits used in experiments were similar size and weight (20-25 and 6-8 g, for strawberries and raspberries, respectively). The green leafy part of each strawberry was removed aseptically, except for strawberries tested for shelf life stability.

Bacterial cultures and growth conditions

Listeria monocytogenes OSY-328 and *Salmonella enterica* subsp. *enterica* serovar Muenchen ATCC 8388 were used throughout the study. Both pathogens were obtained from the culture collection of the Food Safety Laboratory at The Ohio State University. Stock cultures of *L. monocytogenes* were stored at -80°C in tryptose broth

(TB; Becton, Dickinson, and Co., Sparks, MD) containing 40% (vol/vol) glycerol. The culture of *L. monocytogenes* was streaked onto modified oxford agar (MOX; Oxoid) and an isolated colony was transferred and cultured in TB two times prior to use. Experiments were carried out using stationary phase *L. monocytogenes* cultures; this was achieved by growing the bacterium in TB at 37°C for 18 h. The final count in these cell suspensions was approximately 10^9 CFU/ml.

A loopfull of *S.* Muenchen stock culture was transferred to brain heart infusion broth (Becton Dickinson) and incubated at 37°C for 24 h. Aliquots of the grown culture were subsequently transferred to tryptic soy broth (Difco) and incubated at 37°C for 24 h. The final count in these cell suspensions was approximately ~10^9CFU/ml.

Inoculation of fruits

Each strawberry was spot-inoculated using a total of 100 µl of the overnight *L. monocytogenes* or *S.* Muenchen culture to achieve ~10^8 CFU/g-fruit. For the raspberry experiment, a 50-µl inoculum of the overnight *L. monocytogenes* was used. Fruits were then held in laminar-flow hood at room temperature for 2 h before any further treatment. For each experiment, two sets of four fruits (four for ozone treatment and four for control) contaminated with either *L. monocytogenes* or *S.* Muenchen were used.

Ozone generation units

One of two ozone generators, namely LT-1 (Lynntech, Inc., College Station, Tex.) or CD-150S (DEL Industries Inc., San Luis Obispo, CA), was used to produce gaseous ozone. The LT-1 is an electrochemical ozone generator that produces up to 12 to 14 % (wt/wt) ozone in oxygen gas stream. The capacity of the generator is 1 lb/day. The

CD-150S is a corona discharge type ozone generator and the unit produces a gas stream containing up to 5.4% ozone by weight when operating on an enriched oxygen source. The capacity of the unit is 8 lbs/day.

Ozone measurement

The concentration of gaseous ozone was measured using UV-based monitor (model Mini Hi-Con, IN USA Inc., Norwood, Mass.). A pump was connected between the vessel and the monitor to circulate the gas stream into the monitor.

Ozone treatments

Setup 1 (Fig. C.1) was used in the first two experiments (Figs. C.2 and C.3) whereas setup 2 (Fig. C.4) was used in the remainder of the experiments

. In setup 1, strawberries were treated with wet-ozone (from LT-1 generator) in a 4 L capacity stainless steel vessel. Four strawberries, contaminated with *L. monocytogenes*, were placed into the vessel. A vacuum pump was used to apply vacuum in the vessel. Gaseous ozone was delivered into the vessel to reach target pressures (3 and 7.5 psig for the experiment 1 and 2, respectively) and concentrations (45 g/kg and 60 g/kg for the experiment 1 and 2, respectively) within 2-3 min, and then a 10-min holding/treatment time was applied. After treatments, pressure was released from the vessel in 30 sec. Fruits then were tested *L. monocytogenes* population was enumerated.

In the setup 2, strawberries and raspberries were treated with dry-ozone in a 300-liter capacity stainless steel vessel. Four strawberries inoculated either with *L. monocytogenes* or *S.* Muenchen were placed into the vessel. A vacuum pump was used to reduce the pressure in the vessel. Gaseous ozone was delivered in the previously

vacuumed (10 in. Hg) vessel to reach the targeted ozone concentration and pressure. Vessel pressure of 10 psig and ozone concentration of 14-16.5 g/kg was achieved within 10 min, and then samples were held for 10-30 min under these conditions. After treatment, pressure was released and fruits were tested for enumeration of *L. monocytogenes* or *S.* Muenchen. In addition to inoculated pathogens, natural microbiota of strawberries was also tested.

