EFFICACY OF GASEOUS OZONE IN COMBINATION WITH VACUUM COOLING AND PRE-WASHING FOR THE INACTIVATION OF *Escherichia coli* O157:H7 ON FRESH PRODUCE

THESIS

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ABSTRACT

The relationship between the consumption of fresh produce and reduced risk of cardiovascular diseases makes these products a necessary component of healthy diet. However, concerns have been raised because of increased disease outbreaks associated with the consumption of contaminated produce. The development of new and promising decontamination technologies is necessary to provide safe produce to the public and to prevent economic losses.

The objectives of this study were (i) to assess the efficacy of gaseous ozone in combination with vacuum cooling for the inactivation of *Escherichia coli* K12 and *E. coli* O157:H7 spot inoculated on leaves of baby spinach and Romaine lettuce, and (ii) to evaluate the efficacy of a pre-washing step, integrated into the gaseous ozone treatment, to enhance the inactivation of *E. coli* K12 and *E. coli* O157:H7 on baby spinach and cut Romaine lettuce leaves.

The efficacy of gaseous ozone in combination with vacuum cooling was evaluated for inactivation of *Escherichia coli* on ready-to-eat baby spinach and Romaine lettuce leaves. In a preliminary experiment, baby spinach leaves were spot inoculated with *E. coli* K12. Inoculated leaves were vacuum cooled (-28.5 in. Hg) and then treated with gaseous ozone at the following conditions: 1.5 g/kg ozone in gas mix at 10 psig

holding pressure for 30 min holding time. Compared to inoculated non-treated samples, oxygen only (control) and gaseous ozone treatment decreased the microbial populations 0.27 and 1.29 log CFU/g, respectively. Similarly, baby spinach leaves were spot inoculated with *E. coli* O157:H7 and treated at the same conditions described in the preliminary study. Efficacy of the treatment varied with the crop season. Reductions on baby spinach were 1.86 and 1.59 log CFU/g of *E. coli* 0157:H7 in January, 2011 and February, 2012 respectively, and these reductions were significantly higher than the average 0.97 log reduction obtained between May and September, 2011. The color of baby spinach leaves was comparable to non-treated produce. The same gaseous ozone treatment of hearts of Romaine yielded a reduction of 1.47 log CFU/g of *E. coli* O157:H7, however discoloration of the produce was evident. The effect of a fan in the treatment vessel also was evaluated. Fan-off treatments decreased inactivation of *E. coli* O157:H7 significantly compared to fan-on treatments.

The effect of pre-washing and gaseous ozone treatment for the inactivation of *E. coli* on baby spinach and Romaine lettuce was also evaluated. Baby spinach and Romaine lettuce leaves were dip-inoculated with *E. coli* K12 and washed with 1% dimethyl sulfoxide (DMSO) or sterile distilled water. Washed leaves were treated with gaseous ozone, with initial vacuum application (-28.5 in. Hg), at the following conditions: 2.0 g/kg ozone at 10 psig holding pressure for 30 min holding time. There was no significant difference between washing with water and washing with DMSO in terms of microbial inactivation. The various washing treatments did not show any significant difference from each other, nor were the combination treatments different from one another.

However, the combination gave significantly more reduction in the target from any washing alone treatments. Based on the results obtained from this set of experiments, a new washing equipment was constructed. At the same washing and gaseous ozone treatment conditions, efficacy of sterile distilled water, 2% PRO-SAN, and 200 ppm chlorine, alone or in combination with gaseous ozone, were evaluated. Chlorine or PRO-SAN alone decreased the microbial population 1.1 and 1.2 log, respectively. Chlorine and PRO-SAN followed by gaseous ozone treatment decreased the *E. coli* O157:H7 population 2.3 and 2.1 log, respectively, and microbial reductions were significantly higher than any other treatment. Environmental and health concerns related to chlorine increase the appeal of using PRO-SAN alone or in combination with gaseous ozone for the production of microbiologically safe, fresh produce.

DEDICATED TO

My parents;

Emine and Hikmet,
&
My brothers and sister, my lovely nephews and nieces

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Literature Review

OZONE

Physicochemical features

Ozone (O_3) is a triatomic molecule formed from tree oxygen atoms. It has a molecular weight of 48 g/mol and is a highly reactive form of oxygen with an oxidation potential of 2.07 eV. Ozone is formed naturally in the atmosphere and synthetically in industrial settings. Gaseous ozone has a characteristic bluish color at high concentrations and it is colorless at low concentrations. It also has a distinctive, pungent odor. The odor of ozone is detectable by human nose even at the very low concentrations (0.02ppm) (Horváth et al., 1985).

Some selected physicochemical properties of ozone are listed in Table 1.1. The density of ozone is 2.14 g/L which makes it heavier than air which has a density value 1.26 g/L. These values are important when designing ozone treatment chambers for use with ozone as well as ozone/air mixtures, since even ozone distribution at any place in the treatment vessel is desired.

Table 1.1. Selected physicochemical features of ozone (Horváth et al., 1985; Ullmann, 2005)

Features of ozone	**Numeric values**
Melting point (1 atm)	-192.5^{o}C
Boiling point (1 atm)	-111.9^{o}C
Density (gaseous ozone)	2.14 g/L
Molecular weight	48 g/mol
Oxidation potential	2.07 (eV)

The oxidation potentials of some selected chemicals are listed in Table 1.2. The oxidation potential of gaseous ozone is 2.07 eV, which is the second highest oxidation potential after fluorine, making it good candidate as a sanitizer for microbial inactivation.

Table 1.2.Oxidation potential of ozone and other chemicals (Ullmann, 2005)

Chemicals	**Relative oxidation potentials (eV)**
Fluorine	3.06
Hydroxyl radical	2.80
Ozone	2.07
Hydrogen peroxide	1.77
Hypochlorous acid	1.49
Chlorine	1.36

Ozone Solubility in water

Aqueous ozone has been used by industry to treat drinking water, sewage, for the microbial decontamination of food processing equipment and for treatment of food itself. In order to generate aqueous ozone, gaseous ozone has to be dissolved in a liquid medium. The solubility of gasses in a liquid medium is described by Henry's Law, which states that the amount of gas dissolved in the liquid at a specific temperature is proportional to the gas pressure over the liquid medium. Ozone solubility is affected by many variables, as has been reported by many researchers. In the water, ozone is more soluble than nitrogen and oxygen but less soluble than carbon dioxide and chlorine (Khadre et al., 2001). The basic conditions that affect ozone solubility can be classified as follows.

- The temperature of the water
- The organic and inorganic content of the liquid medium
- The pH of the environment
- Size of the gaseous ozone bubbles formed in the water

Ozone is more soluble in water at low than at higher temperatures. Changes in the solubility of ozone as a function of water temperature are listed in Table.1.3.

Table 1.3. Solubility of ozone in water at different temperatures (Ullmann, 2005)

Temperature (°C)	**Ozone solubility in water (kg/m^3)**
0	1.09
10	0.78
20	0.57
30	0.4
40	0.27
50	0.19
60	0.14

In addition to the temperature of the water, pH affects ozone solubility. Higher pH reduces ozone solubility. Organic material present in the medium where aqueous ozone is created decreases its solubility so that the more pure the medium is, the more ozone can be dissolved. This was shown in studies using different types of water such as distilled, double distilled, and tap water (Kim, 1998). For tap water, ozone solubility was lower because of its high pH, compared to that of distilled water. Ozone solubility is also dependent on the size of bubbles formed in the aqueous solution. Finally, good agitation can create higher solubility of ozone in the medium (Katzenelson et al., 1974).

Ozone stability

The stability of ozone is described by the half-life. Based on studies about the stability of ozone, it can be concluded that ozone is not exceptionally stable, especially in the aqueous phase. The gaseous phase is relatively more stable, but must be generated on site prior to use. As with solubility, the surrounding conditions also affect ozone stability.

At higher pH, ozone is less stable. For example, when ozone was dissolved in phosphate buffer at different pH values, solubility decreases when the pH increased from pH 5 to pH 9; at pH 9, no ozone was detected (Khadre et al., 2001).

Ozone decomposition

Decomposition of ozone to oxygen in the aqueous phase starts with the formation of free radicals in a chain reaction and follows a stepwise process of initiation, promotion, and inhibition steps. The decomposition rate is affected by many factors such as temperature, pH, and other environmental conditions. With increasing pH, the decomposition of ozone in water accelerates (Staehelin and Hoigné, 1985; Roth and Sullivan, 1981). It has been reported that above 30°C at pH 8, the decomposition of ozone was accelerated while at a pH <3, the decomposition rate of ozone was hardly affected (Sotelo et al., 1987). It was found that in the reaction of ozone with water, the initiation step involving the hydroxyl radical formation is the main cause for ozone decomposition (Sotelo et al., 1987).

Initiation step: The initiation step is based on the formation of radicals such as hydroxyl, hydroperoxide and superoxide-anion radicals, in a series of reactions. The substances causing increases in the reaction rate are called promoters. Hydroxyl ions, organic substances, UV light at 253.7 nm and hydrogen peroxide are examples of promoters (Vurma, 2009; Khadre et al., 2001; Staehelin and Hoigné, 1985).

Promotion step: Superoxide and hydroperoxides are generated by using the hydroxyl radicals. The substances accelerating the reaction rate are formic acid, primary alcohols,

aryl groups and glyoxyclic acid (Vurma, 2009; Khadre et al., 2001; Staehelin and Hoigné, 1985).

Inhibition step: Hydroxyl radicals are consumed but there is no reformation of superoxide anions. Scavengers slow down this chain-reaction. Scavengers react with hydroxyl radicals and no further reaction with ozone occurs. Some well-studied scavengers are formic acid, bromide ion, acetic acid, carbonate ion, phosphate ion, and tert-butyl alcohol (Tomiyasu et al., 1985; Vurma, 2009; Khadre et al., 2001; Staehelin and Hoigné, 1985).

Ozone generation

Ozone is formed in the atmospheric layers of the stratosphere and troposphere or produced in an industrial set-up. In the atmosphere, ozone is produced from the interaction of UV radiation from the sun with oxygen in the atmosphere. The basic mechanism of ozone formation involves breaking an oxygen molecule (O_2) by solar UV radiation into two, single oxygen atoms which are highly reactive. These single oxygen atoms react with another oxygen molecule (O_2) to produce ozone (O_3) (Horváth et al., 1985).

Of the first and second atmospheric layers closest to the earth's surface, the vast majority (~90%) of ozone is located in the stratosphere. Ozone in this layer prevents harmful UV light (B) from reaching the earth's surface. However, the ozone formed in excess in the troposphere has a negative effect since it is highly reactive and can damage living organisms (NOAA, 2010).

Electrical discharge method

There are different ways to produce ozone synthetically such as corona, spark, arc, and others. The corona electrical discharge method, capable of producing high concentrations of ozone, is mainly used in industrial applications (Horváth et al., 1985). The simple corona ozone generator includes two high voltage electrodes (one higher tension, one lower tension in addition to a ground electrode) with a dielectric material to separate the electrodes from the discharge gap. When the high voltage of alternating current is applied, an electrical discharge forms in the discharge gap between the two electrodes which excites the electrons of oxygen. Excited oxygen electrons split the oxygen molecules to oxygen atoms and these oxygen atoms react with the residual oxygen in the discharge gap to form ozone. In order to effectively generate ozone, the ozone generator has to be supplemented with a cooling unit to remove heat from the generator. Dry air or pure oxygen is preferred for ozone production in this method. When using pure oxygen, 6-12 % ozone is obtained whereas the ozone output is only 3-5 % in the case of dry air (Ozonia, 2009).

Photochemical method

Ozone can also be generated by using UV radiation to break the oxygen molecule. Photochemical methodology is based on splitting oxygen molecules into two oxygen atoms by using UV radiation ranging from 140 to 190 nm, then combining reactive oxygen atom with oxygen molecule to form ozone molecule. Reports of ozone generation using high transmission ultraviolet lamps at 185 nm found the levels of ozone produced to be very low (Ewell, 1946).

Chemical methods

Ozone can additionally be generated as a result of chemical processes. The chemicals that catalyze the decomposition of hydrogen peroxide to ozone include bismuth, chlorates, bromates, and iodates together with metal oxides or peroxides. In these reactions, the formation of ozone is based on the disassociation of oxygen rich compounds. High concentrations of ozone can be formed during the reaction of fluorine and water at the room temperature or below (Horváth et al., 1985).

Electrochemical method

This method was developed and patented by the Lynntech Company to produce ozone from water. This generation takes place by splitting water into its constituents, hydrogen and oxygen, via electrolysis and then removing hydrogen from the system. A large amount of ozone can reportedly be produced using this method. The system components include an anode, gas diffusion cathode, and proton exchange membrane (Murphy and Hitchens, 1999).

Other methods reported to generate ozone are thermal methods, cold plasma, and chemo nuclear methods (Horváth et al., 1985).

Ozone measurement

The measurement of ozone concentration is challenging since there are many different conditions which impact it and methods by which measurement is carried out. The method should be chosen based on the physical state of ozone, gaseous or aqueous, and whether the ozone is in a pure solution or a solution with high amounts of organic

material that could be easily oxidized by ozone. There are many methods for the measurement of ozone and some of the common ones are explained below.

Iodometric determination

Stoichiometry shows the quantitative relationship between the reactants and products in a chemical process. The iodimetric method for ozone concentration measurement was formerly based on ozone/iodine stoichiometry (1:1). However, with new information, it was reported that many factors affect this ratio and the value can vary from 0.65 to 1.5 (Rakness et al., 1996). When gaseous ozone is bubbled through a solution of potassium iodide (KI), iodide ions become oxidized by ozone and iodine is formed as a result of this oxidation process. After the bubbling of ozone is stopped in the solution, the pH is maintained at or below 2 with sulfuric acid (H_2SO_4). The analysis is completed by titration of released iodine with known standard of sodium thiosulfate using starch as an indicator. Lack of reproducibility at low concentration and side reactions are reasons to consider the use of other methods (Horváth et al., 1985).

Indigo method

The indigo method was commonly used to measure ozone concentration in the air when Bader and Hoigne (1981) showed that it could be used to measure ozone concentration in aqueous solutions precisely as well. The basis of the method is to use sulfonated indigo dye which has one c=c bound that can easily react with ozone and the dye becomes colorless. The measurement is based on the color change of indigo observed at 600 nm and pH 4. There are small deviations if the temperature is higher. The method

is most suitable for measuring ozone concentrations between 0.005 and 30 mg/L. Similarly, the measurement of ozone in the gaseous phase could be done by using a UV spectrophotometer at 258 nm (Vurma, 2009).

Regulations and safety concerns

Ozone has been used to treat drinking water for more than a century in Europe. The approval of ozone came later in the United States. In 1982 ozone was approved as Generally Recognized as Safe (GRAS) substance for treatment of bottled drinking water. GRAS affirmation of ozone was declared for the treatment of food products in 1997 (FDA, 1997). There are other applications of ozone in the meat and poultry industries, but based on the definition from the United Stated Department of Agriculture (USDA) and Food and Drug Administration (FDA), the food additive petition must be submitted to the FDA to be able to use ozone in either meat or poultry. In 2001, ozone was approved as a secondary food additive which means that it is not directly added to the food but used in the process of reformation of the food. With this approval by FDA (2001), now aqueous and gaseous ozone are used as an antimicrobial agent in food products including meat and poultry. As opposed to radiation, there is no labeling requirement when food is treated with ozone. In addition to these regulations, the use of ozone is regulated by different governmental organizations such as the National Organic Program or Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), if it is to be used to treat pesticides or organic food.

One of the safety concerns related to the use of ozone relates to the treatment of drinking water. In the presence of bromide in water, ozone oxides these ions to bromate, which is harmful for humans (von Gunten, 2003). There are regulations establishing a maximum limit of bromate in bottled water of 0.01 mg/L (FDA, 2009b).

There are also health effects of ozone that can harm people who are working with it. Occupational Safety and Health Administration (OSHA) and FDA have limitations for ozone in the ambient work environment. FDA rules state that no ozone in the work environment is allowed above 0.05 ppm by volume. FDA and OSHA regulations are based on the time and exposure. Limits are set at 0.1 ppm and less than 2 hr of exposure or 0.05 ppm with the exposure less than 8 hr (Gonçalves, 2009).

Foodborne diseases: Outbreaks, recalls and the role of government bodies

A "foodborne outbreak" is announced when two or more people get sick by the consumption of a contaminated food (CDC, 2011) and it might be caused by chemical, physical, or biological contaminants. The general responsibility of government agencies is to prevent, detect, and control foodborne outbreaks and illnesses.

Fresh produce associated outbreaks are of increasing concern in the United States. From 1973 to 1990, a total of 190 produce associated outbreaks occurred with a reported 16.058 illnesses, 598 hospitalization, and 8 deaths. In this time frame, the number of ill people per outbreak increased from 21 to 43 while the increase in the number of outbreaks went from 2 to 16 per year. The consumption of fresh produce per capita increased 24%, from 573 pound in the 1970's to 711 pound in 1990's (Sivapalasingam et

al., 2004). The five years following 1997, produce related outbreak numbers increased to 279 with 10,533 hospitalization and 7 deaths (McGlynn et al., 2009). A recent study estimates the cost of the outbreaks in Ohio is around 1- 7 billion dollars with an average of 4.1 billion per year which equates to a cost or 91-624 dollar for each resident (Scharff et al., 2009).

The Center for Disease Control and Prevention (CDC) states that foodborne diseases cause 48 million people to get sick with 128,000 hospitalization and 3,000 deaths in the United Stated each year (CDC, 2011). It has been estimated that reducing foodborne diseases just 10% will help keep five million Americans safe from illnesses each year. CDC predicts that challenges in food safety will keep increasing because of the changes in the environment, food production, and supply. Additionally, there might be some new emerging microbial strains of pathogens which have never been associated with outbreaks before and new foods which have not typically been associated with health risks through microbial contamination are also becoming risk factors. Recently, there has been an increase in the number of outbreaks from fresh produce related contaminated with pathogens such as *Escherichia coli* O157:H7, *Salmonella* and *Listeria monocytogenes*. CDC estimates for *E. coli* causes 73,000 infections and 61 deaths per year.

In 2006, the outbreaks related to consumption of *E. coli* O157:H7 contaminated baby spinach leaves caused 205 confirmed cases of illnesses and 3 deaths. In the same year, two more outbreaks resulting from consumption of *E. coli* O157:H7 contaminated

lettuce lead to more than 150 illnesses and more than 50 hospitalizations. Even more recently, additional outbreaks occurred from the consumption of produce contaminated with *E. coli, Listeria* and *Salmonella* as detailed in Table 1.4. However, there were many additional cases of produce contamination with enteric pathogens that did not get reported as outbreaks but were identified as a result of improved traceability and surveillance of the pathogens in fresh produce. Selected produce recalls are listed in Table 1.5. These improvements allow producers to prevent outbreaks before contaminated produce reaches the consumers. The FDA website is regularly updated as to what produce were recalled due to the potential or confirmed contamination with pathogens.

Food safety and government agencies

The Center for Disease Control and Prevention (CDC) is one of the main organizations under the United State's Department of Health and Human Services (HHS). The Food and Drug Administration (FDA) is also an important organization under this government body. Additionally, another well-known food safety agency is Food Safety and Inspection (FSIS), which is a part of the United States Department of Agriculture. While the FDA and USDA are the main regulatory agencies, CDC is a non-regulatory agency.

The FDA and USDA (FSIS) are regulatory agencies and are responsible for food safety issues, factory inspection, and law enforcement. However, CDC is responsible for disease watch, detection of outbreaks, investigations, and educating the public. From a

general perspective, CDC, FDA and USDA (FSIS) work together to ensure the food safety together with local health departments.

