Ozonated Water with Plant Antimicrobials: An Effective Method to Inactivate *Salmonella enterica* on Iceberg Lettuce

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Introduction

The contamination of fresh produce by foodborne pathogens results in 9.5 million illnesses in the United States each year causing $39 billion in medical losses (Schraff 2010). Transfer of pathogens to produce could happen in the field making post-harvest washing treatment an important step. Iceberg lettuce could get contaminated in the farm environment by pathogens such as *Salmonella enterica* which could attach to open stomata, fissures in the cuticle or trichome. Postharvest washing is an important step to reduce contamination by foodborne pathogens on lettuce leaves, since consumption does not usually involve heating.

Chlorine is a commonly used sanitizer by the produce industry. The efficacy of chlorine decreases with reuse and the byproducts of chlorine could have adverse health effects. Alternatives to chlorine are being aggressively explored by the produce industry. Ozone (O_3) is an allotrope of oxygen used for the disinfection of bottled water and waste water treatment (Rice, Robson, Miller, & Hill, 1981). In bacteria, it may act as a protoplasmic oxidant causing progressive oxidation of vital cellular components (Khadre, Yousef, & Kim, 2001). Ozone is approved by the United States Food and Drug Administration for use as a disinfectant or sanitizer in the gas or liquid phase on food including meat and poultry and has Generally Recognized as Safe (GRAS) status (FDA, 1982; USDA, 1984). Ozone is effective against a broad range of Gram-positive and Gram-negative bacteria (Guzel-Seydim, Greene, & Seydim, 2004; Khadre et al., 2001).

Essential oils from herbs and spices have been known to exhibit antimicrobial properties. Numerous essential oils have shown antimicrobial activity *in vitro* against different foodborne pathogens such as *Escherichia coli*, *Campylobacter jejuni*, *S. enterica*, and *Listeria monocytogenes*

(Friedman, Henika, & Mandrell, 2002; Olasupo, Fitzgerald, Gasson, & Narbad, 2003). Essential oils, also fall under the "GRAS" status and can be used as sanitizers for the washing of produce without the risk of adverse health effects to the consumer and the environment. Olive extract and other plant derived compounds have demonstrated antimicrobial activity against *Salmonella* on leafy greens (Moore, Patel, Jaroni, Friedman, & Ravishankar, 2011). Romaine lettuce, iceberg lettuce, mature spinach, and baby spinach showed between 0.7-4.8-log, 0.8-4.8-log, 0.8-4.9-log, and 0.5-4.7-log CFU/g reductions in *S.* Newport, respectively, when washed with oregano oil containing wash water (Moore-Neibel, Gerber, Patel, Jaroni, Friedman, & Ravishankar, 2012).

While oils are generally not miscible in water, the use of a tensioactive agent in wash water could aid in better suspension of the oil and result in better antimicrobial activity. Quillaja saponins are natural tensioactive agents with soap-like properties. While both olive extract and oregano oil have demonstrated antimicrobial activity against foodborne pathogens on organic leafy green surfaces (Moore-Neibel et al., 2012; Moore et al., 2011; Wallace, 2004), the combinations of these compounds with ozonated water could result in lesser treatment time and increased antimicrobial activity.

The objective of this study is to evaluate the efficacy of ozone in combination with plant extracts and essential oils in decreasing *Salmonella* population on iceberg lettuce leaves. Antimicrobials that were tested individually and in combination with ozone include carvacrol, oregano oil, olive extract and Quillaja saponin. Quillaja saponin is obtained from the bark of the South American soaptree. While carvacrol, oregano oil, and olive extract have been known to have antimicrobial properties, the Quillaja saponin has been known to have a detergent effect, hence could possibly cause the detachment of pathogens from the leaf surface. The combination of ozonated water and plant-based antimicrobials can provide a dual hurdle approach to controlling

Salmonella on iceberg lettuce surfaces which could prove to be a more effective sanitation method than washing with chlorinated water. Chlorine and many oxidants have limited activity under high organic loads; hence, the use of plant antimicrobials in combination with ozone could help overcome the limitations of chlorinated water use. This approach will be of benefit specifically for organic iceberg producers who are limited in their sanitization options for washing fresh cut iceberg lettuce. The conventional producers can use this as an alternative method for chlorinated wash.