Enumeration of microorganisms

Treated and untreated fruits were aseptically placed in a sterile polyethylene stomacher bag (PE bags, Fisher Scientific Co., Fair Lawn, NJ). Sterile peptone water (0.1 %, 100 ml for strawberries, 50 ml for raspberries; Difco) was added into the stomacher bag. The bag containing a fruit and diluent was then placed on an orbital shaker (model 361, Fischer Scientific, Pittsburgh, PA) and mixed for 2 min at 250 rpm. Samples were serially diluted in 0.1% peptone water (Difco) and surface-plated onto modified oxford agar (MOX; Oxoid) for enumerating *L. monocytogenes*. To recover potentially injured *L. monocytogenes* cells, samples were also plated onto tryptose Agar (TA; Difco). For enumerating *S.* Muenchen, samples were plated onto tryptose Agar (Difco). Inoculated plates were incubated at 35°C for 48 h and colonies were counted.

The total mesophilic aerobic counts of un-inoculated strawberries treated with or without gaseous ozone, were also determined. These samples were homogenized as described earlier and plated onto TA (Difco). Plates were incubated at 37 °C for 24 h and mesophilic aerobic counts were reported as total count CFU/g strawberry.

RESULTS

High ozone concentration and short treatment time combinations were applied and inactivation of pathogenic and spoilage microorganisms were investigated. Selected treatments were produced more than 2-log reduction of the inoculated pathogens , *L. monocytogenes* and *S.* Muenchen, on fruits. The natural microbial population of fruits was inactivated to undetectable level with selected ozone treatments.

Experiment	Treatment Conditions				Inactivation[c] (log CFU)
	Setup#	Holding Time (min)	Pressure (psig)	Concentration (g/kg)	
1	1	10	3	45±1.5	1.2[a]
2	1	10	7.5	60±1.5	2.4[a]
3	2	10	10	15±1.5	0.6[a]
4[d]	2	30	10	15±1.5	2.0[a]
5[e]	2	30	10	15±1.5	1.7[b]
6	2	30	10	15±1.5	1.0[a]

[a,b] Initial populations of pathogens were around ~10^8 CFU/ g fruit.

[c] Averages for two independent trials.

[d] Inactivation of natural microbiota on strawberries was more than 1.8 log CFU

[e] Inactivation of natural microbiota on strawberries was more than 2.3 log CFU

Table C.1. Changes in *L. monocytogenes* [a] and *S.* Muenchen[b] populations on fruits when the fruits treated with gaseous ozone, and held in the vessel above atmospheric pressure for ≤30 min using setup 2.

Figure C.1. Experimental setup 1 to treat fruits with gaseous ozone under pressure

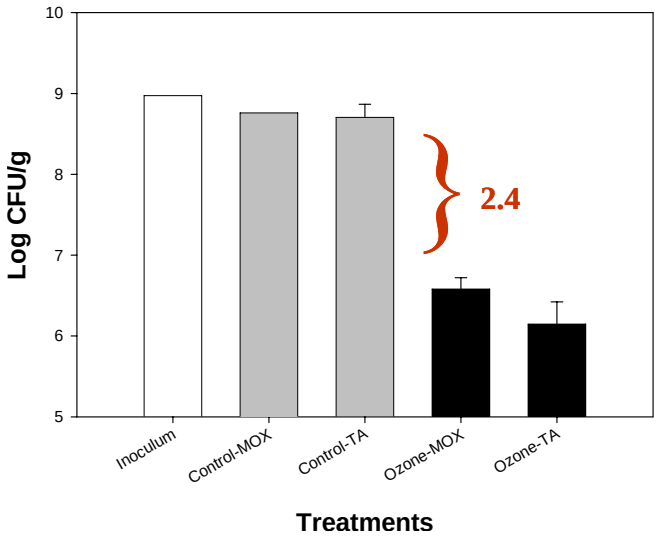

Figure C.2. Changes in population of *L. monocytogenes* OSY-328 on strawberries when treated with 45 g/kg (60 g/m^3) moist ozone at 3 psig for 10 min using Setup 1. *Listeria* populations were enumerated on MOX agar and TA. Values are the average of duplicate samples of two experiments.