Table 1.4. Selected disease outbreaks associated with consumption of contaminated leafy greens

Date	Produce	Microorganism	Number of cases	Hospitalization/ death	Reference
September, 2006	Baby spinach	*Escherichia coli* O157:H7	205	3 deaths	FDA, 2007a
Nov.- Dec., 2006	Shredded lettuce	*Escherichia coli* O157:H7	71	53 hospitalization	CDC, 2006
Nov.- Dec., 2006	Lettuce	*Escherichia coli* O157:H7	81	26 hospitalization	FDA, 2007b
April, 2008	Jalapeño, serrano peppers and tomato	*Salmonella* Saintpaul	1442	286 hospitalization / 2 possible deaths	CDC, 2008
February, 2009	Raw alfalfa sprouts	*Salmonella* Saintpaul	235	Hospitalization (3% of cases)	CDC, 2009
May, 2010	Shredded Romaine lettuce	*Escherichia coli* O145	30	12 hospitalization	CDC, 2010
June, 2011	Cantaloupe	*Salmonella* Panama	20	3 hospitalization	CDC, 2011a
April- July, 2011	Alfalfa and spicy sprouts	*Salmonella* Enteritidis	25	3 hospitalization	CDC, 2011b
May- June, 2011	Sprouts	*Escherichia coli* O104:H4	3842	855 hospitalization/ 53 deaths	Beutin & Martin, 2012
September, 2011	Cantaloupe	*Listeria monocytogenes*	146	142 hospitalization/ 30 deaths	CDC, 2011c
December, 2011	Romaine lettuce	*Escherichia coli* O157:H7	60	30 hospitalization	CDC, 2011d

Table 1.5. Recall of fresh produce initiated by the companies as a result of positive (+) sampling or possibility of pathogen contamination of the produce*.

Date	**The produce item recalled**	**Microorganism**
December, 2011	Sprouts	*Listeria monocytogenes* (+)
December, 2011	Fresh Spinach	*E. coli* O157:H7 (+)
November, 2011	Bagged Salad Products (Romaine Lettuce)	*E. coli* O157:H7 (possible)
October, 2011	Spinach	*Listeria monocytogenes* (+)
September, 2011	Organic Grape Tomatoes	*Salmonella* (+)
August, 2011	Organic Baby Spinach	*Listeria monocytogenes* (+)
June, 2011	Salad	*Listeria monocytogenes* (possible)
April, 2011	Spinach	*Salmonella* (+)

*Information source: http://www.fda.gov/Safety/Recalls/

Bacterial internalization

The increase in the number of outbreaks related to the consumption of fresh produce compels researchers to find out why outbreaks are on the rise despite the fact that there are more precautions than ever to prevent such outbreaks. Besides better outbreak surveillance methods and increased consumption of fresh produce, there are some challenges that make produce sanitization harder with conventional methods. It has been proven that bacteria can infiltrate the internal part of fresh produce through various suggested mechanisms, and this internalization of bacteria makes them more resistant to conventional sanitization methods such as chlorinated and ozonated water washing (Saldana et al., 2011; Takeuchi and Frank, 1999). Conventional methods are used to inactivate microorganisms attached to the surface and are not effective against internalized bacteria. Some good reasons to study the internalization of bacteria are to figure out the routes of internalization so that they could be prevented, and to help develop a new, promising sanitization method which is effective to kill internalized microorganisms.

Researchers studied the effect of light and its chemical stimulus of plants for its impact on internalization of *Salmonella enterica* via stomata in lettuce. To study the effect of light on *Salmonella* infiltration of the lettuce, leaves were soaked in a saline solution in a tub and then kept either in the dark, under a laboratory style neon light, or under a high intensity bulb. The pre-conditioned lettuce leaves were inoculated with a GFP labeled *Salmonella* suspension containing 10^8 CFU/ml. These tubs were kept at the same chosen conditions for 2 hours. To observe if bacterial internalization occurred,

confocal laser microscopy was used. The results showed that stomata, the opening including two guard cells that manages air exchange between the environment and the plant, is one of the main routes for bacterial internalization. It was found that in the presence of light, bacterial attachment and internalization occurred; however, in dark conditions, bacteria were just attached to the surface. The study concludes that when photosynthesis takes place, the stomata open, which increases bacterial internalization. The products of photosynthesis make the internal environment attractive for bacteria. Studies have also shown that the use of a chemical substance, fusiocin, keeps stomata open even in the dark conditions; however, results showed that the internalization is still lower which suggests the role of photosynthesis in internalization beyond simply keeping the stomata open (Kroupitski et al., 2009).

In the other type of internalization studies, researchers focused on infiltration from soil and from leaves. It was found that when bacteria were introduced to the leaf from the phyllosphere, they were able to survive for 14 days with increasing numbers and area of colonization. However, when the same strain of GFP-labeled *E. coli* O157:H7 was introduced to the plant from the rhizosphere, bacteria were detected in only a few spinach leaves in very low numbers which decreased with time. It was also shown that different cultivars of baby spinach have different surface characteristics that might cause a different degree of colonization of *E. coli* O157:H7 (Mitra et al., 2009). Moreover, researcher stab inoculated the bacteria to the internal part of the leaf where they survived for 2 weeks (Mitra et al., 2009). In another study, spinach seeds were inoculated and held for 42 days, after which, the bacteria attached to the surface and the internalized bacteria

were recovered from the plant. Bacteria were ably recovered from the surface, but no internalized bacteria were found. Effects of cultivation method, soil or hydroponic, were assessed and it was concluded that when surface sterilized seedlings were transferred to the hydroponic growth medium contaminated with low numbers of microorganisms, the pathogen was recovered from roots which were surface sterilized. This shows that the microorganism has more affinity for the plant in hydroponic medium rather than in soil. Researchers concluded that the competition between the natural microflora in soil and the pathogen can cause prevention of root internalization of the microorganism (Warriner et al., 2003). Another study was done using 3 different cultivars of lettuce inoculated with *E. coli* 0157:H7 on the lettuce leaves, roots, and soil to determine the presence or absence of microorganisms in the selected environment. The overall result was different from previous studies and claims that there is no internalization that occurs regardless of the cultivar of lettuce, the maturity of plant and the strain of microorganism. It was mentioned that the upper part (abaxial) of the leaves are more prone to bacterial attachment compared to the lower (adaxial) part and also showed a longer survival period. Another study claims that when soil is contaminated with *E. coli* 0157: H7, no internalization of bacteria was observed on the leaves, and just one sample was found to contain bacteria in the root of spinach. The internalization is infrequent and if it happens, the bacteria will not survive more than 7 days (Erickson et al., 2010). These results are consistent with the internalized bacteria recovered from root tissue but not shoot tissue of the spinach plant grown in soil inoculated *with E. coli* O157: H7 (Sharma et al., 2009). For lettuce plants, it was shown that soil, irrigation water, and manure amended soil

contaminated with low level of *E. coli* O157:H7 lead to internalization. This work did not include direct plating, but enrichment was followed (Mootian et al., 2009).

Research about the initial population level and plant age showed that *E. coli* O157:H7 was attached to the lettuce leaves at higher populations when the inoculum size was greater. It was also observed that more attachment occurred at the cut surfaces of lettuce leaves (Takeuchi and Frank, 1999). In another study, it was observed that in greenhouse setups for spinach, a greater initial population increased the survival rate on leaves during cultivation. Surface contamination occurred but only in leaves more than 3 weeks old. From 120 spinach samples, only one resulted in internalization (Pu et al., 2009).

Some steps in fresh produce processing can cause the infiltration of bacteria. Vacuum cooling is one of the most common methods used in the California fresh produce industry to remove field heat. Researchers conducted a study to figure out if vacuum cooling could cause internalization in Romaine lettuce. It was concluded that vacuum cooling causes greater and deeper internalization of GFP labeled *E. coli* O157:H7 around the stomata compared to non-vacuum cooled samples. It is suggested that as a result of negative pressure, the stomata open and cause infiltration. Releasing pressure from the vacuum to atmospheric pressure can also increase internalization (Li et al., 2008). Other reports show that when the produce and washing environments have different temperatures than in the processing environments, internalization can occur (Zhuamg et al., 1995).

The genetic material responsible for the *E. coli*'s pathogenesis was studied to compare it to its effect on plants. It was concluded that T3SS, pili, and flagella which are responsible for bacterial attachment on human mucosa are also responsible for attachment, colonization, and internalization of bacteria on leaves (Saldana et al., 2011). Curli producing *E. coli* strains are able to attach to the leaves at higher numbers. There is a hydrophobic cuticle on the leaf surfaces that makes attachment of curli producing *E. coli* O157:H7 possible, since curli are also hydrophobic (Patel et al., 2011).

The damage and lesions on the produce also increases attachment and internalization of bacteria into the leaves. Harvest and processing of produce must be done with care to prevent tissue damage and possible contamination and growth of the microorganisms (Brandl, 2008).

Fresh produce industry: Challenges and utilization of ozone

Fresh produce is one of the most growing segments of the food industry. The increase in the market has brought other issues to attention. With the increase in the consumption of fresh produce, the number of outbreaks associated with it increased rapidly. New strains of foodborne pathogens emerged and it has become challenging to assure clean final produce, which is fresh and at the same time free from pathogens. Fresh produce are minimally processed fruit and vegetables, and as such, it does not undergo harsh and complicated chemical and physical procedures to make it totally safe. Fresh produce are perishable food commodities. It is common nomenclature to specify "fresh-cut produce," when appropriate. These are value-added products that undergo

cutting which can lead to microbiological and chemical changes affecting the shelf life and overall quality of the produce. There is also the leafy greens category; these can be unprocessed raw produce or value added cut produce. This group includes iceberg, Romaine lettuce, and spinach, but does not include herbs such as cilantro and parsley.

The consumption of fresh produce is in sharp rise. Government, social, and commodity organizations support the consumption of fresh or minimally processed food commodities. It was suggested by the Food and Agriculture Organization (FAO) of the United Nations that consumers eat "5-a-day," a campaign promoting five servings of fresh produce in a day. Fresh produce consumption is also supported by government in the schools since it is believed that with the consumption of fresh produce, there is an increased health benefit. Additionally, the eating habits of people have changed and people want to get ready-to-eat foods rather than prepare them at home. With the globalization and improvements in processing and distribution technology, it is easier now to get produce from far places and even from other countries year round.

There were 82 reported outbreaks of foodborne illness between 1996 and 2008. Leafy greens were responsible for 28 of these outbreaks with 949 illnesses and 5 deaths. *E. coli* O157:H7 caused 85.7% of the leafy green outbreaks (FDA, 2009a). In the 2006 baby spinach outbreak, 3 people died and 205 people were hospitalized. An economic loss as much as 100 million dollars including the loss of consumer confidence in the producers was estimated (Blake, 2011).

An increase in the number of outbreaks lead researchers to search for the possible contamination sources and to explore promising technologies to make produce safe for consumption. Fresh produce can be contaminated preharvest or postharvest by handling, processing, and in the kitchen (Beuchat and Ryu, 1997). Possible pre-harvest contamination sources include using manure instead of chemical fertilizers, labor handling, sewage near to the farming area, wild animals, and contaminated waters. After harvest, equipment used for harvesting and labor handling could be contributing to the contamination (Beuchat and Ryu, 1997). Even though processing steps are aimed at decreasing the microbial load and making it safe and extending the shelf life, there is a possibility of contamination during processing. The equipment used is a reservoir for the microorganisms and also water used for washing might become contaminated. The storage temperature and blades used to cut produce could contribute to the proliferation of microorganism on the produce if they are not handled with caution (Beuchat and Ryu, 1997). The recent outbreaks related to leafy greens increased the care of producers at every step of fresh produce production to prevent any possible changes that will affect product quality and public health. With the new Food Modernization Act, now FDA has a right to initiate the recall process if needed and more documentation from the farmers and companies is required to prove that the produce is handled appropriately (Hamburg, 2009). After many outbreaks related to juice, FDA required HACCP implementation for production with at least 5 log reduction regardless of the method used to achieve this goal (FDA, 2002)

For fresh produce, FDA issued the "Guide to Minimize Microbial Food Safety Hazards for Leafy Greens." After the spinach and lettuce outbreaks in 2006, the leafy green industry, from farmers to consumer, came together and established their own structure to manage the operations, from research to education, to meet the food safety requirements established for leafy greens. It is estimated that 90% of fresh produce is harvested in Arizona and California. These states have their own structures under the Leafy Greens Marketing Agreements. These structures established rules for everyone from small size farmers to big producers and government officials. Appointment to these structures is voluntarily but once gained, producers have to follow dictated rules. Companies are certified by these organizations and are investigated by people educated and licensed by government organizations. If producers do not follow the guidance, they might be de-certified. These organizations look for ways to make fresh produce safe via research or education of the people involved in any step of fresh produce related activities and suggestions are submitted to the USDA for approval (Blake, 2011).

Leafy Greens Processing

To make leafy greens safe, the processing steps from harvesting to the table must be well understood by researcher, producers, and all people involved in or contributing to leafy greens production. Basic fresh produce processing steps and unit operations with timeline are listed in Figure 1.1.

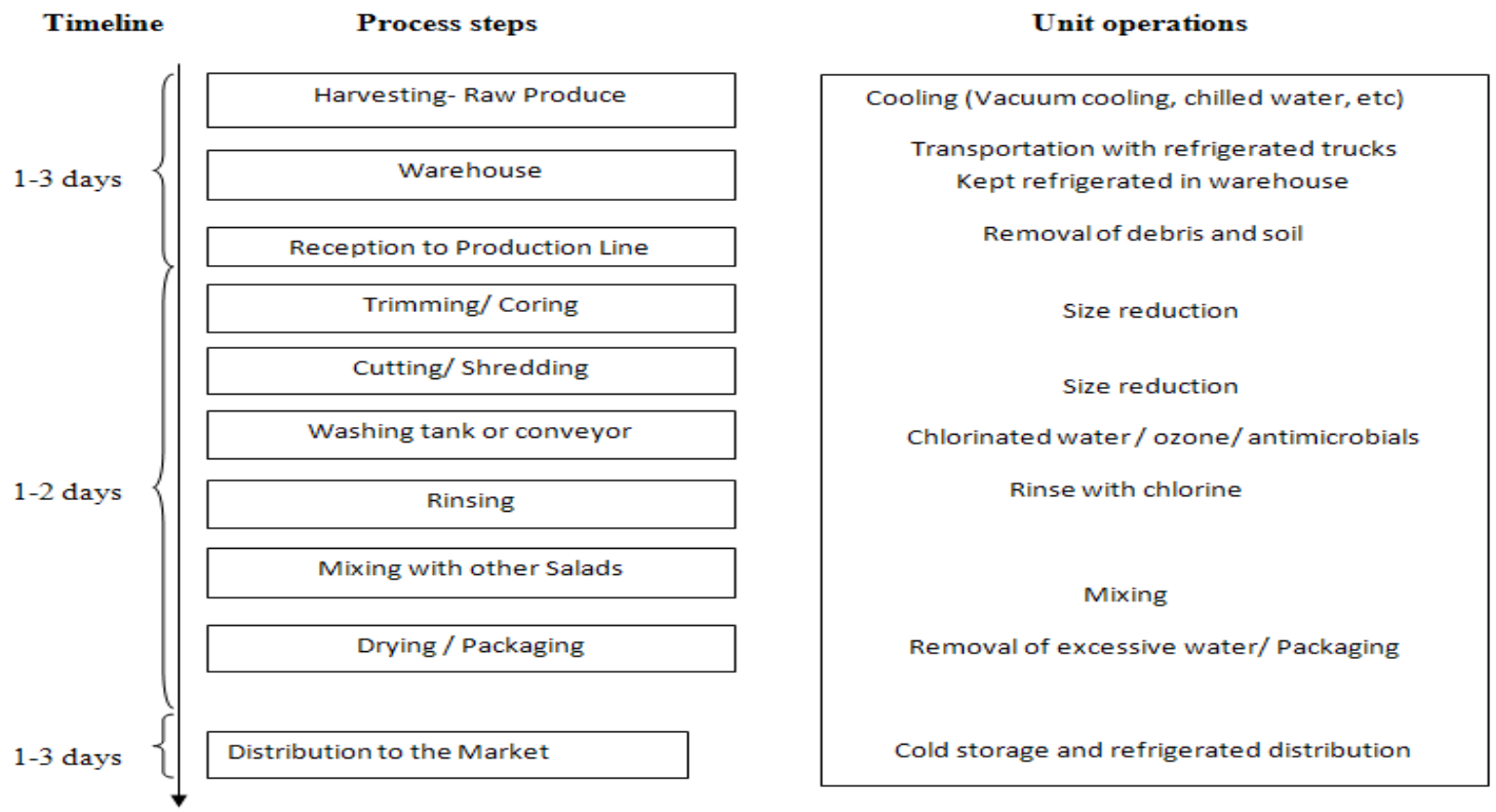

Figure 1.2. Journey of fresh produce from harvest to market (Strickland et al., 2007; Kader, 2002)

Once fresh produce is harvested, the changes in the quality and quantity are to be expected. The produce loss after harvest is reported to be up to 25 and 50% for developed and developing countries, respectively (Kader, 2002). The most important factor affecting microbiological quality and shelf life of produce is temperature. After they are harvested, leafy greens start rapid respiration as a result of catabolism of organic components: protein, lipid, and carbohydrate. During respiration, they release energy that causes an increase in the temperature of the leaves where microorganisms most likely are located. The change and loss of organic material causes deterioration of food in terms of appearance and aroma. High temperature also promotes microbial growth and reduces the shelf life of the produce.

The reactions after harvesting that causes changes on the produce can be slowed but not completely prevented. Best practice is to reduce the temperature as much as possible, as quickly as possible. Vacuum cooling technology has been used for a long time for the cooling of lettuce. This technology works best when the produce is porous and surface area to volume ratio is large. The principle of the vacuum cooling is that once the vacuum is pulled in a closed system, it reduces the pressure in the system. The lower the pressure is, the lower the boiling point of water inside the produce. Water evaporates at the low temperatures and carries heat form the environment into the surroundings. The closed system is usually cooled before starting the vacuum cooling process so that heat loss occurs mostly from the produce rather than the treatment vessel itself. In the leaf, a 10°C temperature increase causes a 2 or 3 times shorter shelf life (Kader, 2002). However, during the vacuum cooling operation, every 5°C decrease in the leaf

temperature caused 1% water loss. Loss of water results in produce weight loss and an undesirable appearance and texture even though a decrease in the lettuce temperature from ambient to 1°C might increase the shelf life by 14 days (Artes and Martinez, 1994). Water loss should be compensated for during processing and it is made by introducing water inside of the vessel during treatment. The rapid cooling of produce, afforded by applying vacuum, is essential for better microbial quality and longer shelf life.

Another critical step in fresh produce processing is cutting. Bacteria attach abundantly to cut surfaces since the leaves release organic materials and nutrients from these openings that attract bacteria. Generally speaking, the sharper the blade, the less the chance of encouraging microbial growth due to nutrient release.

Washing is done by flume water or spraying of sanitizer or water. Chlorine and aqueous ozone are the most commonly used sanitizers for fresh produce washing. Up to 200 ppm of chlorine in the washing solution is typical. Microbial reduction caused by water washing is comparable to that obtained by chlorine washing. Chlorine is capable of inactivating microorganism and preventing cross contamination in wash water. However, there is a concern about the by-products formed during chlorination. Chlorine reacts with organic material in the water and forms trihalomethane (THM) which is a carcinogenic substance. Ozone is a promising alternative which can be used instead of chlorine and has many advantages. Ozone does not form carcinogenic material except if high concentrations of bromide are present in the water. Ozone decomposes to oxygen which is a non-toxic gas. The germicidal activity of ozone is broader than chlorine with the

capability of ozone inactivation of the waterborne parasites, *Cryptosporidium and Giardia,* which are resistant to chlorine treatment.