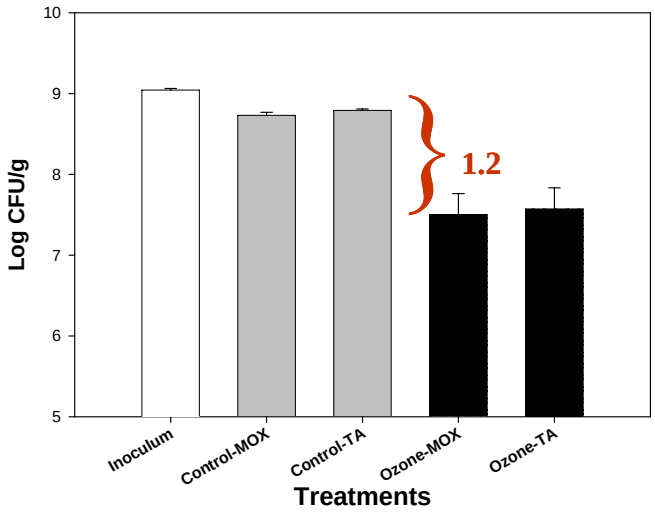

Figure C.3. Changes in population of *L. monocytogenes* OSY-328 on strawberries when treated with 60 g/kg (80 g/m^3) moist ozone at 7.5 psig for 10 min using Setup 1. *Listeria* populations were enumerated on MOX agar and TA. Values are the average of duplicate samples of two experiments.

Figure C.4. Experimental setup 2 to treat fruits with gaseous ozone under pressure

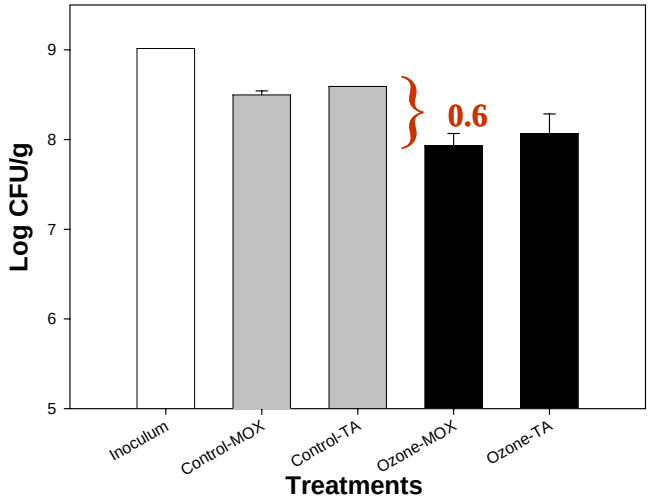

Figure C.5. Changes in population of *L. monocytogenes* OSY-328 on strawberries when treated with 14g/kg (19 g/m^3) dry ozone at 10 psig for 10 min using Setup 2. *Listeria* populations were enumerated on MOX agar and TA. Values are the average of duplicate samples of two experiments.

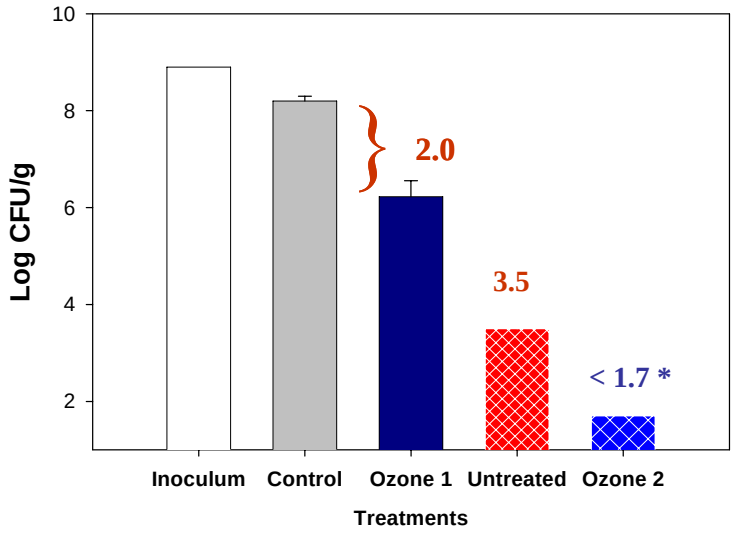

Figure C.6. Changes in population of *L. monocytogenes* OSY-328 (solid white, gray, and black bars) and natural microbiota (hatched red and blue bars) on strawberries when treated with 14-15 g/kg (19-20 g/m^3) dry ozone at 10 psig for 30 min using Setup 2. Values are the average of duplicate samples of two experiments.

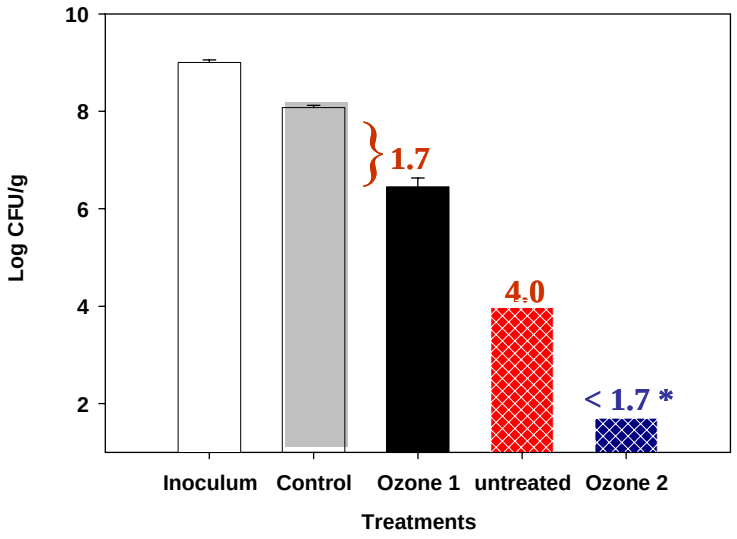

Figure C.7. Changes in population of *S.* Muenchen (solid white, gray, and black bars) and natural microbiota (hatched bars) on strawberries when treated with 16.5 g/kg (22 g/m^3) dry ozone at 10 psig for 30 min using Setup 2. Values are the average of duplicate samples of two experiments.

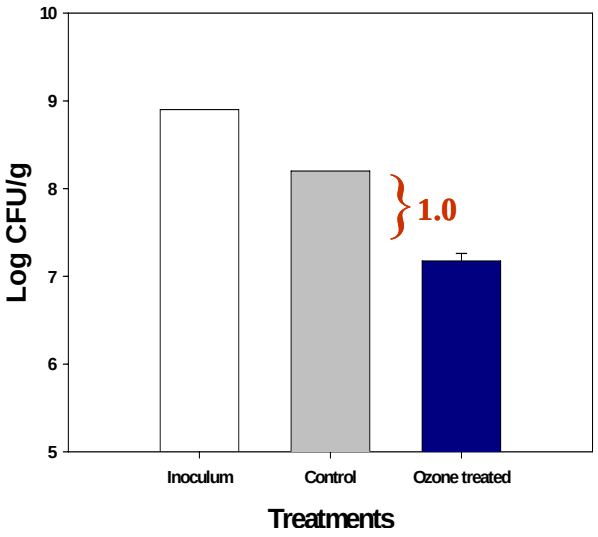

Figure C.8. Changes in population of *L. monocytogenes* OSY-328 on raspberries when treated with ~15 g/kg (20-21 g/m^3) dry ozone at 10 psig for 30 min using Setup 2. Values are the average of duplicate samples of two experiments.

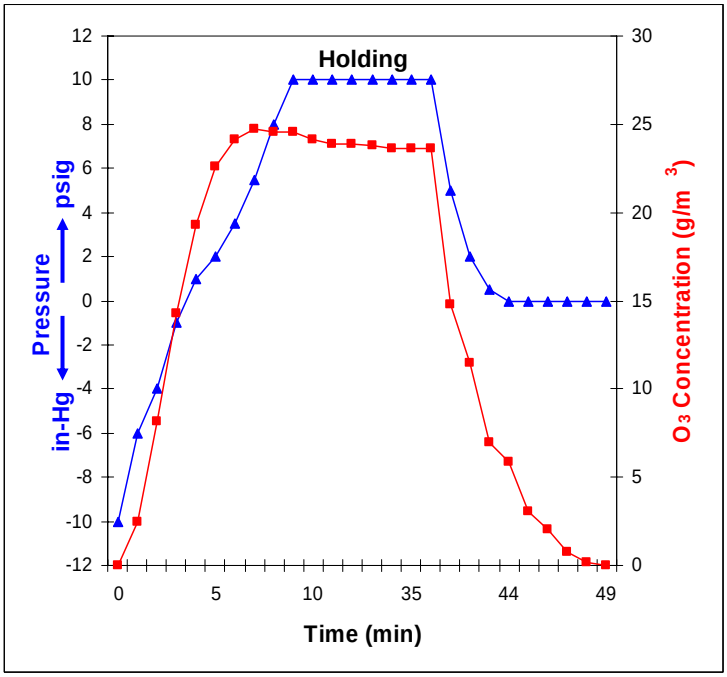

Figure C.9. Changes in typical process parameters including vacuum (in. of Hg), pressure (psig), and ozone concentration (g/m^3) during treatment of fruits inside the treatment vessel during short-term treatments.

2. INACTIVATION OF NATURAL MICROORGANISMS ON STRAWBERRIES WITH LOW OZONE CONCENTRATION AND LONG TREATMENT TIME

Ozone at high concentrations was promising to inactivate pathogens and natural microbiota of strawberries when the fruits were treated under pressure for a short treatment period. Experiments in this part of the study were focused on using low ozone concentration-long time treatments of strawberries and shelflife of these fruits was evaluated. Strawberries were treated with 0.4 g/kg (0.5 g/m^3) ozone and for up to 24 h and microbiological analyses were performed during one-week storage at 20°C. Visual mold growth on strawberries was also observed during storage. Untreated and oxygen treated control strawberries were also microbiologically and visually evaluated.