Escherichia coli O157:H7

Escherichia coli is a member of the *Enterobacteriaceae* family. It is a facultative anaerobe, gram negative, and rod shaped bacterium. Most of the strains of this genus do not cause illnesses; however, they are used as an indicator of fecal contamination in water and food. *E. coli* can be classified in different ways. Biotyping is based on biochemical tests. Serotyping is based on the antigens that the microorganisms carry. Two types of antigens are defined for *E. coli*; these are O and H antigen. The O antigens are associated with the lipopolysaccharide in the outer membrane and there are 170 known O antigens. The H is a flagellar antigen and more than 50 known. Serotyping gives information about the flagellar and somatic antigens that microorganisms have. Another classification is based on the virulence factors. Virulence factors give information about the symptoms of the diseases and what causes the disease. Based on their virulence factors, *E. coli* can be classified as enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteroaggregative (EAggEC) (Jay et al., 2005). *E. coli* O157:H7 is the most common serotype known under the Enterohemorhagic *E. coli* (EHEC) with the symptoms of hemorrhagic colitis in humans. This microorganism was defined as a foodborne pathogen in 1980's (Eppinger et al., 2011). As opposed to the other *E. coli* strains, *E. coli* O157:H7 is not able to ferment sorbitol and does not grow at 44.5°C where other *E. coli* strains grow well. It can also survive in acidic conditions, as indicated by its recovery from an apple cider outbreak. It also can tolerate high salt

concentrations. They are mostly found in the gut of ruminants such as cattle, deer, and goats. Cattle are generally the host for EHEC affecting humans, but EHEC does not affect cattle (Beutin, 2006). *E. coli* O157:H7 is able to produce Shiga toxin1 (stx_1) and Shiga toxin2 (stx_2) as virulence factors. Shiga toxin1 is similar to the one produced by *Shiegellla dysenteria* (Mead and Griffin 1998; Saldana et al., 2011). Besides stx_1 and stx_2, hemolysin and intimin are also other virulence factors. It was found that Shiga toxin2 is more associated with severe human illnesses (Neupane et al., 2011). There are other microorganisms producing Shiga toxins besides *E. coli* O157:H7. They are usually classified as non-O157 STEC. It is believed that less than 100 cells of *E. coli* O157:H7 is capable of producing disease. The incubation period is 3 to 4 days, but it could be as short as 1 day or as much as 10 days. The first symptom of the disease caused by *E. coli* O157:H7 is diarrhea. If the disease continues then bloody diarrhea, thrombocytopenic purpura (TTP), and hemolytic uremic syndrome (HUS) might develop. TTP and HUS are very late steps of the diseases that can cause kidney failure and death. TTP affects the nervous system but in the case of enterohemorhagic infection, there are similar symptoms to HUS (Mead and Griffin, 1998). Researchers have to follow safety precautions when dealing with this pathogen. For lab studies, a surrogate of EHEC that carries the green fluorescence gene is often used as an alternative to the pathogenic bacterium. This microorganism fluoresces under UV light so that differentiation of the microorganism is easier in the presence of natural microbiota found on food samples. There was no difference in the growth pattern of this GFP microorganism compared to the parental strain in terms of microbial growth kinetics and survival (Ma et al., 2011).

Mode of ozone action

Ozone is highly active against bacteria, bacterial spores, fungi, protozoa and viruses in its aqueous and gaseous form. Staehelin and Hoigné (1985) have described the reaction of organic and inorganic matter with ozone in one of the two ways. One of these ways is the direct reaction of molecular ozone with a compound and the other one is the reactions of ozone decomposition products, such as hydroxyl radicals, with organic and inorganic compounds. The microbial inactivation with ozone generally follows the reaction of ozone with cellular components like the outer coat of bacterial spores, enzymes inactivation, and the destruction of nucleic materials (Khadre et al., 2001). Inactivation variability is further described below.

Bacteria

Broadwater et al. (1973) studied the inactivation kinetics of *E. coli*, *B. cereus*, *B. megaterium*, and spores of *B. cereus* and *B. megaterium* in reponse to ozone treatment. The researchers found that the concentration of ozone required to kill these three microorganisms was 0.12 mg/l, 0.19 mg/l, and 0.19 mg/l for *B. cereus*, *E. coli* and *B. megaterium,* respectively. In the ozone demanding organic medium, it is hard to kill microorganism since organic matter consumes ozone. A relatively low concentration of ozone is required to kill vegetative forms of microorganism in a saline solution. In this study, it was observed that when the cells of *E. coli* and *B. cereus* treated in their growth medium, it was not possible to kill them in 5 minutes even at ozone concentrations of 0.79 g/ml. After washing bacterial cells twice with physiological saline before treatment, ozone treatment almost eliminated cell populations within 5 minutes. In their vegetative

form, microorganisms are protected by their cell wall only. In their spore form they are protected with many outer layers such as a thick cortex and spore coat. It requires higher ozone concentration to inactivate the spore form of these microorganisms. Ozone treatment of spores of *B. cereus* and *B. megaterium* consumed 2.29 g/ml of ozone to inactivate the spore forms of both species.

In another study, different strains of *Bacillus* species were used to measure the sporicidal efficacy of gaseous ozone. In the first set of experiments, *B. subtilis* IF0 3134 and *B. cereus* spores were used and the treatment was run with ozone concentrations of 3 mg/L and at different relative humidity values. The findings from this study showed that the relative humidity is important for rapid inactivation of the spores. At conditions lower than 50% relative humidity, the decrease in the numbers of spores was not appreciable while over 80% relative humidity, the sporicidal activity was fast. Depending on the strain, *B. cereus* spores did not need any lag phase for inactivation; however, for the strains *B. subtilis*, there was a lag phase before an exponential decrease in the number of spores. The effect of ozone concentration was evaluated by using spores of *B. subtilis* NCTC 10073 and the D value, inactivation of 90% of microorganisms, was 68 minutes for 0.5 mg/L of ozone concentration and 13 minutes at the concentration 3 mg/L. Depending on the spore carrier, the glass fiber filter or filter paper, inactivation can be enhanced or decreased. Spores were more resistant against ozone than on filter paper (Ishizaki et al., 1986).

Kim and Yousef (2000) studied the effectiveness of ozone against spoilage and pathogenic microorganisms. *Pseudomonas fluorescens*, *E. coli* O157:H7, *Leuconostoc mesenteroides,* and *L. monocytogenes* were the microorganisms of interest. In this study it was observed that at first the initial inactivation of populations was fast and decreased with time. A 5 to 6 log microbial reduction was obtained for ozone concentration at 2.5 ppm for 40 second treatment time. *E. coli* O157:H7 was more resistant to ozone treatment whereas *L. monocytogenes* was less resistant. The inoculum size effected the efficacy of ozone,with the highest initial population of microorganism (7.7 log), the microbial reduction was less than 1 log, but once the initial population was reduced to half of this value (7.4 log), the reduction achieved was more than 6 log.

Khadre and Yousef (2001) observed the efficacy of ozone against different strains of *Bacillus* spores at the ozone concentration of 11 μg/L and they observed reductions ranging from 6.1 to 1.3 log. *B. stereotermophilus* had the greatest resistance while *B. cereus* had the least. Based on the outcomes of this study, it was suggested that *B. stereotermophilus* could be used to measure the efficacy of equipment sterilization methods. In their study, Restaino et al. (1995) compared the resistance of gram positive and gram negative bacteria against aqueous ozone treatment and observed that gram negative bacteria were more resistant to ozone than gram positives with the exception of *L. monocytogenes.*

Fungi

Beuchat et al. (1999) studied the effectiveness of ozone to inactivate conidia of *Aspergillus parasiticus* and *Aspergillus flavus* in phosphate buffer. These microorganisms produce afflatoxins, which are carcinogenic and mutagenic, as metabolites. At pH 7.0 and 5.5, *A. parasiticus* had D values of 2.08 and 1.71 log while *A. flavus* had D values of 1.72 and 1.54 log, respectively. Restaino et al. (1995) observed the effect of ozonated, deionized water to inactivate yeast and fungi. A population of 4.5 log of *Candida albicans* and *Zygosaccha romyces bailii* cells was inactivated instantly by ozone treatment. At the same conditions, but with 5 minutes of exposure, the inactivation of *Aspergillus niger* spores was less than 1 log with 0.188 mg/L ozone. In another study, the efficacy of ozone against the molds on oranges during a storage period was evaluated. *Penicillium digitatum* and *Penicillium italicum* were the target microorganisms inoculated on oranges stored at 12.8°C and treated with ozone at 0.72ppm for 14 days. Sporulation of mold was significantly inhibited in the environment which permitted ozone penetration and contact with the microorganism. The packages provided the opportunity for ozone to penetrate inside. No spores were observed while the control oranges had a 2 and 3 sporulation index for *P. italicum* and P. *digitatum*, respectively. These results show that ozone is effective at inhibiting sporulation of mold on oranges when it can penetrate the external packaging material in storage conditions (Palou et al., 2003). Ozone was also effective as a treatment for blueberries inoculated with *Rhizopus stolonifer* compared to SO_2 fumigation without any quality change observed (Sarig et al., 1996). Inactivation of 5 log *Aspergillus*, in peptone solution, requires 1.8 g/L ozone and

0.4 g/L for *Penicillium*. *Aspergillus spp.* requires higher ozone concentration to experience the same lethality observed in *Penicillium spp.* (Zhao and Cranston, 1995).

Protozoa

Recently, protozoan parasites have garnered interest since they can cause foodborne disease outbreaks due to the consumption of contaminated food and water. *Cryptosporidium, Giardia,* and *Cyclospora* are responsible for the largest parasite outbreaks. They might also be a problem with fresh salads but are more commonly reported in conjunction with drinking water contamination (Dawson, 2005). The consumption of *Cryptosporidium* contaminated water caused a huge outbreak in the US with 403,000 cases reported (Mc Kenzie et al., 1994). Since the resistance of these microorganisms against chlorine drinking water has been reported, recent studies have shown that ozone treatment can be an effective treatment (Erickson and Ortega, 2006; Dawson, 2005). It is reported that ozone is more effective than chlorine against these microorganism in water systems (Erickson and Ortega, 2006). When purified *Cryptosporidium parvum* oocysts were treated with 1 ppm ozone for 5 minutes, more than a 90 percent (1 log) increase in inactivation was achieved compared to chlorine dioxide and chlorine. *Giardia* cyst and *Cryptosporidium parvum* oocysts were 30 times more resistant to ozone, by comparison (Korich et al., 1990; Owens et al., 2000). It was suggested that ozone can be used to treat *Cryptosporidium parvum* oocysts in drinking water as opposed to chlorine dioxide and chlorine which might not achieve a complete disinfection (Korich et al., 1990). Finch et al. (1993) studied the resistance of *Giardia lamblia* and *Giardia muris* cysts to ozone treatment and found no significant difference

between the two parasites. The inactivation of *Cryptosporidium parvum* oocysts by ozone at 22°C in 0.05 M phosphate buffer with different pH values was investigated and the ozone concentration and contact time were found to have equal importance in inactivation. There was no significant difference between inactivation rates at pH at 6 and at 8 (Gyurek et al., 1999). The mode of action for ozone during the inactivation of ooycts and cysts was explained by using the same concentrations of ozone with different treatment times, and observing the structural changes on the cysts that resulted (Widmer et al., 2002). With the longer treatment times, changes occurred in the cysts' wall structure. An additional 60 seconds of exposure to ozone caused harsh protein degradation, cyst wall disappearance, and a trophozoite antigen. The effect of initial population was investigated and it was observed that 1.11 g/ml ozone was required to inactivate 10^4 oocysts in 6 minutes while 2.27 g/L was needed to inactivate 5×10^5 in 8 minutes (Peeters et al., 1989).

Viruses

Viruses cause the majority of foodborne outbreaks and inactivation of them with ozone has been studied extensively. Dawson et al. (2005) studied the survival of MS2, a surrogate of human noroviruses, on produce and in water at 4°C, 8°C, and 22°C. The survival of the virus in buffer after 9 days at 22°C was found. Less than a 1 log reduction was achieved in 50 days at 4°C and 8 °C. Similar results were observed with fresh produce washed with chlorine and the reduction was less than 1 log on produce and fruit. This shows viruses survive longer than the shelf-life of fresh produce and that chlorine treatment does not eliminate viruses. Ozone has been tested against viruses since chlorine

was not effective enough to remove MS2. Murine norovirus is mainly used as a surrogate of human noroviruses. Inactivation of murine noroviruses with ozone was studied at two different pH levels and temperatures. For the given conditions, regardless of pH (5.6 and 7) and temperature (5°C and 20°C), 1 mg/L ozone in demand free buffer treated for 2 min inactivated the 99% (2 log) of the murine norovirus (Lim et al., 2009). Ozone was also tested against bacteria and viruses at the same conditions, 20°C at pH 7. The resistance of microorganisms to ozone treatment was found in the following, decreasing order of *Bacillus subtilis* spores > *Legioneila pneumophila* serogroup 6 > hepatitis A virus (HAV) > *Escherichia coli* > poliovirus 1 (PV1). Bacteriophage f2 was tested with ozone and 5 to 7 log reductions were achieved with the ozone concentrations of 0.09 and 0.8 mg/L, respectively, in 5 seconds. Norwalk virus, Poliovirus 1, and Bacteriophage MS2 were treated with 0.37 mg/L ozone at 5°C for up to 5 minutes. The inactivation rate was higher than 3 log in 10 seconds of contact time for Norwalk viruses (Shin and Sobsey, 2003). Feline calicivirus [FCV] and murine norovirus [MNV], surrogates of human norovirus, were artificially seeded on green onions and lettuce and in sterile water and efficacy of ozone was evaluated. Ozone concentrations at 5 ppm were enough to kill more than 6 log $TCID_{50}$/ml in water and 2 log $TCID_{50}$/ml for produce samples. As opposed to the FCV, the inactivation of MNV was greater than 2 log $TCID_{50}$/ml after 1 min of ozone treatment. These findings show that food matrix, which is organic, plays major role for ozone activity against viruses. Khadre and Yousef (2002) investigated ozone inactivation of human rotavirus; 25 mg/L ozone reduce the infectivity of virus by more than 8 log $TCID_{50}$/ml.

Intervention technologies for fresh produce safety

Chlorine dioxide

Chlorine dioxide is a strong oxidizer and sanitizer, having an oxidation potential that is 2.5 times higher than the chlorine in a conventional hypochlorite solution. Additionally, chlorine dioxide does not produce harmful chlorine by-products. In Europe, use of chlorine dioxide for fresh produce treatment is not allowed; however, in the United Sates it could be used at up to 3mg/L in water for the treatment of whole produce and 1mg/L for tomatoes. Food industries use chlorine dioxide mainly in the treatment of drinking water. However, recent studies related to fresh produce safety revealed that bacterial internalization occurs in leafy greens and conventional sanitizers are not capable of reducing internalized microorganisms. Chlorine dioxide is a gaseous sanitizer with potential penetration into the internal parts of leafy greens to inactivate infiltrated microorganisms. Aqueous solutions of chlorine dioxide are as effective as chlorine without producing trihalomethanes (THM), carcinogenic by-products of chlorine treatment, and without inducing quality deterioration in the produce (López-Gálvez et al., 2010). Many studies have been conducted to test the efficacy of chlorine dioxide against pathogens in fresh produce. Mahmoud and Linton (2008) studied the efficacy of gaseous chlorine dioxide on the quality and shelf life of lettuce. Lettuce leaves were spot inoculated with *Escherichia coli* O157:H7 and *Salmonella enterica* and treated with 5 mg/L of gaseous chlorine dioxide at 22°C and at a relative humidity between 90 and 95%. A 5 log reduction, equivalent to FDA suggestions, was achieved for *E. coli* O157:H7 and *S. enterica* by using 14.5 and 19 minutes of treatment time, respectively.

During storage at 4°C for 7 days, the population of natural microflora was lower than those of control lettuce leaves and the treatment at given conditions negatively changed the visual quality of the produce. Kaye et al. (2005) studied the efficacy of gaseous chlorine dioxide to reduce the population pathogens on fresh-cut lettuce, cabbage, and carrot. A concentration of 4.1 mg/L of chlorine dioxide, and with relative humidity between 48 to 85% at 21±1°C, held for 30 minutes, caused a 4.42, 5.15 and 1.58 log reduction of *Salmonella* on cabbage, carrot and lettuce, respectively. The same treatment yielded a 3.13, 5.62 and 1.57 log reduction of *Escherichia coli* O157:H7 and 3.60, 5.58 and, 1.53 log reduction of *L. monocytogenes* on cabbage, carrot and lettuce. The log reductions achieved at this concentration of chlorine dioxide did not change the quality of lettuce and carrots adversely, but fresh cut cabbage did show some deterioration (Mahmoud et al., 2008). The efficacy of chlorine dioxide was also tested on cantaloupe spot inoculated with *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* Poona. After treatment with 5 mg/L of gaseous chlorine dioxide at 22°C and relative humidity 90-95% for 10 minutes, 4.6 and 4.3 log reductions of *Escherichia coli* O157:H7 and *L. monocytogenes* were obtained. Gaseous chlorine dioxide treatment reduced the initial microflora more than 2 logs and extended the shelf life of the cantaloupe from 3 days to 9 days without significant color change to the produce. When a similar chlorine dioxide treatment was tested on strawberries, a 4.7, 4.3 and 4.6 log reduction was achieved for *L. monocytogenes, Salmonella enterica*, and *E. coli* O157:H7, respectively (Mahmoud et al., 2007). Singh et al. (2002) observed the greater effectiveness of gaseous chlorine dioxide

over aqueous ozone in the treatment of shredded lettuce leaves, but the decolorization of lettuce leaves was observed with gaseous chlorine dioxide.

Irradiation

Radiation is a process that has a high potential for improving the safety of fresh produce. It utilizes ionizing radiation, in the form of x-rays, gamma rays, and accelerated electron beams. Although radiation had been used before to treat fresh produce in order to decrease spoilage microorganism and insects, the dosages allowed were low (FDA, 2008). FDA approved the use of radiation for fresh spinach and fresh iceberg lettuce at higher doses, up to 4 kGy. Additionally, irradiation treatment up to 8 kGy had been allowed for seeds in order to control microbial pathogens (Farkas, 2006). There are some differences between the types of radiation sources, but the effect that they cause is similar. While the penetration ability of electron beams is low, gamma and x-ray have higher penetration capabilities. X-rays are not as effective as other sources since they are generated from e-beams and in this process they lose 90% of their energy via heat loss (Farkas, 2006; Komolprasert, 2007). There are two mechanisms by which radiation inactivates microorganisms, direct and indirect effects. Direct damage takes place by the interruption of DNA sequence while indirect damage is caused by free radical formation in water via interaction of radiation sources (Farkas, 2006). Since vegetative cells include 80% water in their cytoplasm, the second mechanisms of radiation inactivation was more effective in microbial reduction (Gomes et al., 2011).

Another advantage of radiation is the lack of heat formation. At levels up to 10 kGy, the nutritional quality is protected while extending the shelf-life of the product. (Komolprasert, 2007).

Much research has been conducted to observe the efficacy of radiation on fresh produce. Niemira (2007) observed the efficiency of irradiating on baby spinach and Romaine lettuce leaves that were vacuum inoculated (internalized) with *E. coli* O157:H7. At the highest doses used (1.5 kGy), the inactivation of *E. coli* O157:H7 observed was 4 log for Romaine lettuce and 3 log for baby spinach leaves. The same research group investigated the efficacy of radiation on a variety of lettuce and results showed that the type of leaf is a variable impacting effectiveness. The highest log reduction achieved was 5 log on iceberg lettuce with a dose of 1.5 kGy ionizing irradiation (Niemira, 2007; Niemira, 2008). In another study, when *Escherichia coli* O157:H7 inoculated cilantro was treated with a 3.85 kGy irradiation, the treatment caused a 6.7 log reduction in pathogen's population (Foley et al., 2004). Jeong et al. (2010) showed the ability of radiation to penetrate the internal parts of leaves. With a 1 kGy dose of low-energy X ray radiation, they detected 0.2 kGy dose of radiation at the center. From this value, there was an expected inactivation of 5 log of *E. coli* O157:H7 in the center of produce without overexposing the upper parts of the leaves. . Irradiation was tested against *Salmonella* and *Listeria* under modified atmosphere packaging conditions. The formation of ozone as a result of radiolysis in the packaging atmosphere of oxygen was observed and caused a germicidal effect. With a 0.7 kGy dose of irradiation at modified atmosphere packaging conditions of 100% oxygen, more than a 5 log reduction of the tested microorganisms

can be achieved without any quality deterioration (Gomes et al., 2011). The effect of the irradiation treatment at 1 kGy on 13 different fresh produce samples was evaluated. The conclusions from this study claimed that there was no vitamin C loss in most produce. The aroma of the samples was found to be better than those untreated after 14 days storage, and there was no textural change at this dose (Fan and Sokorai, 2008).