MATERIALS AND METHODS

Fruits

Fresh and unblemished strawberries were purchased at a local supermarket on the day of testing. The fruits were placed in small carton container (provided by the retailer), refrigerated, and transferred to biological hood about 1 h before testing. All the strawberries used in the experiments were similar size and weight (20-25 g).

Ozone generation units

The ozone generator (Ozat; model CSF-7, Ozonia Inc., Elmwood Park, N.J.) was used to produce gaseous ozone in the experiments. The generator was used at 4% power (to produce low ozone concentration) and 0% power for oxygen treatment (control experiment).

Ozone measurement

Concentration of gaseous ozone was measured using a UV absorption monitor (Mini Hi-Con). A pump was connected between the monitor and vessels to provide the gas flow into the meter.

Treatments

Changes of the berries natural microbiota, in response to ozone treatment, were investigated. Strawberries were treated with ~0.5g/m^3 (0.4 g/kg) in a 300-liter capacity stainless steel vessel. Approximately 10-15 strawberries placed in each carton container and total of four containers used in the study. Two containers were ozone-treated, one was oxygen-treated, and the fourth received no treatment. The humidity was adjusted to 95-100% using a thermohygrometer (Model OKTAN 03313-70, Cole-Parmer Instrument Company, Vernon Hills, Ill.). After reaching the target humidity, gaseous ozone was delivered into the vessel to reach the lowest detectable ozone concentration by the monitor. After reaching the target concentration, the strawberries containers were treated for 24 h. Fluctuations in ozone concentration in the vessel were unavoidable. For the samples treated with oxygen and the non-treated samples, humidity, treatment time and temperature were same as the samples treated with ozone. Strawberries were analyzed

visually and microbiologically before the experiment, immediately after treatment, and after 3 and 7 days of treatment. Treated and control packages were stored at 20°C and ~95-100 % RH.

Enumeration of microbiota

Total mesophilic aerobic count and yeast-mold count were determined. Strawberries samples were weighed and aseptically placed in stomacher bags (PE bags, Fisher Scientific Co., Fair Lawn, N.J.). Sterile peptone water (0.1 %, Difco) was added as a diluent into the stomacher bag. The bag was then placed on an orbital shaker (model 361, Fischer Scientific) and mixed for 2 min at 250 rpm. Bag fluid was serially diluted and plated onto tryptic soy agar (TSA; Difco) for mesophilic aerobic bacteria and on acidified potato dextrose agar (Acidified-PDA; Difco) for yeast and mold. Plates were incubated at 37 °C for 24-48 h for mesophilic aerobic and at 20°C for 5 days for yeast and mold counts. Experimental procedure is illustrated in Fig. C.10.

RESULTS

Strawberries were treated with low ozone concentration (0.5 g/m^3) and long time (up to 24 h) and microbiological analyses were performed during one-week storage at 20 °C and 95-100 % RH. Natural microbiota of untreated and ozone or oxygen treated strawberries were monitored visually and microbiologically. Approximately 2-log reductions on natural microbiota of strawberries were achieved immediately after the ozone treatments, compared to untreated and oxygen treated samples. These treatments delayed the initiation of mold growth on ozone treated strawberries by up to 2 days.