Electrolyzed water

Electrolyzed water is a novel sanitizer utilized for the treatment of fresh produce. It is easy to generate electrolyzed water with salt, and it can be produced on site, making it safe for the environment. Compared to conventional chlorine, electrolyzed water does not have a corrosive effect on food processing equipment or cause damage to human skin. Once electrolyzed water reacts with organic material, it returns to the natural water form. The basic mechanism by which electrolyzed water is produced is that a diluted salt solution travels through a chamber where an anode and a cathode are charged with current and separated by a membrane. On the anode side, negatively charged ions produce acidic electrolyzed water, whereas, on the cathode side, positively charged ions produce alkali electrolyzed water (Bialka and Demirci., 2011; Huang et al., 2008). The acidic water generated from the anode side has pH values between 2.3 and 3.7, and high oxidation capacity from the free chlorine content. On the other side, alkali electrolyzed water has a pH value in the range of 10-11.5 with reducing potential and a plenty of dissolved hydrogen. Some studies showed the effect of electrolyzed water on the reduction of pathogens on fresh produce. Lin et al. (2005) studied the effect of alkali and acidic electrolyzed water to inactivate aerobic microorganisms on produce samples.

Acidic electrolyzed water was able to reduce the population by 1.7 log with a 9 minute wash without affecting the quality. It was concluded that electrolyzed water was more effective than water treatment alone. It is also effective for the degradation of pesticide residues on the produce. The longer the immersion of produce, the greater is the degradation of pesticides expected. No loss of C vitamin content was observed (Hao et al., 2011). Electrolyzed water with 50 mg/L free chlorine was more effective than the sodium hypochlorite solution of 15, 30, and 100 ppm without any change in produce surface color, tissue texture, and appearance which indicates that chlorine application can be reduced by using electrolyzed water (Graca et al, 2011; Izumi, 1999). According to another study, an insignificant difference in pathogen population reduction was obtained via electrolyzed water compared to chlorine, but safety concerns are making to use of electrolyzed water valuable for fresh produce treatment (Issa-Zacharia et al., 2011).

Bacteriophages

Recently, another technique for fresh produce treatment is under investigation and could be a promising technology/biological control method fresh produce. Bacteriophages could be used to control spoilage and pathogenic microorganisms on food. They can be effective at the temperatures at which foods commodities are transported and stored. Bacteriophages need a host cell for replication and could be classified as lytic or lysogenic bacteriophages. Lytic phages are able to regenerate inside the host cell and cause lysis of the cell, releasing new phages to the environment. Lysogenic phages are known for transfering the genes between microorganisms since they insert into the host genome. Lytic phages are the most applicable for the treatment of

fresh produce and are less likely to transfer toxin producing or drug resistance genes between microorganisms (CFR, 2002; Goodriche and Bisha, 2011; Monk et al., 2010). Bacteriophages are usually specific to their host. They will affect only a limited number of strains rather than broad range of microorganisms. As a result of studies conducted to observe the efficacy of phages, the use of bacteriophages in the treatment of fresh, ready-to-eat meat and poultry was approved by the FDA. Since many preservatives are not allowed in organic produce, bacteriophages could be a good alternative for their treatment. The efficacy of bacteriophages is related to the concentration used, food matrix being treated, and other factors. High relative concentrations of the phage, compared to the host, may also lead to lysis from without, wherein the phage does not have to enter the host for destruction. They cause the death of a pathogen by overwhelming the cell and detrimentally increasing membrane permeability.

Studies revealed how bacteriophages could be used effectively to decontaminate fresh produce. Guenther et al. (2009) studied the effect of bacteriophages on the reduction of two strains of *Listeria* inoculated on a set of foods including sliced cabbage and lettuce leaves after 6 days storage at 6 °C. Produce samples were inoculated with low population of this microorganism and two different concentrations of phages A511 and P100. After a 6 day of storage, *Listeria* population decrease by approximately 2.6 log for lettuce leaves and about 4 log for sliced cabbage compared to the untreated, inoculated produce samples. Researchers also concluded that the higher initial load of bacteriophages can yield higher log reductions. This study was consistent with the result obtained by Leverentz et al. (2003) who studied the effectiveness of phage cocktails on

the reduction of *Listeria monocytogenes* on fresh-cut apples and honeydew melons. The combination of bacteriophages with nisin provided a 5.7 and 2.3 log reductions of microorganisms compared to the control. Combination treatment can be used to increase the efficacy of the bacteriophages. Sharma et al. (2009) showed the efficacy of *E. coli* specific bacteriophages in the treatment of cantaloupe and fresh iceberg lettuce. Produce samples inoculated with low populations of target microorganism and then treated with high concentration of bacteriophage cocktails. Lettuce leaves were stored for 2 days at 4°C while cantaloupe was stored up to 7 days at the temperatures of 4°C and 20°C. For lettuce leaves, the reduction from the treatment compared to the control were 2.6, 1.8, and 2.2 log CFU/cm^2 for 0, 1, and 2 days, respectively. For cantaloupe samples stored at 4°C, log reductions achieved were 3.3, 3.2, and 4.1 log CFU/ml at 2, 5, and 7 days, respectively, compared to the control samples.

Treatments with ozone in combination with other agents

Advanced oxidation processes results in the formation of hydroxyl radicals, which are more oxidative than ozone itself, so that increases in microbial reductions could be achieved. The combination treatment of ozone with UV and hydrogen peroxide (H_2O_2) can produce hydroxyl radicals and increase the microbial reduction. Ozone can also be used in combination with chlorine and heat to get increased microbial lethality. Some example of increased reactivity of ozone with other methods will be explained.

Combination with chlorine

When *B. subtilis* spores were treated with ozone and then a free chlorine treatment, ozone enhanced the disinfection; however, when ozone followed free chlorine treatment, it did not enhance the process. It was suggested that since free chlorine is not able to damage the cell wall as much as ozone, ozone was used for the initial disruption of cell wall and followed by free chlorine application to enhance the diffusion of chlorine (Cho et al., 2003). In another study, minimally processed lettuce leaves inoculated with spoilage microorganisms, were treated with ozone, chlorine, and their combination. By application of chlorine and ozone, a 1.4 and 1.1 log reduction was achieved, respectively, however with the combination process a 2.5 log reduction was achieved and the shelf life was extended at least 5 days longer than with either to ozone or chlorine treatments alone (Garcia et al., 2003). Morphological changes were observed on the *Giardia lamblia* treated with chlorine and ozone. Once one of the sanitizers was applied, it changed the permeability of the cyst walls and made it easier for penetration of a second sanitizer, enhancing the damage (Li et al., 2004).

Combination with hydrogen peroxide

Hydrogen peroxide can be combined with ozone to initiate the hydroxyl radical formation in order to enhance the germicidal activity. *Cryptosporidium parvum* and *Giardia lamblia* are waterborne parasites that cause disease outbreaks and are resistant to chlorine treatment. *Bacillus subtilis* spores were used as a surrogate for *Cryptosporidium parvum* oocysts and it was observed that ozone and hydrogen peroxide had been used to

treat drinking water, and could potentially be a good alternative to an ozone-only treatment of drinking water (Cho and Yoon, 2006).

Combination with heat

Perry et al. (2008) investigated the efficacy of ozone, heat, and a combination treatment for the microbial reduction of *Salmonella enterica* serovar Enteritidis inoculated in shell eggs. The microbial reductions were 0.11 and 3.1 for ozone and heat, respectively. However, when the experiment was run with the combination of ozone followed by heat treatment at the same conditions, they were able to achieve a 4.2 log reduction. The synergistic effect of ozone with heat was evaluated on beef inoculated with enterotoxin producing strains of *Clostridium perfringens*. When 5 ppm of ozone was applied and followed by heat treatments at 45°C and 55°C, the initial population decreased from 5.6 log CFU/g to 4.1 log CFU/g and 3.5 log CFU/g at the respective temperatures. The spore population decreased from 2.9 log to 2.0 log and 1.7 log for 5 ppm of ozone followed by heat treatments at 55°C and 75°C, respectively. If ozone is applied at first, it decreases the resistance of vegetative cells and spores against heat treatment (Novak and Yuan, 2003).

Combination with UV

The efficacy of ozone in combination with UV was tested against *Salmonella enterica* serovar Enteritidis inoculated on shell eggs. Once inoculated eggs were treated with ozone they were then subjected to UV irradiation. The microbial populations for this combination were reduced by 4.6, 4.1, and 2.1 log compared to untreated, ozone only-

treated and UV only-treated egg samples. UV causes the photolytic destruction of ozone and creates hydroxyl radicals which are highly reactive against microorganisms (Rodriguez-Romo and Yousef, 2005). To evaluate the efficacy of UV and ozone against *Bacillus subtilis* spores, ozone and UV were applied simultaneously and sequentially. Inactivation of the spores of this microorganism with the combined treatment was greater than with UV, ozone alone, or the sequential application. A greater decrease (0.8 log) in the population was achieved with the combined process (Jung et al., 2008). The combination of UV and ozone was suggested for the treatment of fresh produce. Some fresh produce wash water samples were treated with ozone, UV radiation, and with the combination. UV and ozone treatments alone caused 4.0 and 5.9 log reductions, respectively. The combination treatment resulted in maximum 6.6 log reduction and required lower levels of sanitizers (Selma et al., 2008).

Chapter 2

Efficacy of gaseous ozone treatment in combination with vacuum cooling for the inactivation of *Escherichia coli* O157:H7 on baby spinach and Romaine lettuce

ABSTRACT

The goal of this study was to assess the efficacy of gaseous ozone, in combination with vacuum cooling, for the inactivation of *Escherichia coli* K12 and *E. coli* O157:H7 GFP B6-914 that were spot inoculated on leaves of baby spinach and Romaine lettuce. In the first part of the study, ready-to-eat bagged baby spinach leaves were inoculated with *E. coli* K12 and air dried. In the second set of experiments, ready-to-eat bagged baby spinach leaves and hearts of Romaine lettuce were spot inoculated with *E. coli* O157:H7. After attachment of target microorganisms to the produce samples, the experiments were conducted under similar conditions. The treatment started with vacuum application (-28.5 in Hg) in order to mimic industrial vacuum cooling practices for fresh produce. Subsequently, the treatment vessel was pressurized with a mixture of ozone and oxygen. Ozone treatment conditions are: 1.5g/kg ozone in ozone/oxygen mixture, 10 psig holding pressure, and 30 minutes holding time. Population of *E. coli* K12 inoculated baby spinach leaves decreased by 1.29 and 0.27 log CFU/g for ozone and oxygen (control) treatments, respectively, compared to inoculated non-treated (control). For baby spinach leaves spot

inoculated with *E. coli* O157:H7, the log reductions obtained showed discrepancy based on the timeline that experiments were conducted. In January and February of 2011 and 2012, the average microbial reduction in was 1.86 and 1.59 log CFU/g, respectively, and the difference was not significant. However, experiments completed between May and September 2011 gave significantly lower log reductions compared to January and February runs.

The efficacy of having a fan in the treatment chamber during treatment was also evaluated. The log reduction for the fan-off treatment was a 0.67 log CFU/g and it was significantly lower than the log reduction obtained with the fan-on. In the final set of experiments, the efficacy of the same treatment conditions was examined on hearts of Romaine lettuce that was spot inoculated with *E. coli* O157:H7. This treatment decreased the microbial population a 1.47 log CFU/g. In conclusion, gaseous ozone treatment of fresh produce, in combination with vacuum cooling, seems a promising technology and can be utilized by the fresh produce industry for the production of microbiologically safe ready-to-eat fresh produces.

INTRODUCTION

The fresh produce industry has been growing rapidly due to research that shows there is a positive correlation between the consumption of fresh produce and the reduced risk of cardiovascular diseases and cancer (Block et al., 1992; Riboli and Norat, 2003). The consumption of fresh produce has been supported by the U.S government with the new school nutrition standards that promotes increased contribution of fresh produce to

school meals (DeNoon, 2012). Based on consumer demand, people are willing to buy ready-to-eat meals including fresh produce rather than prepare them at home (Zink, 1997).

Disease outbreaks associated with the consumption of fresh produce pose a threat to public health, consumer confidence in producers, and sales of fresh produce. The reported number of outbreaks associated with the consumption of contaminated fresh produce was 82 between 1996 and 2008. Contaminated leafy greens were responsible for 28 of these outbreaks with 949 illnesses and 5 deaths. Of those outbreaks, *Escherichia coli* O157:H7 was responsible for % 85.7 of them (FDA, 2009a). In 2006, an outbreak occurred as a result of *E. coli* O157:H7 contaminating baby spinach leaves which ended in 3 deaths and 205 hospitalizations (FDA, 2007). When considering loss of consumer confidence some believe the industries economic losses are as high as 100 million dollars (Blake, 2011).

Contamination of fresh produce with pathogens is on the rise despite the increasing precautions and the stricter safety standards. With the implementations of new standards established for fresh produce safety, it may be possible to control pathogen contaminations or distribution of contaminated produce to the market. Improved detection and traceability enables processors to locate contaminated produce before reaching consumers. However, there is no way to prevent economic loss caused by contaminated produce other than to design new methods for product handling and treatment.

Chlorine has been used for the treatment of fresh produce and is effective to prevent cross contamination between washing water and fresh produce. However, it is not effective to inactivate internalized bacteria in produce. Environmental concerns have risen as a result of overuse of chlorine in industry. Chlorine reacts with organic matter in process water and forms carcinogenic by-products that pose a risk to public health (Rook, 1974).

The development of new technologies for improving fresh produce safety is necessary in order to protect the public and prevent economic losses. Gaseous ozone is an effective sanitizer with very high oxidation potential and diffusion capability. It is also more stable in gaseous phase than aqueous phase and its use has been approved as an additive and antimicrobial agent on food (FDA, 2001). Ozone as a sanitizer is more stable in the gaseous than aqueous phase and could be utilized for safe fresh ready-to-eat leafy greens.

MATERIALS AND METHODS

Bacterial strains, culture conditions and preparation of inoculum

Bacterial strains used for these experiments were *Escherichia coli* K12 and *Escherichia coli* O157:H7 GFP B6-914. *E. coli* K12 is a non-pathogenic strain of *E. coli* used as a surrogate for pathogenic *E. coli* O157:H7 in these studies. The second strain used in the selected experiments was *E. coli* O157:H7 GFP B614. This strain does not produce Shiga toxin1 (stx_1) or Shiga toxin2 (stx_2). Green fluorescence protein (GFP) was inserted via plasmid in order to differentiate the target microorganism from natural

microflora. Under UV light, *E. coli* O157:H7 GFP glows while the natural microbiota of the fresh produce does not. Moreover, the ampicillin and cycloheximide genes are inserted into *E. coli* O157:H7 GFP B6-914 to make it resistant to these antibiotics. It has been reported that the microbial growth kinetics and survival of *E. coli* O157:H7 GFP are more similar to the parent strain than some other GFP labeled microorganisms (Ma et al., 2011; Fratamico et al., 1997).

E. coli K12 and *E. coli* O157:H7 were kept in frozen conditions at – 80°C. In preparation for experiments, a loop of frozen culture of *Escherichia coli* K12 was inoculated in LB broth and incubated overnight at 37°C. This was followed by another transfer into fresh LB broth for second overnight incubation. Incubated culture was harvested by centrifugation at 8000 rpm for 10 min and cell pellets were suspended in 1 ml peptone water (%0.1 wt/vol); population in this suspension was ~10^9 CFU/ml. In case of *E. coli* O157:H7, the inoculum was incubated in a growth medium containing 100µg/ml each of ampicillin and cycloheximide (Sigma-Aldrich, St. Louis, Mo) but the other conditions including incubation time, harvesting and suspending in a peptone water were similar to those used for *E. coli* K12.

The antibiotic stock solution used was prepared to produce 100µg/ml of antibiotics in broth and agar media. This was done by mixing 0.1 g of ampicillin sodium salt with 1 ml distilled water. Stock solutions of antibiotics were sterilized with sterile 0.22 µm filters. To prepare cycloheximide stock solution, 0.1 g of the antibiotic was mixed with 4 mL ethanol and 6 mL of distilled water (Atlas, 2004). The stock solutions

of these antibiotics were kept refrigerated before use in the broth and agar microbiological media**.**

Inoculation of fresh produce leaves

Fresh produce samples used in these studies were baby spinach and Romaine lettuce leaves purchased from the local grocery (Columbus, Ohio) on the same day of the experiment. For the gaseous ozone treatment of baby spinach and Romaine lettuce leaves, 44 and 100 g sample sizes were used, respectively. Half of the leaves were used to enumerate the initial population (control) whereas the other half was used for treatment. *E. coli* K12 and *E. coli* O157:H7 cell suspensions were spot-inoculated on baby spinach leaves and hearts of Romaine, from abaxial and adaxial sides, and inoculated products were air-dried on the lab bench. Produce samples were inoculated with 20 spots of 5 µL (100 µL) which yielded an initial population of ~10^7 CFU/g.

Treatment conditions and equipment setup

To treat samples with gaseous ozone, two trays of fresh produce were placed in a custom 300-L stainless steel treatment vessel (Fig.2.1. and Fig.2.2.). Before the process started, the treatment vessel was cooled by an external chiller (Model NESLAB RTE-10, Thermo Electron Corporation, Newington, N.H.) which uses propylene glycol as a cooling medium. The vessel temperature was between -4°C and -6°C before vacuum and gaseous ozone were applied. This low temperature of the vessel also helped to decrease the temperature of fresh produce leaves quickly during vacuum cooling. The vacuum level was approximately -28.5 in. Hg achieved by a vacuum pump (HS 652 Varian Inc.,

Lexington, Ma.). During the vacuum cooling, water was supplied to the vessel to compensate for the water loss. The temperature of fresh produce leaves decreased from 12±2°C to 4±7°C by the end of vacuum application. Thereafter, the vessel was pressurized with gaseous ozone to achieve the desired ozone concentration and pressure level. The final concentration of gaseous ozone was 1.5 g/kg ozone in oxygen. The ozone concentration during processing was monitored by a high capacity ozone monitor (Mini-Hicon; IN USA, Inc., Norwood, MA). The final treatment pressure was approximately 10 psig and was kept constant during holding time. After 30 minutes, the vessel contents were sent to a thermal destruct for decomposition of the ozone gas to oxygen. This exhaust process was completed by repressurizing the vessel to one psig with air for 30 minutes. After ozone gas inside the vessel was exhausted, the process was complete and the samples were subjected to microbial analysis.

Ozone generation and measurement

Gaseous ozone was produced from pure oxygen by using a high capacity ozone generator (CSF-7, Ozonia, Elmwood Park, NJ). The ozone concentration during process was monitored by a high capacity ozone monitor (Mini-Hicon; IN USA, Inc., Norwood, MA). Excess ozone was exhausted from the vessel through ozone destruction unit to decompose the ozone to oxygen at high temperature (454°C).

Enumeration of microorganism

The population of microorganisms was counted before and after treatment. Eleven grams of leaves were placed in a stomacher bag and mixed with 99 mL of sterile peptone

water (0.1% wt/vol), and homogenized by using a lab stomacher (STO-400, Tekmar, Cincinnati, Ohio) for two minutes and serial dilutions prepared from this initial dilution. To enumerate *E. coli* K12 on the leaves, Sorbitol-MacConkey (SMAC) agar was used throughout the study. Selected dilutions of homogenized sample were spread plated on SMAC agar. The colonies were enumerated after incubation of inoculated plates at 37 °C for 24 hours.

Experiments completed with *E. coli* O157:H7 were carried out using the same procedure but with modifications to the growth media. After homogenization for 2 minutes, selected dilutions were spread plated on LB agar supplemented with ampicillin and cycloheximide. The inserted fluorescence gene makes it easy to differentiate this microorganism from the natural background microflora of fresh produce under UV light.

Data analysis

There were at least three independent replicates, with two sample trays per replicate, for each treatment. Average microbial reductions, expressed in logarithmic values, were used in the statistical analyses. Comparisons between several groups were performed by one-way analysis of variance (ANOVA) using IBM SPSS with LSD post-hoc analysis. Additionally, student's t-test was performed to compare means of two groups using the statistical software, SPSS (version 19.0.0, IBM, Chicago, IL). Differences at $p<0.05$ were considered significant.