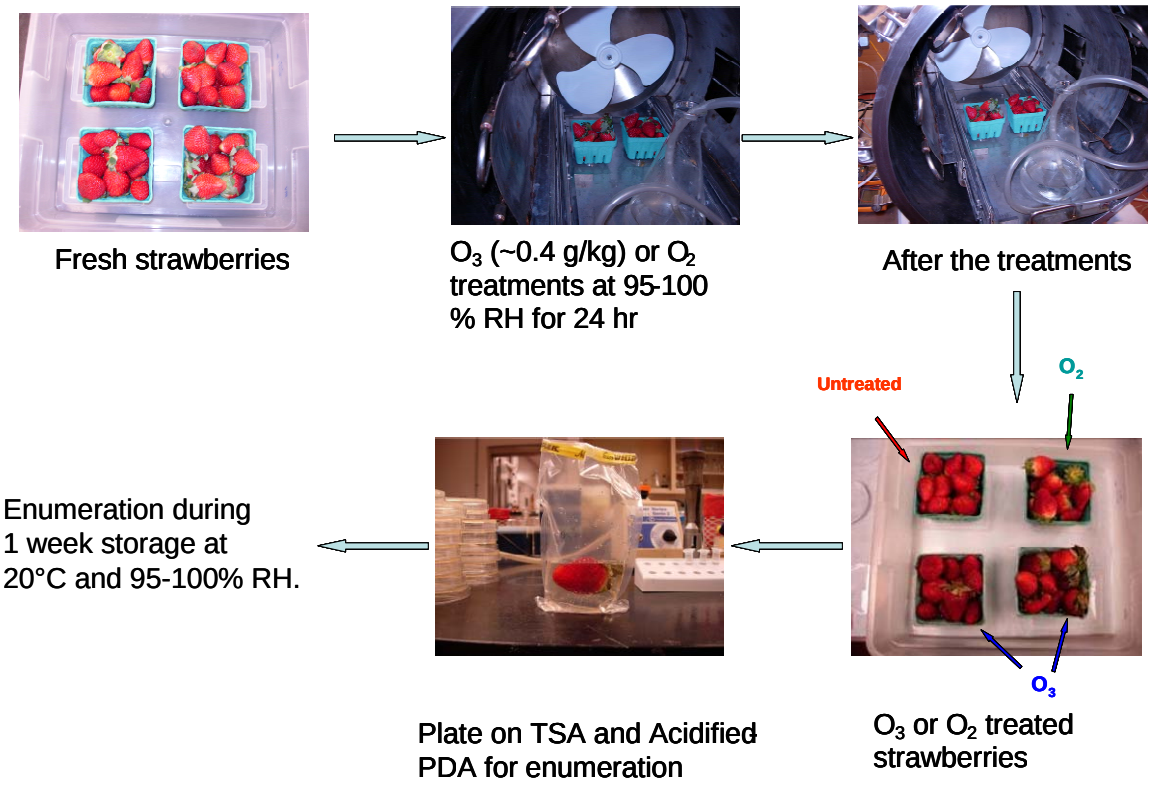

Figure C.10. Experimental procedure

Control

Oxygen treated

Ozone treated

Figure C.11. Strawberries that were treated with ozone (0.4 g/kg) or oxygen for 24 h and after 3 days storage at 20 °C and 95-100 % RH.

Control

Oxygen treated

Ozone treated

Figure C.12. Strawberries that were treated with ozone (0.4 g/kg) or oxygen for 24 h and after 7 days storage at 20 °C and 95-100% RH.

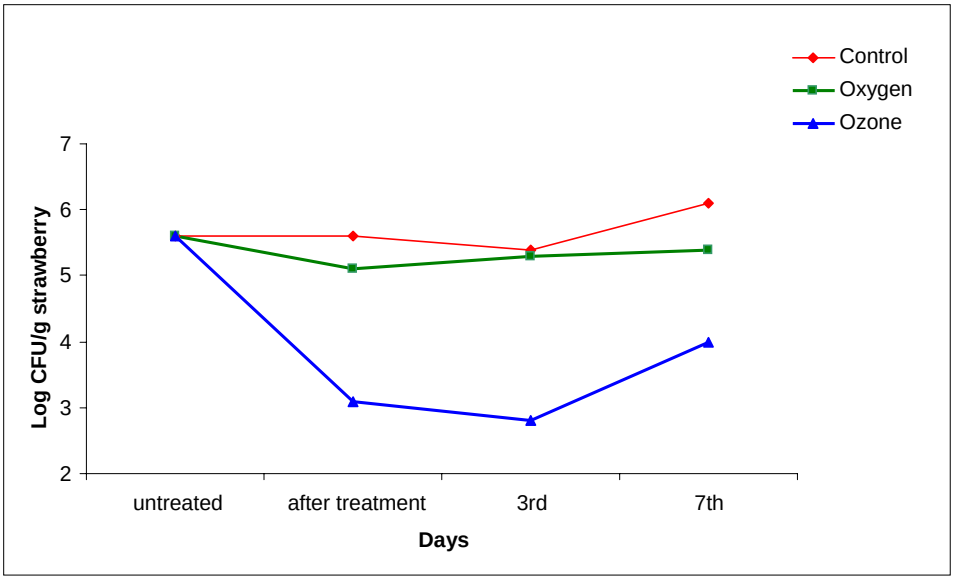

Figure C.13. Changes in mesophilic aerobic count of strawberries that were treated with ozone (0.4 g/kg) or oxygen for 24 h and stored for 7 days at 20 °C and 95-100% RH.

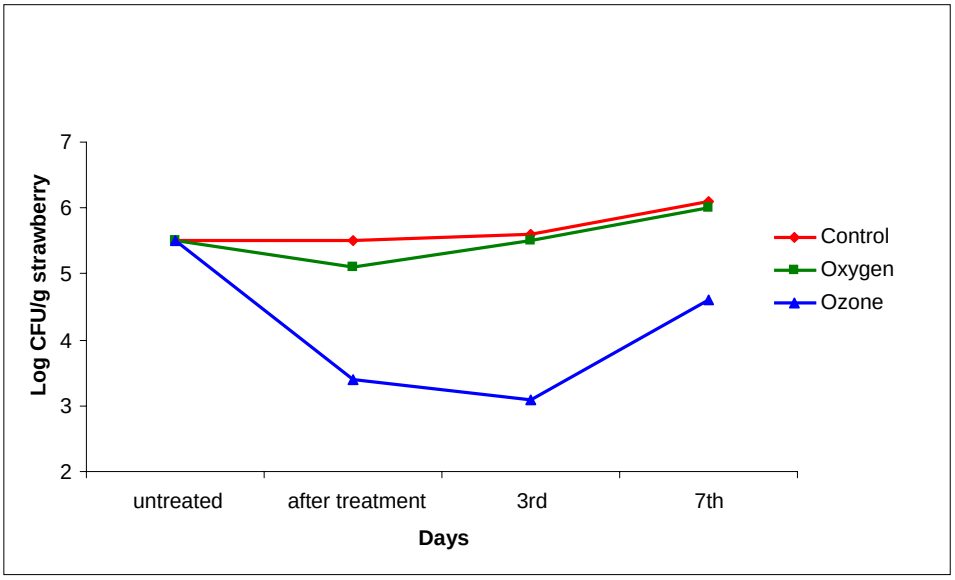

Figure C.14. Changes in yeast and mold count of strawberries that were treated with ozone (0.4 g/kg) or oxygen for 24 h and stored for 7 days at 20 °C and 95-100% RH.

3. TREATMENT OF STRAWBERRIES WITH OZONE AT VERY LOW CONCENTRATION FOR LONG TIME

Ozone concentration at 10 ppm vol/vol level (16 mg/kg) was used for long time (up to 24 h) to treat strawberries and natural microbiota was monitored.

METHODS

Fruits

Fresh strawberries were purchased at a local supermarket on the day of testing. The fruits were placed in small carton container (provided by the retailer), refrigerated, and transferred to biological hood about 1 h before testing. All the strawberries used in the experiments were similar in size and weight (20-25 g).

Ozone generation and measurement units

Del Ozone generator (Model: Infinity CD-150S; Del Industries, San Luis Obispo, CA) and the 300-liters stainless steel treatment chamber was used in the experiments. The concentration of gaseous ozone was measured using an UV absorption monitor (LC-L2-2000, IN USA, Inc. IN).

Treatments

Changes in berries natural microbiota, in response to ozone treatment, were investigated. Strawberries were treated with low ozone concentration (10 ppm, vol/vol = 20 mg/m^3) in a 300-liter capacity stainless steel vessel. Approximately 10-15 strawberries placed in each carton container and total four containers used in the study. Two containers were ozone-treated, one was oxygen-treated, and the fourth received no treatment. The humidity was adjusted to 95-100 % using a hygrometer (Oaktan). After reaching the target humidity, gaseous ozone was delivered into the vessel to reach the target ozone concentration by the monitor. After reaching the target concentration, the strawberries in containers were treated for 2, 4, 6 and 24 hr.

For the samples treated with oxygen and the non-treated samples, humidity, treatment time and temperature were same as the samples treated with ozone. Strawberries were analyzed visually and microbiologically before the experiment, immediately after treatment, and after 3 and 7 days of treatment. Treated and control packages were stored at 20°C and ~95-100% RH.

Enumeration of microbiota

The total mesophilic aerobic and yeast and mold counts were determined. Strawberries samples were weighed and aseptically placed in stomacher bags (Fisher Scientific Co.). Sterile peptone water (0.1 %, Difco) was added as a diluent into the stomacher bag. The bag was then placed on an orbital shaker (model 361, Fischer Scientific) and mixed for 2 min at 250 rpm. Bag fluid was serially diluted and plated onto TSA (Difco) for aerobic mesophilic bacteria and on Acidified-PDA (Difco) for yeast

and molds. Plates were incubated at 37 °C for 24-48 h for mesophilic aerobic count and at 20°C for 5 days for yeast and mold enumeration.

RESULTS

Ozone treatments at very low concentration (16 mg/kg) and long time periods (up to 24 h) were applied on strawberries and natural microbiota were monitored during storage. These treatments did not delay the visual mold growth on strawberries. Ozone at very low concentration was not effective on reducing the microbial population of strawberries during storage. Visual qualities were similar of both ozone treated and control strawberries.