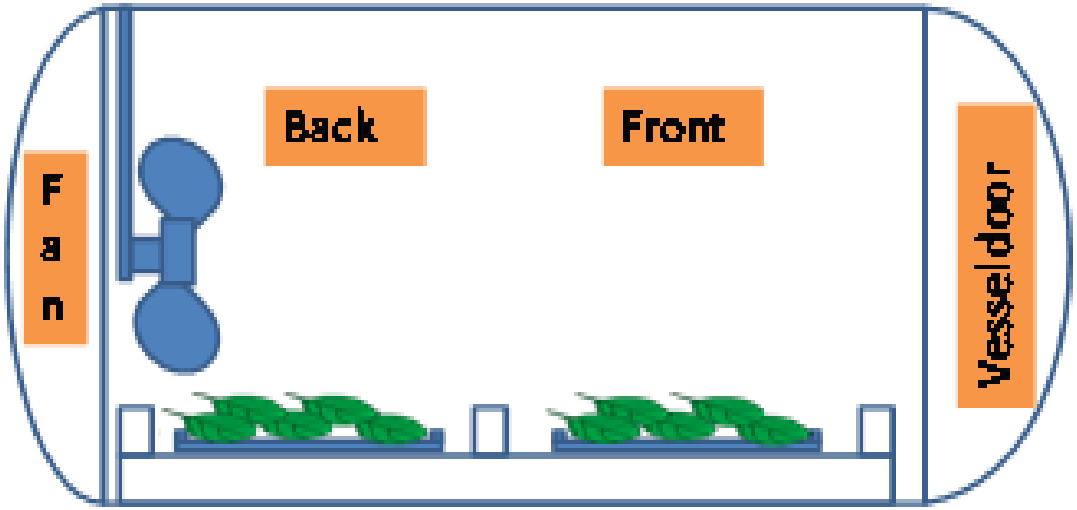

Figure 2.1. Location of the fan and sample trays inside the treatment vessel during gaseous ozone treatment

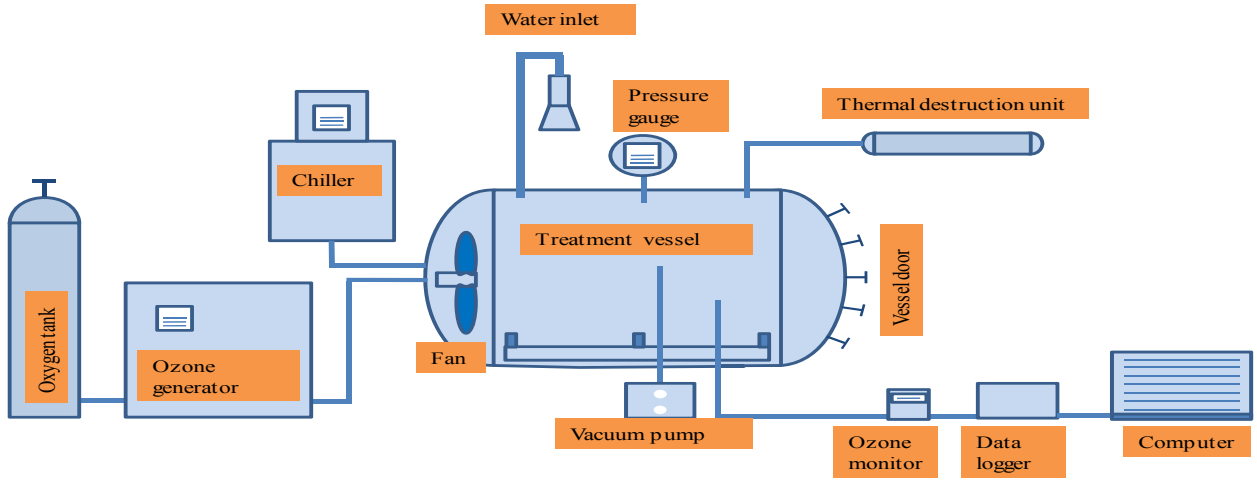

Figure 2.2. Schematic of the treatment vessel and the related components used for gaseous ozone treatment of produce samples

RESULTS AND DISCUSSION

Inactivation of *Escherichia coli* K12 using gaseous ozone treatment in combination with vacuum cooling

The efficacy of gaseous ozone was evaluated on baby spinach and hearts of Romaine lettuce samples. The initial average microbial populations of *Escherichia coli* K12, inoculated on non-treated leaves, was 6.9 log CFU/g (Figure 2.3). When treatment vessel was pressurized with oxygen (control), the average log reduction obtained was 0.27 log CFU/g. Gaseous ozone treatment of *E. coli* K12 inoculated baby spinach leaves decreased the microbial population 1.29 log CFU/g. The microbial populations after oxygen only (control) treatment were not significantly different from the non-treated control samples. Gaseous ozone treatment gave significantly higher log reduction compared to non-treated and oxygen-treated inoculated baby spinach leaves. The microbial reduction created by oxygen only treatment was insignificant compared to the inoculated non-treated (control) samples and this small decrease might be caused by pressure inside the vessel or oxidative stress on microorganisms. However it was clear that microbial inactivation inside the vessel is achieved by oxidative effects of ozone.

Inactivation of *Escherichia coli* O157:H7 on baby spinach leaves using gaseous ozone treatment in combination with vacuum cooling

Another set of experiments were done by spot inoculation of baby spinach leaves with *E. coli* O157:H7. The log reductions obtained from this process varied based on the season experiments were carried out (Figure 2.4). There were three different sets of

experiments run under the same conditions but in different seasons: January, 2011; May-Sep, 2011; February 2012.

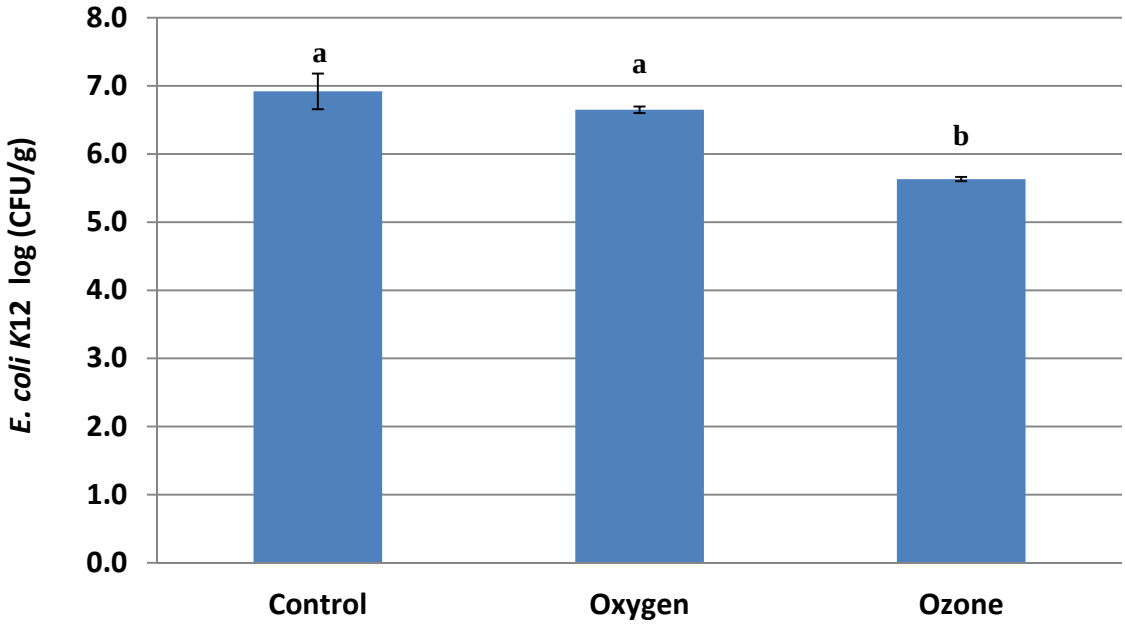

Figure 2.3. Survival of* Escherichia coli K12 *spot inoculated on baby spinach leaves with selected treatments*: Non-treated (control), oxygen only (control) or 1.5 g/kg gaseous ozone treatment. Treatment started with the application of vacuum (-28.5 in. Hg) in the vessel for cooling and then vessel was pressurized with oxygen only or gaseous ozone and oxygen, to reach desired ozone concentration (1.5 g ozone/ 1 kg ozone + oxygen mix) and process pressure (10 psig). Holding time was 30 minutes. Average log reductions are averages from at least 3 independent treatments. Different letters on bars represents significant difference (p <0.05). Error bars indicates ± standard error of the mean.*

Microbial inactivation of *E. coli* O157:H7 spot inoculated on baby spinach was 1.86 log CFU/g in January, 2011. However in the following months; May - September, 2011; the average log reduction obtained was 0.97 log CFU/g. When experiments were completed in February, 2012, an average of 1.59 log CFU/g of *E. coli* O157:H7 was inactivated on baby spinach leaves. Microbial reductions obtained in January and February were not significantly different from each other, however, the inactivation of *E. coli* O157:H7 obtained from May – September 2011 was significantly lower. Changes in the season caused changes in the microbial reductions achieved. Ninety percent of leafy greens production is in California and Arizona. Produce companies obtain their produce from different states in different seasons (Blake, 2011). During studies conducted in the lab, it was observed that baby spinach leaves were more uniform and bigger in size in January and February. In the following months; May to September; the leaves were smaller in size and damage such as cracks was more obvious with purchased leafy greens. With the larger leaves, fewer leaves were required to reach same weight of produce sample for experiments. The smoother surfaces of leaves may make it easier for ozone to diffuse between the spinach leaves to inactivate microorganisms. However, small leaf size and cracks on the leaves might have increased the microbial internalization to the cut edge during vacuum cooling and caused possible decrease in the efficacy of gaseous sanitizer.

Compared to experiments done with *E. coli* K12, drying time was reduced to one hour from two hours for these experiments. The reason for this change was that long drying time on the bench affected the visual quality of baby spinach leaves and it might

have been caused by the relative humidity difference between drying environment and spinach leaves. The microbial strain used in this set of experiments was *E. coli* O157:H7 with a green fluorescence gene insertion instead of *E. coli* K12. It was also theorized that with the use of bacterium with GFP gene, possible overestimation of microbial count of *E. coli* K12 might be prevented since only GFP inserted microorganisms will give fluorescence under UV light in the presence of natural microbiota of the leaves.

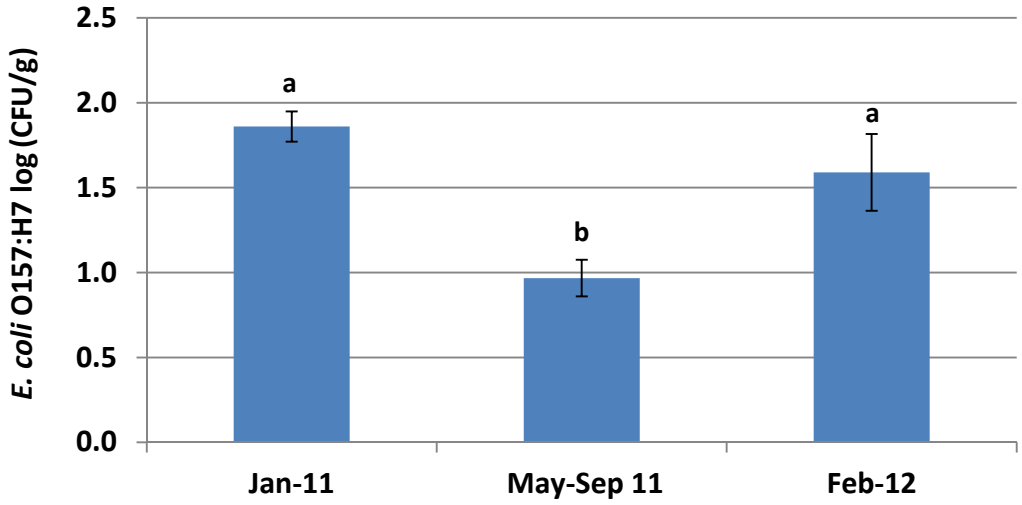

Figure 2.4 Inactivation of* Escherichia coli O157:H7 *spot inoculated on baby spinach leaves with gaseous ozone treatment in different seasons of the year. *Treatment started with the application of vacuum (-28.5 in. Hg) in the treatment vessel for cooling and then vessel pressurized with the gaseous ozone and then oxygen, respectively to reach desired ozone concentration (1.5 g/kg) and process pressure (10 psig). Holding time was 30 minutes. Average log reductions from at least 3 independent treatments. Different letters on bars represents significant difference ($p < 0.05$). Error bars indicates ± standard error of the mean.*

The effect of a fan inside the treatment vessel was also evaluated. For selected experiments, the fan installed inside the treatment vessel was turned off and experiments were completed in the following conditions: initial vacuum application (-28.5 in. Hg) and 1.5 g/kg ozone concentration at 10 psig holding pressure for 30 minutes holding time. When the fan was turned off, the average log reduction obtained on baby spinach leaves inoculated with *E. coli* O157:H7 was 0.69 log CFU/g. This reduction was significantly lower than the average log reductions obtained for fan-on experiments, 1.86 log CFU/g, run in the same season of the year under the same conditions (Figure 2.5). The fan is intended to maintain better distribution of the gaseous ozone in the vessel when it is mixed with oxygen. Since gaseous ozone is denser than oxygen, it could be possible that ozone is settling at the bottom of the vessel after a while. Integration of fan into the vessel seems beneficial to achieve higher log reductions.

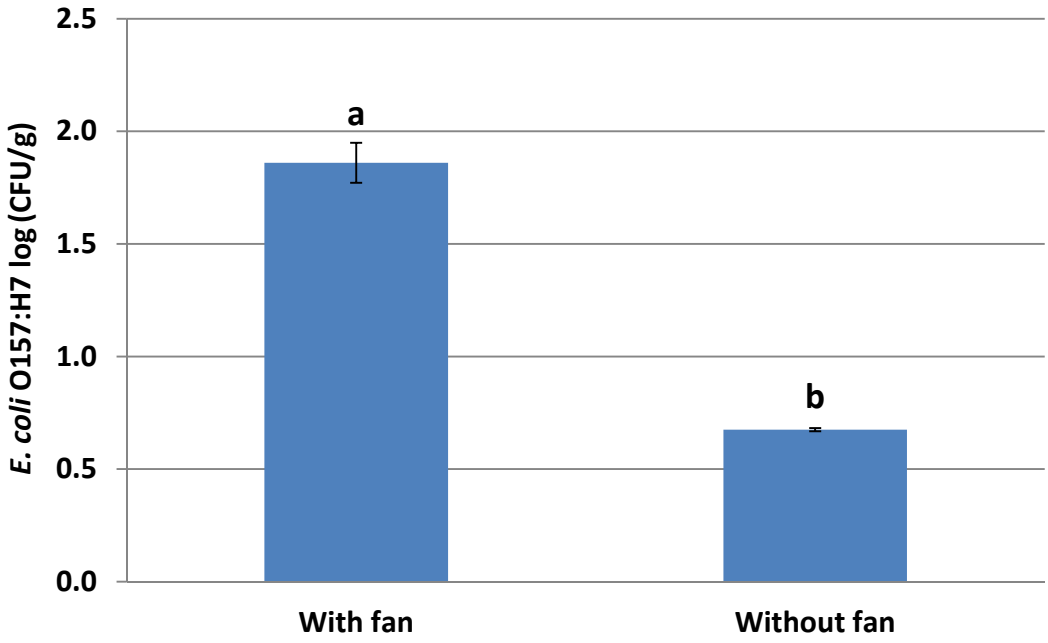

Figure 2.5. The effect of the fan used in the vessel on the inactivation of* Escherichia coli O157:H7 *with gaseous ozone treatment. *Treatment started with the application of vacuum (-28.5 in. Hg) for cooling and then the vessel was pressurized with the gaseous ozone to reach desired ozone concentration (1.5 g/kg) and process pressure (10 psig). Holding time was 30 minutes. Average log reductions are from 2 or more independent treatments. Different letters on bars represents significant difference (p <0.05). Error bars indicates ± standard error of the mean.*

Inactivation of *Escherichia coli* O157:H7 on hearts of Romaine leaves using gaseous ozone treatment in combination with vacuum cooling

In the last set of experiments, the efficacy of gaseous ozone for the treatment of hearts of Romaine lettuce spot inoculated with *E. coli* O157:H7 was evaluated. Inoculated leaves were treated with gaseous ozone following vacuum application (-28.5 in. Hg) in the following treatment conditions: 1.5 g/kg ozone at 10 psig holding pressure

for 30 minutes holding time. This treatment inactivated microbial population of 1.47 log CFU/g (Table 2.1). However some discoloration was observed on the hearts of Romaine (Fig. 2.6.). Since the produce sample was ready-to-eat, degradation of color pigments and texture might have started even in storage conditions. It is also easier for gaseous ozone to penetrate inside the leaf from cut edges and react with the color pigment, chlorophyll. This process might be improved by using freshly harvested non-prewashed lettuce samples.

Table 2.1. Inactivation of* Escherichia coli *O157:H7 spot inoculated on hearts of Romaine lettuce

Run #	*Escherichia coli* O157:H7 log (CFU/g)		Average log reductions
	Sample tray location in treatment vessel		
	Front	Back	
1	1.35	1.48	1.42
2	1.43	1.59	1.51
Average log reductions	1.39	1.54	1.47±0.10

Treatment started with the application of vacuum (-28.5 in. Hg) for cooling and then the vessel was pressurized with the gaseous ozone to reach desired ozone concentration (1.5 g/kg) and process pressure (10 psig). Holding time was 30 minutes. Average log reductions are from 2 independent treatments. Different letters on bars represents significant difference ($p < 0.05$). Error bars indicates ± standard error of the mean.

In our study, gaseous ozone treatment of baby spinach leaves in combination with vacuum cooling was more effective in inactivating the inoculated microorganism, compared to log reductions achieved from conventional chlorinated water wash (Garcia at al., 2003; Keskinen et al., 2009; Niemira and Cooke, 2010; Niemira, 2007). The visual quality of baby spinach leaves was hardly affected by the gaseous ozone treatment (Fig.2.6). Since the baby spinach samples were purchased from a local grocery, it is expected that freshly harvested baby spinach samples would give better visual color and textural properties compared to the already processed bagged ready-to-eat baby spinach samples used in these studies.

In summary, since the average microbial inactivation achieved on baby spinach leaves by oxygen only treatment was not significant, it can be concluded that the microbial inactivation obtained inside the treatment vessel was due to ozone. Results also showed that gaseous ozone in combination with vacuum cooling is a promising technology and can be utilized by industry; however, seasonal changes on produce might affect sanitizer efficacy. Results also showed that hearts of Romaine lettuce gave relatively high log reduction, but the color change was evident. Freshly harvested product might give better results. Treatments with the fan-on seem feasible to enhance the antimicrobial action of ozone inside the vessel. The gaseous ozone treatment of fresh produce samples in combination with vacuum cooling is a promising technology and can be utilized by the fresh produce industry for the production of microbiologically safe, ready-to-eat fresh produce.

Figure 2.6. Color of baby spinach and hearts of Romaine lettuce before and after treatments with ozone-vacuum colling combination

A. Baby spinach leaves before treatment
B. Baby spinach leaves after treatment
C. Hearts of Romaine before treatment
D. Hearts of Romaine after treatment

Chapter 3

Effect of pre-washing and gaseous ozone treatment for the inactivation of *Escherichia coli* O157:H7 on baby spinach and Romaine lettuce

ABSTRACT

The objective of this study was to evaluate the efficacy of a washing step integrated into the gaseous ozone treatment of fresh produce to enhance the inactivation of *Escherichia coli* K12 and *E. coli* O157:H7 GFP B6-914 on baby spinach and cut Romaine lettuce leaves. Baby spinach and Romaine lettuce samples were dip-inoculated, air dried, and treated with combination of washing and a gaseous ozone treatment. In the first part of the study, Romaine lettuce and baby spinach leaves were washed with either sterile distilled water or 1% dimethyl sulfoxide (DMSO). The log reductions obtained were 0.59 and 0.55 log CFU/g for sterile distilled water and 1% DMSO wash, respectively. Combinations of washing followed by gaseous ozone treatment, were also tested. After washing with sterile distilled water or 1% DMSO, a gaseous ozone treatment (2.0 g ozone/kg gas mixture with 10 psig vessel pressure for 30 minutes holding time with an initial -28.5 in Hg vacuum applications) was applied. The combination treatments inactivated 1.26 and 1.07 log CFU/g of *E. coli* K12, respectively. The same experiments

were repeated with baby spinach leaves and 0.49 and 0.33 log CFU/g reductions were achieved for sterile distilled water and a 1% DMSO wash, respectively. The combination treatments of sterile, distilled water or 1% DMSO wash followed by gaseous ozone treatment decreased the *E. coli* K12 count 1.24 and 1.10 log CFU/g, respectively on baby spinach leaves.