Control

Oxygen treated

Ozone treated

Figure C.15. Strawberries immediately after treatments with ozone (10 ppm vol/vol; 16 mg/kg) or oxygen for 24 h.

Control

Oxygen treated

Ozone treated

Figure C.16. Strawberries that were treated with ozone (10 ppm vol/vol; 16 mg/kg) or oxygen for 24 h and after 3 days storage at 20 °C and 95-100% RH.

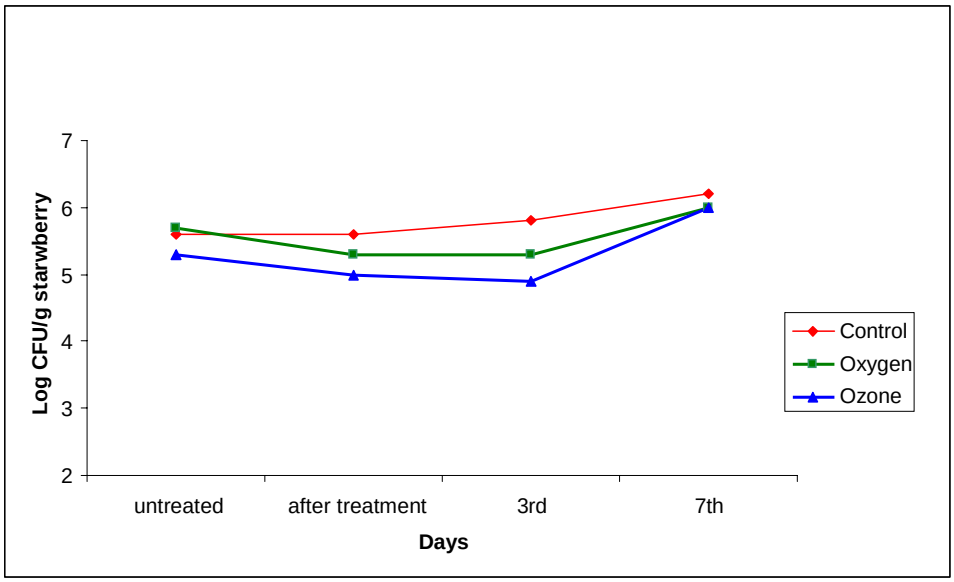

Figure C.17. Changes in mesophilic aerobic count of strawberries that were treated with ozone (10 ppm vol/vol; 16 mg/kg) or oxygen for 24 h and stored for 7 days at 20 °C and 95-100% RH.

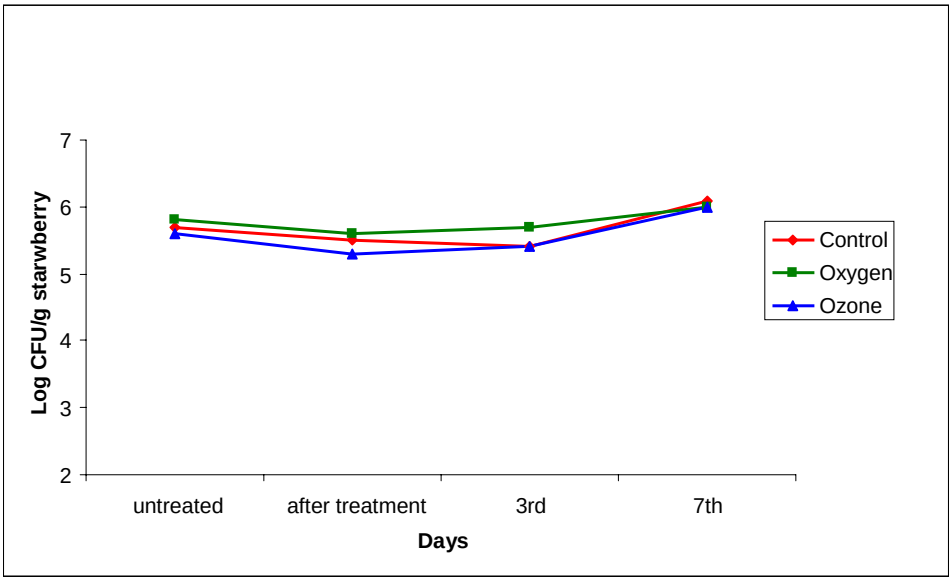

Figure C.18. Changes in yeast and mold count of strawberries that were treated with ozone (10 ppm vol/vol; 16 mg/kg) or oxygen for 24 h and stored for 7 days at 20 °C and 95-100% RH.

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