The second part of the study was carried out with a new washing container design. This washing system was assembled with two spray nozzles that could be rotated with a handle, enabling the gentle handling of produce to prevent damage and possible internalization. Baby spinach samples were dip-inoculated with *E. coli* O157:H7 and washed with a selected sanitizer followed by gaseous ozone treatment. The sanitizers chosen for these set of experiments were the commercial sanitizers PRO-SAN and chlorine, as well as sterile distilled water. The washing process alone with sterile distilled water, 200 ppm chlorine, and 2% PRO-SAN inactivated 0.64, 1.12, and 1.23 log CFU/g of *E. coli* O157:H7, respectively. Combination treatments of these washes followed by gaseous ozone treatment decreased the microbial count of the target microorganism by 1.70, 2.28 and 2.07 log CFU/g, respectively. Data was also collected on the effect of ozone treatment alone on dip-inoculation baby spinach leaves. This treatment decreased *E. coli* O157:H7 populations by 1.70 log CFU/g. The results from the second part of study indicate that (i) the average log reduction achieved from sterile distilled water wash only was significantly lower than that from the 200-ppm chlorine or the 2%-PRO-SAN wash; (ii) the sterile distilled water wash followed by gaseous ozone treatment gave significantly higher log reduction compared to washing with 200 ppm chlorine or a 2%

PRO-SAN alone; (iii) a 2% PRO-SAN wash followed by gaseous ozone treatment is not significantly different from a 200 ppm chlorine wash followed by gaseous ozone treatment; however, it yielded a reduction of 2.07 logs, which is significantly higher than all other treatments, which suggests its potential applicability within industry. Some discoloration on the product was observed, but all experiments were conducted with pre-bagged baby spinach and Romaine lettuce leaves that might have contributed to enhanced degradation of color pigments. The log reductions achieved by combination treatments could be a possible alternative for the industry standard of chlorine wash treatment, and the results of this study may even be improved by using freshly harvested, non-processed baby spinach and Romaine lettuce leaves.

INTRODUCTION

Fresh produce is an indispensable part of a healthy diet. There has been continuous growth in the production and sale of fresh produce as a result of an increase in consumer demand (Sivapalasingam et al., 2004). The increase in demand requires mass production, quick delivery, and global trade of fresh produce. This increase has also brought about some issues concerning the safety of produce. There have been a number of disease outbreaks recently that were associated with the consumption of contaminated produce.

In 2006, there were three disease outbreaks causing three deaths and more than 300 confirmed cases of illnesses related to the consumption of *E. coli* O157:H7 contaminated leafy greens, including baby spinach and lettuce (FDA, 2007a; FDA,

2007b; CDC, 2006). After these outbreaks, a surge in public interest about food safety forced the producers and processors to develop new mitigation strategies for fresh produce safety. New rules and regulations have been established by government agencies and fresh produce growers; however, complete prevention of outbreaks has not yet been achieved.

Increased traceability of leafy greens enables producers to withdraw products from market in case of health related emergency situations associated with these products. However, the economic loss cannot be avoided. Contributing to this challenge is the fact that bacteria can be internalized by fresh produce and it is challenging to inactivate internalized microorganisms with conventional sanitization methods. A chlorinated water wash is mainly used by the industry because of its simple implementation and it is relatively inexpensive. This sanitizer is effective at preventing cross contamination between wash water and product, but its reaction with organic compounds produces carcinogenic by-products which are considered a risk to public health (Rook, 1974).

It could be beneficial to wash produce before sanitizer application to enhance inactivation of microorganisms. By washing produce, pesticides residues, soil, and debris could be removed from produce and also microbial removal could be achieved. Sanitizer application following washing may enhance effectiveness of sanitizers since organic material reacts with the sanitizers to decrease their efficacy.

The efficacy of ozone has been assessed in aqueous and gaseous phases. In the gaseous phase, ozone has a longer half-life with enhanced antimicrobial activity (Vurma, 2009). It was reported that applying vacuum to the process environment before gaseous ozone application, increased the penetration capability to the internal parts of eggs (Yousef and Rodriguez-Romo, 2004). Recent studies have been focused on using gaseous sanitizers, such as ozone or chlorine dioxide, to inactivate internalized bacteria on fresh fruit and vegetables. The enhanced efficacy of ozone was reported for its combination with chlorine, heat, UV, and hydrogen peroxide. Gaseous ozone could be a good alternative to inactivate pathogenic microorganisms on fresh produce when applied after the pre-washing step in produce processing.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and preparation of inoculum

Bacterial strains used for these experiments were *Escherichia coli* K12 and *E. coli* O157:H7 GFP B6-914. *E. coli* K12 is a non-pathogenic strain of *E. coli* (Levy et al., 1980). It is often used as a surrogate for pathogenic *E. coli* O157:H7 in studies. The second strain, *E. coli* O157:H7 GFP B614, does not produce Shiga toxin1 (stx_1) or Shiga toxin2 (stx_2). A green fluorescence protein (GFP) encoding plasmid was inserted into the genome of this strain to differentiate it under UV light from other microorganisms which are present on fresh produce as natural microbiota. Moreover, ampicillin and cycloheximide genes were inserted into *E. coli* O157:H7 GFP B6-914 making it resistant to these antibiotics which were used as selective agents in the growth medium. It has been reported that the microbial growth kinetics and survival of *E. coli* O157:H7 GFP are

more similar to the parent strain than some other GFP labeled microorganisms. (Ma et al., 2011; Fratamico et al., 1997).

E. coli K12 and *E. coli* O157:H7 were kept frozen at –80°C. The frozen stock of *E. coli* K12 was prepared by mixing 50% fresh culture in Luria-Bertani broth (LB broth; Difco, Becton Dickinson, Sparks, Md.) with 50% glycerol. To prepare a fresh culture from a frozen stock, a loop of *E. coli* K12 was inoculated in Luria Bertani broth (LB broth; Beckton Dickinson, Sparks, Md.) and incubated overnight at 37°C. This overnight incubation was followed by another transfer of *E. coli* K12 into fresh LB broth for second overnight incubation. Cells were harvested using centrifugation at 8000 rpm for 10 min. and the recovered microorganisms were re-suspended in 1 ml peptone water (0.1% wt/v) to obtain a cell suspension containing ~10^9 CFU/ml. The preparation of *E. coli* O157: H7 differs slightly in that the culture was incubated in a growth medium including 100 µg/ml each ampicillin and cycloheximide (Sigma-Aldrich, St. Louis, Mo). Other conditions remained the same.

The antibiotic stock solution were prepared to obtain 100 µg/ml of each antibiotic in broth and agar media. The ampicillin stock solution was prepared by mixing 0.1 g of ampicillin sodium salt with 1 ml distilled water, and the solution was sterilized with 0.22 µm filters. To prepare the cycloheximide stock solution, 0.1 g of the antibiotic was mixed with 4 mL ethanol and 6 mL of distilled water (Atlas, 2004). The stock solutions of these antibiotics were kept refrigerated before use in microbiological media**.**

Inoculation of fresh produce leaves

Romaine lettuce (cut) and baby spinach leaves were purchased from the local grocery (Columbus, Ohio) on the day of the experiments. Romaine lettuce and baby spinach leaves were used in the first set of experiments, which evaluated the efficacy of water only and 1% DMSO wash followed by gaseous ozone treatment to inactivation of *E. coli* K12. For all other experiments, baby spinach leaves only were used.

Combination treatments for inactivation of *Escherichia coli* K12 on baby spinach and Romaine lettuce leaves

The inoculation method used throughout the study was dip inoculation. After fresh cultures of *E. coli* K12 were suspended in peptone water (~10^9 CFU/ml), inoculum was transferred into an inoculation container (Figure 3.1.) with 3 L of peptone water. An agitator was used to evenly distribute the microorganism. Fresh produce leaves were kept immersed into this inoculation medium and agitated for 15 minutes. Inoculated produce samples were held on a bench to dry for 45 minutes to facilitate bacterial attachment before selected treatments were applied. The initial population of the target microorganism on the leaves was ~10^5 CFU/g after drying.

Combination treatments for inactivation of *Escherichia coli* O157:H7 on baby spinach leaves

The procedure described previously was followed with modifications. Inoculation time was reduced from 15 minutes to 3 minutes to prevent possible internalization of

microorganism and to minimize damage to the leaves which could also cause internalization. The inoculated leaves were dried for an hour and the sample size was reduced to 150 g, compared to 250g used in the previous experiments. The initial population of the *E. coli* O157:H7 on baby spinach leaves was ~10^5 CFU/g after drying.

Treatment conditions

Washing

To evaluate the possible increase in microbial inactivation of the target microorganisms, the washing step was combined with gaseous ozone treatment. The washing procedure evolved based on the results. By using the results from 1% DMSO and sterile, distilled water washing of lettuce and baby spinach leaves, it was decided that the washing treatment conditions should be modified for later experiments. However, all the experiments run after those initial trials followed this same procedure so that conditions were held constant.

At the initial step, fresh produce samples were washed by using 1% DMSO or sterile distilled water. For this process step, a sterile salad spinner (Fig.3.2) was filled 3L of either sterile distilled water or a 1% DMSO solution prepared in distilled water. The washing process took three minutes and agitation was achieved using a stirring motor. The excess water left on the produce was removed by using a sterile salad spinner. Half of the leaves were used to evaluate the microbial reduction from washing while the other half was used for gaseous ozone treatment after washing. Based on the results obtained from this set of experiments, the new washing equipment was constructed. In later

experiments, to stimulate the industrial fresh produce processing, an autoclavable, 15 L plastic bucket was designated as a washing container (Fig. 3.3). Two spray nozzles were attached to the underside of the lid of this washing bucket in order to mimic the spray washing of produce. Spray nozzles also created agitation of the water and sample without causing any damage to the produce. A handle was attached to the washing bucket in order to rotate the nozzles to achieve even application of the spray washing solutions on whole produce. Five L of sterile distilled water, 200 ppm bleach, or 2% PRO-SAN solutions were prepared with sterile, distilled water and used for the washing process. After the washing process was completed, excess water was removed using a sterile salad spinner. After Half of each washed sample was carried into the ozone treatment vessel on sample trays for gaseous ozone application.

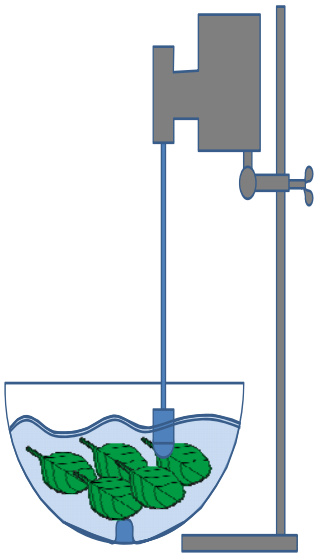

Figure 3.1. Schematic of the apparatus used for inoculation of leafy greens with bacteria

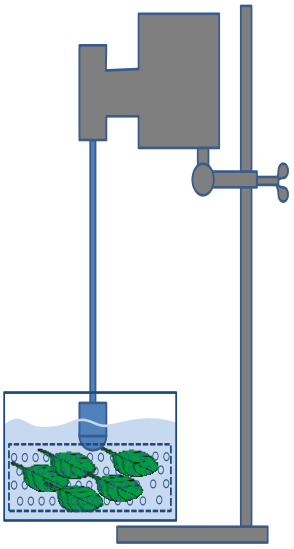

Figure 3.2. Schematic of apparatus used for washing leafy greens post inoculation

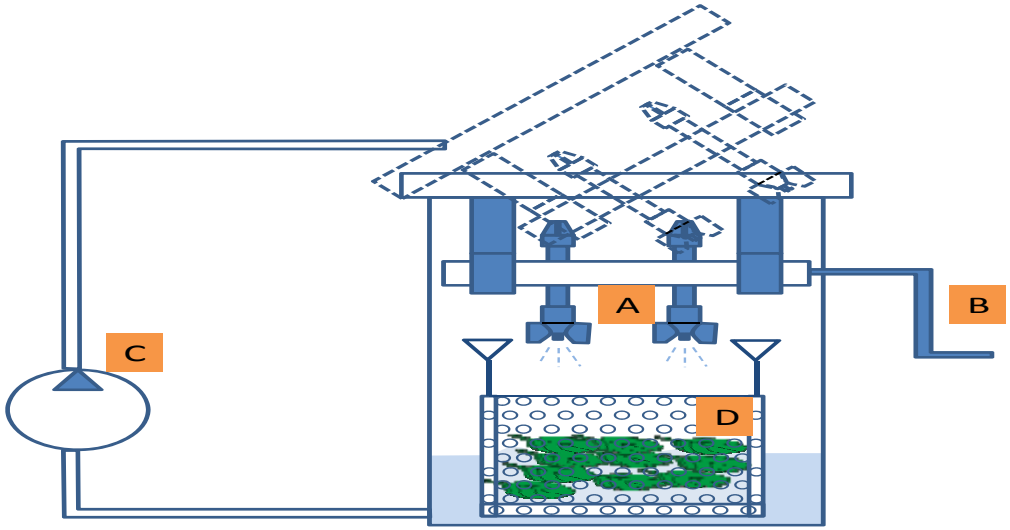

Figure 3.3 Schematic of a custom washing container designed to mimic industrial washing of leafy greens

A: *Spray nozzles*

B: *Handle for rotation of nozzles*

C: *Peristaltic pump*

D: *Sample mesh tray*

Gaseous ozone treatment

To treat samples with gaseous ozone, two trays of fresh produce were placed in a custom 300-L stainless steel treatment vessel. Before the process started, the treatment vessel was cooled down by using an external chiller (Model NESLAB RTE-10, Thermo Electron Corporation, Newington, N.H.) which uses propylene glycol as a cooling medium. The vessel temperature reached between -4°C and -6°C before vacuum and gaseous ozone were applied. This low temperature helped to decrease the temperature of fresh produce leaves quickly during vacuum cooling. The actual process started with the application of vacuum cooling to simulate the industrial cooling of fresh produce. The vacuum level was approximately -28.5 in. Hg achieved by a high capacity of the vacuum pump (HS 652 Varian Inc., Lexington, Ma.). During the vacuum cooling, water was supplied to the vessel to compensate for water loss from fresh produce during vacuum cooling. The temperature of fresh produce leaves decreased from 12±2°C to 4±7°C by the end of vacuum application. When vacuum was achieved, the vessel was pressurized with gaseous ozone for a specific time and then by providing gaseous oxygen until the desired ozone concentration and pressure level was achieved. The final concentration of gaseous ozone was 2.0 g/kg ozone in oxygen (2.0 g O_3 / 1.0 kg O_3+O_2). Ozone gas was produced by using a high capacity ozone generator (CSF-7, Ozonia Inc., Elmwood Park, N.J.). The ozone concentration during processing was monitored by a high capacity ozone monitor, (Mini-Hicon; IN USA, Inc., Norwood, MA). The final treatment pressure was approximately 10 psig and it was kept constant during holding time. After 30 minutes, the gaseous ozone that remained in the vessel was vented to a thermal destruct unit for

decomposition of the gas to oxygen. This exhaust process was completed by repressurizing the vessel to one psig with air for 30 minutes. After ozone gas inside the vessel was exhausted, the process was complete and the samples were taken out for microbial analysis.

Enumeration of microorganisms

The target microorganism was enumerated by using plate count techniques. In the study, the population of microorganisms was counted before and after washing treatments, and after gaseous ozone treatment. Twenty five grams of either spinach or lettuce leaves were placed in a stomacher bag and mixed with 225 mL of sterile peptone water (0.1% w/v). The samples were then homogenized using a lab stomacher (STO-400, Tekmar, Cincinnati, Ohio) for two minutes and serial dilutions were prepared. To enumerate *E. coli* K12 on the leaves, Sorbitol-MacConkey (SMAC) agar was used. Plates were incubated at 37 °C for 24 hours.

Experiments conducted with *Escherichia coli* O157:H7 were carried out following the same procedure but with modifications to the growth media. Dilutions were spread plated on LB agar supplemented with ampicillin and cycloheximide. The colonies formed on the plates were enumerated under UV light after incubation at 35°C for 24 hours. The fluorescence gene in *E. coli* O157:H7 GFP makes it easy to differentiate this microorganism from the natural background microbiota of fresh produce.

Data analysis

There were at least three independent replicates with two sample trays per replicate for each treatment. Average microbial reductions from two trays were converted to logarithmic values before statistical analyses. Comparisons between several groups were performed by one-way analysis of variance (ANOVA) using the statistical software SPSS with LSD post-hoc analysis. Differences at $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Inactivation of *Escherichia coli* K12 by sequential application of washing and gaseous ozone treatment

In this study, gaseous ozone treatment of Romaine lettuce and baby spinach leaves was integrated with pre-washing of leafy greens to observe the efficacy of these combination treatments to enhance microbial inactivation. In the first portion of the study, baby spinach and Romaine lettuce leaves were dip inoculated with *Escherichia coli* K12. Dip inoculated leaves were washed with sterile, distilled water or 1% dimethyl sulfoxide (DMSO) and then subjected to gaseous ozone treatment (2.0 g/kg ozone concentration at 10 psig vessel pressure for 30 minutes holding time with initial -28.5 in. Hg vacuum application). Results for these treatments are shown in Figure 3.4. Washing Romaine lettuce leaves with sterile, distilled water or 1% DMSO decreased the microbial count by 0.59 and 0.55 log CFU/g, respectively. The log reductions obtained for sterile, distilled water and 1% DMSO wash treatment were not significantly different ($p>0.05$). The combination treatments, sterile distilled water or 1% DMSO wash followed by gaseous ozone treatment decreased the microbial populations of *E. coli* K12 on lettuce leaves by 1.26 and 1.07 log CFU/g, respectively. The log decreases obtained for combination treatments were significantly higher ($p<0.05$) than that from wash only steps but not different from each other ($p>0.05$). Baby spinach leaves were dip inoculated with *E. coli* K12 and all experiments were repeated at the same conditions as explained previously for Romaine lettuce leaves. Log reductions achieved for inoculated baby spinach leaves were 0.49 and 0.33 log CFU/g for sterile, distilled water wash and a 1% DMSO wash, which were not significantly different ($p>0.05$) from each other (Fig.

3.5). The combination treatments of these two washing steps with gaseous ozone treatment inactivated 1.24 and 1.10 log CFU/g of target microorganisms on the leaves. The combination treatments were more effective ($p<0.05$) than wash only treatments of baby spinach but not significantly different from each other. It has been reported that the efficacy of gaseous ozone is higher on smooth produce surfaces; therefore, higher log reductions were expected for baby spinach samples (Han et al., 2002). However, there was no significant difference ($p>0.05$) between wash only and combination treatments for baby spinach leaves compared to wash only and combination treatments for Romaine lettuce leaves.

Washed produce surfaces were wet and the leaves stuck to each other in the sample tray before gaseous ozone treatment. That could prevent gaseous ozone diffusion on and in the leaves. Additionally, there were significant damage to the leaves of Romaine and baby spinach during the washing step that might have caused higher internalization of the target microorganism. Moreover, the long inoculation time, which was 15 minutes, could have led to more internalization of bacteria and more water could have been taken up internal tissues that, in turn, prevented gaseous ozone penetration to internal tissues.

DMSO is a universal solvent and is used as a detergent to dissolve drugs. It also has a great penetration capability into the cell which is why it is used to carry drugs. It's bacteriostatic and bacteriocidal activities were tested against different microorganisms and at low concentrations, and a bacteriostatic behavior was reported (Ansel et al., 1969; Ghajar and Harmon., 1968; Basch and Gadebusch, 1968). At higher concentrations, DMSO inactivated most of the target microorganisms with irreversible damage.

However, a 1% concentration, similar to the level used in this study, did not increase the inactivation of target microorganisms compared to a sterile, distilled water wash. For this study, sterile distilled water followed by gaseous ozone treatment gave a 1.26 and 1.24 log CFU/g reductions for baby spinach and lettuce leaves. This might be a good alternative to a chlorine wash treatment for produce since similar log reductions are reported for chlorine only wash and does not include the risk of possible formation of carcinogenic by-products.

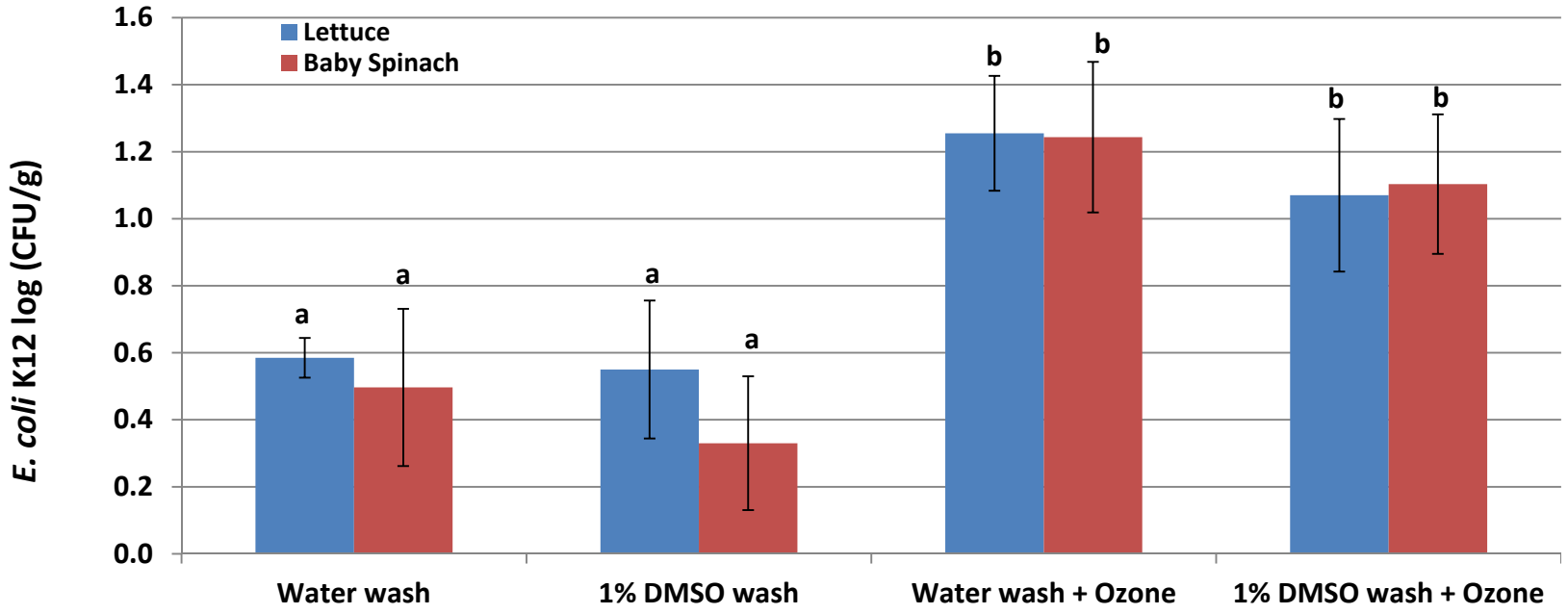

Figure 3.4. Inactivation of* Escherichia coli *K12 on dip inoculated Romaine lettuce and baby spinach leaves with the sequential application of washing and gaseous ozone treatment. *Washing with either sterile distilled water or 1% DMSO followed by vacuum application (-28.5 in. Hg) in conjunction with 2 g ozone/ 1 kg (Ozone + Oxygen mix) at 10 psig vessel pressure for 30 minutes holding time. Number of experiments n = 3 and different letters on bars indicate significant difference. Error bars indicates ± standard error of the mean.*

Inactivation of *Escherichia coli* O157:H7 by sequential application of washing and gaseous ozone treatment

In the second part of this study, baby spinach leaves were dip-inoculated with *Escherichia coli* O157:H7, air dried and then washed with 200 ppm chlorine, 2% PRO-SAN, or sterile distilled water. The washing step was followed by gaseous ozone treatment at the previously described conditions. PRO-SAN is a commercial, organic acid sanitizer made from food grade ingredients: citric acid, sodium dodecylbenzene sulfonate, as well as other inert ingredients. The average log reductions achieved for sterile distilled water, 200 ppm chlorine and 2% PRO-SAN wash were 0.64, 1.12, and 1.23 log CFU/g, respectively (Fig. 3.5). Combination treatments with gaseous ozone decreased the microbial count of the target microorganism by 1.70, 2.28 and 2.07 log CFU/g, respectively. Independent from these experiments, a set of dip inoculated baby spinach leaves were treated with gaseous ozone without including any washing steps. The average log reduction obtained was 1.70 log CFU/g for this treatment. The inactivation achieved by washing baby spinach leaves with 200 ppm or 2% PRO-SAN was significantly higher than ($p<0.05$) sterile distilled, water wash. Water is used to remove soil and debris on produce and it dilutes microorganisms on the produce rather than causing their inactivation. However, chlorine and PRO-SAN are FDA approved sanitizers that are highly effective for the decontamination of produce and may also prevent cross contamination from the wash water and cause some microbial inactivation. The log reductions obtained for 200 ppm chlorine solution were consistent with those reported in literature (Garcia at al., 2003; Keskinen et al., 2009); however, there is no study published showing the efficacy of PRO-SAN wash on *E .coli* O157:H7 on baby spinach.

Two percent PRO-SAN wash inactivated more microorganisms, but the the log reduction was not significantly different ($p>0.05$) from that achieved by the chlorine wash. It can be concluded that PRO-SAN could be used as a sanitizer for baby spinach wash with the same microbial inactivation efficacy of chlorine without forming any dangerous by-products in the wash medium. However, washing of baby spinach leaves with PRO-SAN produces foam that must then be removed from leaves. PRO-SAN was not perfectly soluble in water at room temperature. To increase the efficacy of PRO-SAN, in its powder form, it has to be dissolved completely in the washing medium of produce.

The combination treatments of sterile, distilled water wash, 200 ppm chlorine wash, and 2% PRO-SAN wash with gaseous ozone inactivated 1.70, 2.28, and 2.07 log CFU/g of *E. coli* O157:H7, respectively. There was no significant difference ($p>0.05$) between 2% PRO-SAN or 200 ppm chlorine washing followed by gaseous ozone treatment. However, these combination treatments were more effective ($p<0.05$) than sterile, distilled water wash followed by gaseous ozone treatment. When dip inoculated baby spinach samples were washed, air dried, and treated with gaseous ozone without any washing, it produced a 1.70 log CFU/g *E. coli* O157:H7 inactivation, which is significantly higher ($p<0.05$) than any kind of washing process. However, log reduction obtained for this treatment was significantly lower than that obtained by 200 ppm chlorine and 2% PRO-SAN washing with only exception a combination of sterile distilled water wash and sequential gaseous ozone treatment. Washing inoculated baby spinach leaves with 200 ppm chlorine and 2% PRO-SAN followed by gaseous ozone treatment gave significantly ($p<0.05$) higher inactivation of *E. coli* O157:H7 compared to

all other treatments but the log reductions achieved for these two treatments were not significantly different from each other.

Washing treatment did not cause any color change. However, after gaseous ozone treatment, some discoloration was observed. The discoloration mostly occurred on the leaves located at the bottom and top of the sample tray where gaseous ozone had an easy access. However it is noted that all leafy greens tested were commercially processed products that were purchased on the same day of treatments. After harvest, the degradation of pigments and textural changes happens even in the cold storage and this might have affected the visual quality of the samples during treatments. The better visual quality after treatments might be expected with freshly harvested non-treated leafy greens.

By washing produce, pesticides residues, soil, and debris could be removed from produce and also some microbial removal could be achieved. Sanitizer application following washing may enhance effectiveness of sanitizers since organic material reacts with the sanitizers to decrease their efficacy. It has also been proposed that harsh attachment and biofilms could be softened by washing, and that might have increased the efficacy of the sanitizer. However, no synergistic effect was observed by combining washing and gaseous ozone treatment but the combination enhanced microbial inactivation when compared to treatment with gaseous ozone only.

It can be concluded that a sterile, distilled water wash followed by gaseous ozone treatment could be an alternative treatment for 200 ppm chlorine wash because it gives a

significantly higher reduction. A 2% PRO-SAN wash followed by gaseous ozone treatment seemed to be the best choice compared to all other treatments by giving a 2.07 log reduction, although it is not significantly different from a 200 ppm chlorine wash followed by gaseous ozone treatment.

In summary, microbial reduction caused by 1% DMSO did not show any promising effect on microbial reduction compared to water only wash. A 2% PRO-SAN wash could be a good replacement for chlorine since it gives similar log reductions compared to the 200 ppm chlorine wash, but without any danger of by-product formation and quality change on baby spinach leaves. Additionally, 2% PRO-SAN wash followed by gaseous ozone treatment could be a promising technology since it achieved a 2.07 log reduction, which was higher than all other treatments except chlorine. From the results, it can be concluded that the combination treatments enhanced the microbial reduction, although there was no synergistic effect between sequential application of washing and gaseous ozone. The 2% PRO-SAN only wash or combination wash with gaseous ozone, could be a good replacement for conventional chlorine treatment of fresh produce without any risk of producing carcinogenic by-products.

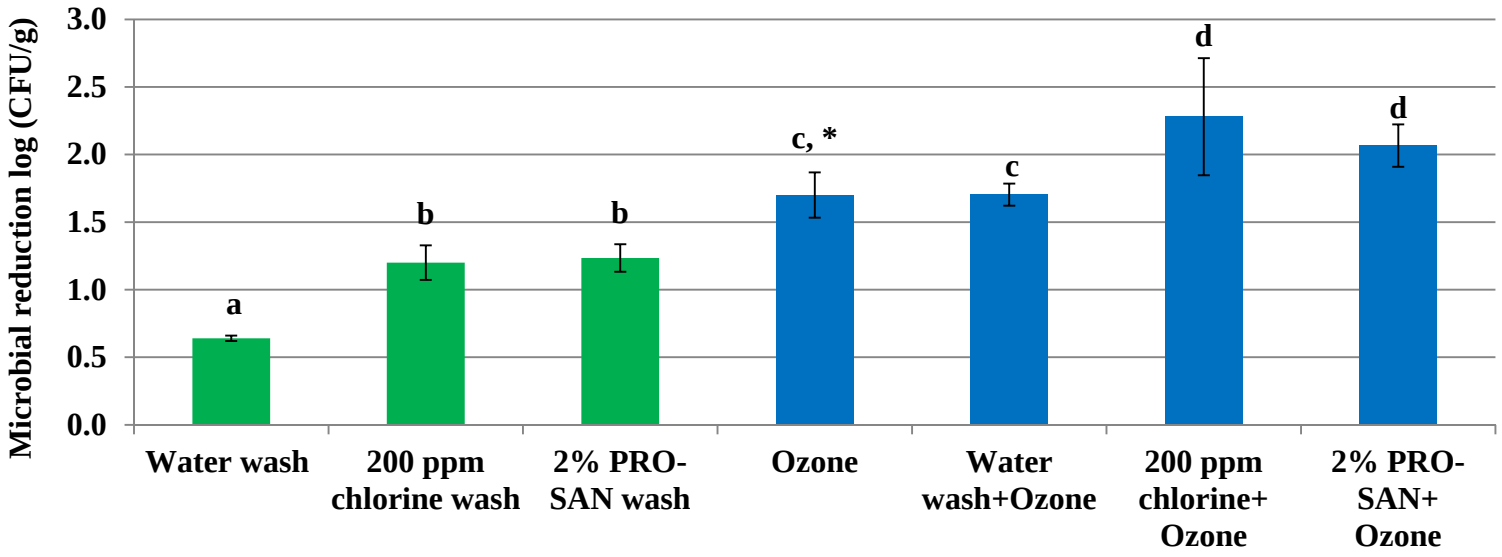

Figure 3.5. Inactivation of* Escherichia coli O157:H7 *on dip inoculated baby spinach leaves with the sequential application of washing (sterile distilled water/ 200 ppm chlorine/ 2% PRO-SAN) and gaseous ozone treatment. *Washing with either sterile distilled water, 2% PRO-SAN or 200 ppm chlorine followed by vacuum application (-28.5 in. Hg) in conjunction with 2 g/kg ozone at 10 psig vessel pressure for 30 minutes holding time.* n = 3 *and different letters on bars indicate significant difference. Error bars represents ± standard error of the mean. *Experiments done at a different time from other experiments*

REFERENCES

Ansel, H.C., Norred, W.P., Roth, I.L., 1969. Antimicrobial activity of dimethyl sulfoxide against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus megaterium*. Journal of pharmaceutical sciences 58, 836-839.

Artes, F., Martinez, J., 1994. Effects of vacuum cooling and package films on the shelf life of 'Salinas' lettuce. International Conference: Refrigeration and quality of fresh vegetables. International Institute Refrigeration Brest. France, pp. 311–317.

Atlas, R.M., 2004. Handbook of microbiological media, third ed. CRC, Boca Raton, FL.

Basch, H., Gadebusch, H., 1968. In vitro antimicrobial activity of dimethylsulfoxide. Applied and Environmental Microbiology 16, 1953.

Beuchat, L.R., Ryu, J.H., 1997. Produce handling and processing practices. Emerging Infectious Diseases 3, 459.

Beuchat, L., Chmielewski, R., Keswani, J., Law, S., Frank, J., 1999. Inactivation of aflatoxigenic Aspergilli by treatment with ozone. Letters in applied microbiology 29, 202-205.

Beutin, L., 2006. Emerging enterohaemorrhagic *Escherichia coli*, causes and effects of the rise of a human pathogen. Journal of Veterinary Medicine, Series B 53, 299-305

Blake, C., 2011. *Leafy greens industry still reeling from E. coli-spinach outbreak. Western Farm Press*. Available at: http://westernfarmpress.com/vegetables/leafy-greens-industry-still-reeling-e-coli-spinach-outbreak

Block, G., Patterson, B., Subar, A., 1992. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutrition and cancer 18, 1-29.

Brandl, M., 2008. Plant lesions promote the rapid multiplication of *Escherichia coli* O157:H7 on postharvest lettuce. Applied and Environmental Microbiology 74, 5285.

Broadwater, W.T., Hoehn, R., King, P., 1973. Sensitivity of three selected bacterial species to ozone. Applied and Environmental Microbiology 26, 391.

CDC, 2006. Multistate Outbreak of *E. coli* O157 Infections, November-December 2006. Available at: http://www.cdc.gov/ecoli/2006/december/121406.htm

CDC, 2008. Investigation of Outbreak of Infections Caused by *Salmonella* Saintpaul. Available at: http://www.cdc.gov/salmonella/saintpaul/jalapeno/

CDC, 2009. Investigation of an Outbreak of *Salmonella* Saintpaul Infections Linked to Raw Alfalfa Sprouts. Available at: http://www.cdc.gov/salmonella/saintpaul/alfalfa/

CDC, 2010. Investigation Update: Multistate Outbreak of Human *E. coli* O145 Infections Linked to Shredded Romaine Lettuce from a Single Processing Facility. Available at: http://www.cdc.gov/ecoli/2010/ecoli_O145/index.html

CDC, 2011. CDC Estimates of Foodborne Illness in the United States. Available at: http://www.cdc.gov/foodborneburden/PDFs/FACTSHEET_A_FINDINGS_updated4-13.pdf

CDC, 2011a. Investigation Update: Multistate Outbreak of *Salmonella* Panama Infections Linked to Cantaloupe. Available at: http://www.cdc.gov/salmonella/panama0311/062311/index.html#highlights

CDC, 2011b. Investigation Update: Multistate Outbreak of Human *Salmonella* Enteritidis Infections Linked to Alfalfa Sprouts and Spicy Sprouts. Available at: http://www.cdc.gov/salmonella/sprouts-enteritidis0611/070611/index.html

CDC, 2011c. Multistate Outbreak of Listeriosis Linked to Whole Cantaloupes from Jensen Farms, Colorado. Available at: http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/120811/index.html

CDC, 2011d. Investigation Announcement: Multistate Outbreak of *E. coli* O157:H7 Infections Linked to Romaine Lettuce. Available at: http://www.cdc.gov/ecoli/2011/ecoliO157/romainelettuce/120711/index.html

CFR, 2002. Food Additives Permitted for Direct Addition to Food for Human Consumption; Bacteriophage Preparation. Available at: http://www.gpo.gov/fdsys/pkg/FR-2011-03-23/html/2011-6792.htm

Cho, M., Chung, H., Yoon, J., 2003. Quantitative evaluation of the synergistic sequential inactivation of *Bacillus subtilis* spores with ozone followed by chlorine. Environmental science & technology 37, 2134-2138.

Cho, M., Yoon, J., 2006. Enhanced Bactericidal Effect of O3/H2O2 Followed by Cl2. Ozone: Science and Engineering 28, 335-340.

Dawson, D., 2005. Foodborne protozoan parasites. International journal of food microbiology 103, 207-227.

Dawson, D., Paish, A., Staffell, L., Seymour, I., Appleton, H., 2005. Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus. Journal of applied microbiology 98, 203-209.

Demirci, A., Bialka, K.L., 2011. Electrolyzed Oxidizing Water. Nonthermal Processing Technologies for Food, 366-376.

DeNoon, D.J., 2012. USDA Issues New School Lunch Nutrition Standards. Available at: http://www.medscape.com/viewarticle/757516

Eppinger, M., Mammel, M.K., Leclerc, J.E., Ravel, J., Cebula, T.A., 2011. Genomic anatomy of *Escherichia coli* O157:H7 outbreaks. Proceedings of the National Academy of Sciences 108, 20142-20147.

Erickson, M.C., Ortega, Y.R., 2006. Inactivation of protozoan parasites in food, water, and environmental systems. Journal of Food Protection 69, 2786-2808.

Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L., Payton, A.S., Liao, J., Ma, L., Doyle, M.P., 2010. Infrequent internalization of *Escherichia coli* O157:H7 into field-grown leafy greens. Journal of Food Protection, 500-506.

Ewell, A.W., 1946. Recent ozone investigations. Journal of Applied Physics 17, 908-911.

FAO., 2006. More fruit and vegetables. Available at: http://www.fao.org/ag/magazine/0606sp2.htm

Fan, X., Sokorai, K., 2008. Retention of Quality and Nutritional Value of 13 Fresh-Cut Vegetables Treated with Low-Dose Radiation. Journal of Food Science 73, 367-372.

Farkas, J., 2006. Irradiation for better foods. Trends in Food Science & Technology 17, 148-152.

FDA, 1982. GRAS status of ozone. Federal Register 47, 50209–50210.

FDA, 1997. Substances Generally Recognized As Safe; Proposed Rule, Federal Register 62(74):18937-18964.

FDA, 2001. Secondary direct food additives permitted in food for human consumption. Federal Register 66(123):33829–30.

FDA, 2002. Guidance for Industry: Exemptions from the Warning Label Requirement for Juice - Recommendations for Effectively Achieving a 5-Log Pathogen Reduction; Final Guidance. Available at: http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodLabelingNutrition/ucm058962.htm

FDA, 2007a. FDA Finalizes Report on 2006 Spinach Outbreak. Available at: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2007/ucm108873.htm

FDA, 2007b. FDA and States Closer to Identifying Source of *E. coli* Contamination Associated with Illnesses at Taco John's Restaurants. Available at: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2007/ucm108827.htm

FDA, 2008. Irradiation: A Safe Measure for Safer Iceberg Lettuce and Spinach. Available at: http://www.fda.gov/downloads/ForConsumers/ConsumerUpdates/UCM143389.pdf

FDA, 2009a. Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards of Leafy Greens; Draft Guidance. Available at: http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ProduceandPlanProducts/ucm174200.htm

FDA, 2009b. Guidance for Industry: Bottled Water: Residual Disinfectants and Disinfection Byproducts; Small Entity Compliance Guide. Available at: http://www.fda.gov/food/guidancecomplianceregulatoryinformation/guidancedocuments/chemicalcontaminantsandpesticides/ucm163194.htm

FDA, 2012. Recalls, Market Withdrawals, & Safety Alerts. Available at: http://www.fda.gov/Safety/Recalls/

Finch, G., Black, E., Labatiuk, C., Gyurek, L., Belosevic, M., 1993. Comparison of *Giardia lamblia* and *Giardia muris* cyst inactivation by ozone. Applied and Environmental Microbiology 59, 3674.

Foley, D., Euper, M., Caporaso, F., Prakash, A., 2004. Irradiation and chlorination effectively reduces *Escherichia coli* O157:H7 inoculated on cilantro (Coriandrum sativum) without negatively affecting quality. Journal of Food Protection 67, 2092-2098.

Fratamico, P.M., M.Y., Deng, T.P., Strobaugh,and S.A. Palumbo., 1997. Construction and characterization of *Escherichia coli* O157:H7 strains expressing firefly luciferase and green fluorescent protein and their use in survival studies. Journal of Food Protection 60, 1167–1173.

Garcia, A., Mount, J., Davidson, P., 2003. Ozone and chlorine treatment of minimally processed lettuce. Journal of Food Science 68, 2747-2751.

Ghajar, B.M., Harmon, S.A., 1968. The effect of dimethyl sulfoxide (DMSO) on permeability of *Staphylococcus aureus*. Biochemical and biophysical research communications 32, 940-944.

Gomes, C., Moreira, R.G., Castell-Perez, E., 2011. Radiosensitization of *Salmonella* spp. and *Listeria* spp. in Ready-to-Eat Baby Spinach Leaves. Journal of Food Science 76, E141–E148.

Gonçalves, A.A.,2009. Ozone: an emerging technology for the seafood industry. Brazilian Archives of Biology and Technology 52, 1527-1539.

Goodridge, L.D., Bisha, B., 2011. Phage-based biocontrol strategies to reduce foodborne pathogens in foods. Bacteriophage 1, 130-137.

Graca, A., Abadias, M., Salazar, M., Nunes, C., 2011. The use of electrolyzed water as a disinfectant for minimally processed apples. Postharvest Biology and Technology 61, 172-177.

Guenther, S., Huwyler, D., Richard, S., Loessner, M.J., 2009. Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. Applied and Environmental Microbiology 75, 93.

Gyürék, L.L., Li, H., Belosevic, M., Finch, G.R.,1999. Ozone Inactivation Kinetics of cryptosporidium in Phosphate Buffer. Journal of Environmental Engineering 125, 913.

Hamburg, M.A., 2009. Food Safety Modernization Act: Putting the Focus on Prevention. Available at: http://www.foodsafety.gov/news/fsma.html

Han, Y., Floros, J., Linton, R., Nielsen, S., Nelson, P., 2002. Response surface modeling for the inactivation of *Escherichia coli* O157:H7 on green peppers (Capsicum annuum) by ozone gas treatment. Journal of Food Science 67, 1188-1193.

Hao, J., Liu, H., Chen, T., Zhou, Y., Su, Y.C., Li, L., 2011. Reduction of Pesticide Residues on Fresh Vegetables with Electrolyzed Water Treatment. Journal of Food Science 76, C520-C524.

Horváth, M., Bilitzky, L., Hüttner, J., Bocsy, S., 1985. Ozone, Elsevier, New York.

Huang, Y.R., Hung, Y.C., Hsu, S.Y., Huang, Y.W., Hwang, D.F., 2008. Application of electrolyzed water in the food industry. Food Control 19, 329-345.

Ishizaki, K., Shinriki, N., Matsuyama, H.,1986. Inactivation of *Bacillus* spores by gaseous ozone. Journal of applied microbiology 60, 67-72.

Issa-Zacharia, A., Kamitani, Y., Miwa, N., Muhimbula, H., Iwasaki, K., 2011. Application of slightly acidic electrolyzed water as a potential non-thermal food sanitizer for decontamination of fresh ready-to-eat vegetables and sprouts. Food Control 22, 601-607.

Izumi, H., 1999. Electrolyzed Water as a Disinfectant for Fresh-cut Vegetables. Journal of Food Science 64, 536-539.

Jay, J.M., Loessner, M.J., Golden, D.A., 2005. Modern food microbiology, Springer Verlag, 637-650.

Jeong, S., Marks, B.P., Ryser, E.T., Moosekian, S.R., 2010. Inactivation of *Escherichia coli* O157:H7 on lettuce, using low-energy x-ray irradiation. Journal of Food Protection 73, 547-551.

Jung, Y.J., Oh, B.S., Kang, J.W., 2008. Synergistic effect of sequential or combined use of ozone and UV radiation for the disinfection of *Bacillus subtilis* spores. Water research 42, 1613-1621.

Kader, A.A., 2002. Postharvest technology of horticultural crops. In: Kader, A.A. (Ed.). University of California Agriculture and Natural Resources Publication 3311, Oakland, CA. p. 39-47

Katzenelson, E., Kletter, B., Shuval, H., 1974. Inactivation kinetics of viruses and bacteria in water by use of ozone. Journal American Water Works Association 66, 725-729.

Keskinen, L.A., Burke, A., Annous, B.A., 2009. Efficacy of chlorine, acidic electrolyzed water and aqueous chlorine dioxide solutions to decontaminate *Escherichia coli* O157:H7 from lettuce leaves. International journal of food microbiology 132, 134-140.

Khadre, M., Yousef, A., 2001. Sporicidal action of ozone and hydrogen peroxide: a comparative study. International journal of food microbiology 71, 131-138.

Khadre, M., Yousef, A., Kim, J.G., 2001. Microbiological aspects of ozone applications in food: a review. Journal of Food Science 66, 1242-1252.

Khadre, M., Yousef, A., 2002. Susceptibility of human rotavirus to ozone, high pressure, and pulsed electric field. Journal of Food Protection 65, 1441-1446.

Kim, J.G., 1998. Ozone as an antimicrobial agent in minimally processed foods. Ph.D.dissertation. The Ohio State University, Columbus, Ohio.

Kim, J.G., Yousef, A., 2000. Inactivation kinetics of foodborne spoilage and pathogenic bacteria by ozone. Journal of Food Science 65, 521-528.

Kim, J.G., Yousef, A.E., Dave, S., 1999. Application of ozone for enhancing the microbiological safety and quality of foods: A review. *J*ournal *of Food Protection*. 62, 1071-1078.

Komolprasert, V., 2007. Packaging for foods treated by ionizing radiation. Packaging for nonthermal processing of food , 87-116.

Korich, D., Mead, J., Madore, M., Sinclair, N., Sterling, C.R., 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Applied and Environmental Microbiology 56, 1423-1428.

Kroupitski, Y., Pinto, R., Brandl, M., Belausov, E., Sela, S., 2009. Interactions of *Salmonella enterica* with lettuce leaves. Journal of applied microbiology 106, 1876-1885.

Leverentz, B., Conway, W.S., Camp, M.J., Janisiewicz, W.J., Abuladze, T., Yang, M., Saftner, R., Sulakvelidze, A., 2003. Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. Applied and Environmental Microbiology 69, 4519-4526.

Levy, S.B., Marshall, B., Rowse-Eagle, D., Onderdonk, A., 1980. Survival of *Escherichia coli* host-vector systems in the mammalian intestine. Science 209, 391.

Li, H., Tajkarimi, M., Osburn, B.I., 2008. Impact of vacuum cooling on *Escherichia coli* 0157:H7 infiltration into lettuce tissue. Applied and Environmental Microbiology 74, 3138-3142.

Li, Y., Smith, D.W., Belosevic, M., 2004. Morphological changes of *Giardia lamblia* cysts after treatment with ozone and chlorine. Journal of Environmental Engineering and Science 3, 495-506.

Lim, M.Y., Kim, J.M., Lee, J.E., Ko, G.P., 2009. Characterization of ozone disinfection of murine norovirus. Applied and Environmental Microbiology 76, 1120.

Lin, C.S., Wu, C., Yeh, J.Y., Saalia, F.K., 2005. The evaluation of electrolysed water as an agent for reducing microorganisms on vegetables. International Journal of Food Science & Technology 40, 495-500.

López-Gálvez, F., Allende, A., Truchado, P., Martínez-Sánchez, A., Tudela, J.A., Selma, M.V., Gil, M.I., 2010. Suitability of aqueous chlorine dioxide versus sodium hypochlorite as an effective sanitizer for preserving quality of fresh-cut lettuce while avoiding by-product formation. Postharvest Biology and Technology 55, 53-60.

Lowe, R., Baines, D., Selinger, L.B., Thomas, J.E., McAllister, T.A., Sharma, R., 2009. *Escherichia coli* O157:H7 strain origin, lineage, and Shiga toxin 2 expression affect colonization of cattle. Applied and Environmental Microbiology 75, 5074.

Ma, L., Zhang, G., Doyle, M.P., 2011. Green Fluorescent Protein Labeling of Listeria, *Salmonella*, and *Escherichia coli* O157:H7 for Safety-Related Studies. PloS one 6, e18083.

Mac Kenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R., Rose, J.B., 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. New England journal of medicine 331, 161-167.

Mahmoud, B.S.M., Bhagat, A., Linton, R., 2007. Inactivation kinetics of inoculated *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* on strawberries by chlorine dioxide gas. Food Microbiology 24, 736-744.

Mahmoud, B., Linton, R., 2008. Inactivation kinetics of inoculated *Escherichia coli* O157:H7 and *Salmonella enterica* on lettuce by chlorine dioxide gas. Food Microbiology 25, 244-252.

Mahmoud, B., Vaidya, N., Corvalan, C., Linton, R., 2008. Inactivation kinetics of inoculated *Escherichia coli* O157: H7, *Listeria monocytogenes* and *Salmonella* Poona on whole cantaloupe by chlorine dioxide gas. Food Microbiology 25, 857-865.

McGlynn, W.G., Brandenberger, L.P., Castillo, A., 2009. CAST Commentary- Food Safety and Fresh Produce: An Update. Available at: http://www.jifsan.umd.edu/docs/workshops/producesafety/outbreaks/CAST%20Commentary%20Fresh%20Produce.pdf

Mead, P.S., Griffin, P.M., 1998. *Escherichia coli* O157: H7. The Lancet 352, 1207-1212.

Mitra, R., Cuesta-Alonso, E., Wayadande, A., Talley, J., Gilliland, S., Fletcher, J., 2009. Effect of route of introduction and host cultivar on the colonization, internalization, and movement of the human pathogen *Escherichia coli* O157:H7 in spinach. Journal of Food Protection 72, 1521-1530.

Monk, A., Rees, C., Barrow, P., Hagens, S., Harper, D., 2010. Bacteriophage applications: where are we now? Letters in applied microbiology 51, 363-369.

Mootian, G., Wu, W.H., Matthews, K.R., 2009. Transfer of *Escherichia coli* O157:H7 from soil, water, and manure contaminated with low numbers of the pathogen to lettuce plants. Journal of Food Protection 72, 2308-2312.

Murphy, O.J., Hitchens, G.D., 1999. Electrochemical production of ozone and hydrogen peroxide

Neupane, M., Abu-Ali, G.S., Mitra, A., Lacher, D.W., Manning, S.D., Riordan, J.T., 2011. Shiga toxin 2 overexpression in *Escherichia coli* O157:H7 strains associated with severe human disease. Microbial pathogenesis 51, 466-470.

Niemira, B.A., 2007. Relative efficacy of sodium hypochlorite wash versus irradiation to inactivate *Escherichia coli* O157:H7 internalized in leaves of Romaine lettuce and baby spinach. Journal of Food Protection 70, 2526-2532.

Niemira, B., 2008. Irradiation compared with chlorination for elimination of *Escherichia coli* O157:H7 internalized in lettuce leaves: influence of lettuce variety. Journal of Food Science 73, 208-213.

Niemira, B.A., Cooke, P.H., 2010. *Escherichia coli* O157:H7 biofilm formation on Romaine lettuce and spinach leaf surfaces reduces efficacy of irradiation and sodium hypochlorite washes. Journal of Food Science 75, M270-M277.

NOAA, 2010. Twenty Questions and Answers About the Ozone Layer: 2010 Update. Available at: http://www.esrl.noaa.gov/csd/assessments/ozone/2010/twentyquestions/

Novak, J.S., Yuan, J.T.C., 2004. Increased inactivation of ozone-treated *Clostridium perfringens* vegetative cells and spores on fabricated beef surfaces using mild heat. Journal of Food Protection 67, 342-346.

Owens, J.H., Miltner, R.J., Rice, E.W., Johnson, C.H., Dahling, D.R., Schaefer III, F.W., Shukairy, H.M., 2000. Pilot-scale ozone inactivation of *Cryptosporidium* and other microorganisms in natural water. Ozone: Science & Engineering 22, 501-517.

Ozonia, Inc., 2009. OZAT CFS OZONATORS. Available at: http://www.degremont-technologies.com/IMG/pdf/OZAT-CFS_US_Ozonia.pdf

Palou, L., Smilanick, J.L., Crisosto, C.H., Mansour, M., Plaza, P., 2003. Ozone gas penetration and control of the sporulation of *Penicillium digitatum* and *Penicillium italicum* within commercial packages of oranges during cold storage. Crop protection 22, 1131-1134.

Patel, J., Sharma, M., Ravishakar, S., 2011. Effect of curli expression and hydrophobicity of *Escherichia coli* O157:H7 on attachment to fresh produce surfaces. Journal of applied microbiology

Peeters, J.E., Mazas, E.A., Masschelein, W.J., de Maturana, I.V.M., Debacker, E.,1989. Effect of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. Applied and Environmental Microbiology 55, 1519-1522.

Perry, J., Rodriguez-Romo, L., Yousef, A., 2008. Inactivation of *Salmonella enterica* serovar enteritidis in shell eggs by sequential application of heat and ozone. Letters in applied microbiology 46, 620-625.

Pu, S., Beaulieu, J.C., Prinyawiwatkul, W., Ge, B., 2009. Effects of plant maturity and growth media bacterial inoculum level on the surface contamination and internalization of *Escherichia coli* O157:H7 in growing spinach leaves. Journal of Food Protection 174; 72, 2313-2320.

Rakness, K.L., 2005. Ozone in drinking water treatment: process design, operation, and optimization, Amer Water Works Assn.

Restaino, L., Frampton, E.W., Hemphill, J.B., Palnikar, P.,1995. Efficacy of ozonated water against various food-related microorganisms. Applied and Environmental Microbiology 61, 3471-3475.

Riboli, E., Norat, T., 2003. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. The American Journal of Clinical Nutrition 78, 559S.

Rodriguez-Romo and, L.A., Yousef, A.E., 2005. Inactivation of Salmonella enterica serovar Enteritidis on shell eggs by ozone and UV radiation. Journal of Food Protection 68, 711-717.

Rook, J.J.,1974. Formation of haloforms during chlorination of natural waters. Water Treatment and Examination, 2, 234-243.

Roth, J.A., Sullivan, D.E., 1981. Solubility of ozone in water. Industrial & Engineering Chemistry Fundamentals 20, 137-140.

Saldaña, Z., Sánchez, E., Xicohtencatl-Cortes, J., Puente, J.L., Giron, J.A., 2011. Surface structures involved in plant stomata and leaf colonization by Shiga-toxigenic *Escherichia coli* O157: H7. Frontiers in microbiology 2

Sarig, P., Zahavi, T., Zutkhi, Y., Yannai, S., Lisker, N., Ben-Arie, R., 1996. Ozone for control of post-harvest decay of table grapes caused by *Rhizopus stolonifer*. Physiological and Molecular Plant Pathology 48, 403-415.

Scharff, R.L., McDowell, J., Medeiros, L., 2009. Economic cost of foodborne illness in Ohio. Journal of Food Protection 72, 128-136.

Selma, M.V., Allende, A., López-Gálvez, F., Conesa, M.A., Gil, M.I., 2008. Disinfection potential of ozone, ultraviolet-C and their combination in wash water for the fresh-cut vegetable industry. Food Microbiology 25, 809-814.

Sharma, M., Ingram, D.T., Patel, J.R., Millner, P.D., Wang, X., Hull, A.E., Donnenberg, M.S., 2009. A novel approach to investigate the uptake and internalization of *Escherichia coli* O157:H7 in spinach cultivated in soil and hydroponic medium. Journal of Food Protection 72, 1513-1520.

Sharma, M., Patel, J.R., Conway, W.S., Ferguson, S., Sulakvelidze, A., 2009. Effectiveness of bacteriophages in reducing *Escherichia coli* O157:H7 on fresh-cut cantaloupes and lettuce. Journal of Food Protection 72, 1481-1485.

Singh, N., Singh, R., Bhunia, A., Stroshine, R., 2002. Efficacy of chlorine dioxide, ozone, and thyme essential oil or a sequential washing in killing *Escherichia coli* O157:H7 on lettuce and baby carrots. Lebensmittel-Wissenschaft und-Technologie 35, 720-729.

Sivapalasingam, S., Friedman, C.R., Cohen, L., Tauxe, R.V., 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. Journal of Food Protection 67, 2342-2353.

Sotelo, J.L., Beltran, F.J., Benitez, F.J., Beltran-Heredia, J., 1987. Ozone decomposition in water: kinetic study. Industrial & Engineering Chemistry Research 26, 39-43.

Staehelin, J., Hoigne, J., 1985. Decomposition of ozone in water in the presence of organic solutes acting as promoters and inhibitors of radical chain reactions. Environmental science & technology 19, 1206-1213.

Strickland, W., Sopher, C.D., Rice, R.G., Battles, G.T., 2007. Six Years of Ozone Processing of Fresh Cut Salad Mixes. Available at: http://www.chimia.it/pdf/Paper%206.09.pdf

Sy, K.V., Murray, M.B., Harrison, M.D., Beuchat, L.R.,2005. Evaluation of gaseous chlorine dioxide as a sanitizer for killing *Salmonella*, *Escherichia coli* O157: H7, *Listeria monocytogenes*, and yeasts and molds on fresh and fresh-cut produce. Journal of Food Protection 68, 1176-1187.

Takeuchi, K., Frank, J.F., 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. Journal of Food Protection 63, 434-440.

Tomiyasu, H., Fukutomi, H., Gordon, G., 1985. Kinetics and mechanism of ozone decomposition in basic aqueous solution. Inorganic chemistry 24, 2962-2966.

Ullmann, F., Gerhartz, W., Yamamoto, Y.S., Campbell, F.T., Pfefferkorn, R., Rounsaville, J.F., 2005. Ullmann's encyclopedia of industrial chemistry, VCH Verlagsgesellschaft.

Von Gunten, U., 2003. Ozonation of drinking water: Part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. Water research 37, 1469-1487.

Vurma, M., 2009. Development of ozone-based processes for decontamination of fresh produce to enhance safety and extend shelflife. Ph.D. dissertation. The Ohio State University, Columbus, Ohio.

Yousef, A.E., Rodriguez-Romo, L.A., 2004. Methods for decontaminating shell eggs

Warriner, K., Ibrahim, F., Dickinson, M., Wright, C., Waites, W.M., 2003. Interaction of *Escherichia coli* with growing salad spinach plants. Journal of Food Protection 174; 66, 1790-1797.

Widmer, G., Clancy, T., Ward, H.D., Miller, D., Batzer, G.M., Pearson, C.B., Bukhari, Z., 2009. Structural and biochemical alterations in *Giardia lamblia* cysts exposed to ozone.

Zhao, J., Cranston, P.M., 1995. Microbial decontamination of black pepper by ozone and the effect of the treatment on volatile oil constituents of the spice. Journal of the science of food and agriculture 68, 11-18.

Zhuang, R., Beuchat, L., Angulo, F.,1995. Fate of *Salmonella montevideo* on and in raw tomatoes as affected by temperature and treatment with chlorine. Applied and Environmental Microbiology 61, 2127.

Zink, D.L., 1997. The impact of consumer demands and trends on food processing. Emerging infectious diseases 3, 467.