Materials and Methods

Bacterial culture: *Salmonella enterica* serovar Newport SN78 (dual antibiotic resistant) was used for this experiment. The antibiotic resistance pattern of the strain provided efficient traceability in soil and composts. The frozen stock culture was initially revived through two transfers into brain heart infusion broth (BHIB) followed by isolation on xylose lysine desoxycholate (XLD) agar containing 100µg/ml ampicillin and 25µg/ml streptomycin. Growth rate of this isolate is similar to those of non-resistant serotypes. For each experiment, a 20 h overnight culture was prepared by inoculating 100 µL of inoculum in 30 ml of tryptic soy broth (TSB) containing 100µg/ml ampicillin and 25µg/ml streptomycin and incubating at 37°C in a shaking incubator at 200 rpm. The culture was centrifuged at 4000xg and the supernatant was discarded. The pellet was washed in 30 ml phosphate buffered saline (PBS) twice and the final suspension of washed cells was used to inoculate iceberg lettuce leaves.

Inoculum preparation: Inoculum was prepared by adding 20 ml of the PBS- *S.* Newport suspension to 180 ml of PBS in a sterile 1 L stomacher bag.

Produce preparation and inoculation: The iceberg lettuce used for this study was obtained from a local grocery store in Tucson, Arizona. The four outer leaves of the iceberg lettuce were removed and discarded. Whole leaf samples (10 g ± 0.5 g each) were used. The iceberg lettuce portions were inoculated by immersing 10 g portions of iceberg lettuce into the PBS- *S.* Newport suspension for 2 minutes each. After immersion, the excess culture suspension was allowed to drain from the leaf and the leaves were allowed to dry in a biohood for 1 h to aid in attachment of *S.* Newport to leaf surface.

Antimicrobial wash solution preparation: The test plant antimicrobials used in this study consisted of oregano oil made from pure *Origanum vulgare* (Lhasa Karnak Company, Berkeley, California), its active component carvacrol (98% pure, molecular weight 150.2,CAS no. 499-75-2, Sigma, St. Louis, MO), olive extract (CreAgri, Hayward, CA) and Quillaja saponin made from *Quillaja saponaria* (Sigma Aldrich, St. Louis, MO). The essential oil and its' active component were tested at concentrations of 0.1 and 0.5% while the plant extract was tested at concentrations of 1 and 5%. Saponin was tested at concentrations of 0.0001%. All plant antimicrobials were prepared by mixing thoroughly and dissolving in 2 L PBS.

Ozonation equipment: Ozone gas was generated using an ozone generator (ForeverOzone™ OG-5G-BB). The unit consists of a 5 KV transformer, a 25 LPM air pump, 110 CFM, 120 mm AC fan for cooling and a corona discharge generator producing 5000 mg/h of ozone. Ozone was generated into the produce wash suspension using a modified sparger made out of perforated tubing. The "perforated tube" sparger was designed in the Ravishankar lab. Clear PVC tubing, 30 cm (Nalgene 180 clear plastic tubing, I.D.,1/8 inch x O.D.3/16 inch x Wall 1/32 inch, Nalgene Nunc Int. Corp; Rochester, NY) was perforated using a 12 gauge insulin syringe (Walgreens, Tucson, AZ). Maximum perforations were created in the middle of the tube with the

concentration of perforations tapering towards the edges to provide even distribution of ozone bubbles. The ozone generator was connected to the perforated tube sparger using a T connector (Thermo Scientific, Hudson, NH).

Ozone measurement: Residual ozone in wash waters was measured using a spectrophotometer for a concentration range of 0.01 to 0.1 mg O_3/L using the Indigo Colorimetric Method (Standard methods for the examination of waters and waste waters, 20th edition) (APHA, 2005). Briefly, 10 ml of Indigo reagent 1 (20 ml potassium indigo trisulfonate stock solution, 10 g sodium dihydrogen phosphate, 7 ml concentrated sulfuric acid) was added to two sterile 100 ml measuring cylinders. The ozonated water was added to one cylinder and regular deionized water was added to the blank cylinder. The absorbance difference was measured between the two solutions at 600 nm to determine ozone content in aqueous ozone.

Ozone-plant antimicrobial Treatment: The 2 L aliquot of PBS antimicrobial suspension was placed in a 5 L sterile stomacher bag. The bag was cooled to a temperature of 1-4°C using a combination of ice and dry ice for the entire duration of the ozonation treatment. The water was chilled to promote better retention of ozone in the aqueous phase. The perforated ozone sparger was placed at the bottom of the bag and the water was circulated using an aquarium pump (Top Fin® Power Head 50, PetSmart, Tucson, AZ) for better dispersion of the ozone molecules. The wash suspension was ozonated for 30 min before leaf treatment. Leaf treatments were performed for 60, 90 and 120 minutes when wash water suspension contained individual plant antimicrobials (oregano oil, olive extract) and for 60 and 90 minutes when carvacrol was used. When combinations of plant antimicrobials with saponin were evaluated for antimicrobial efficacy, ozonation was carried out for a duration of 20 min.

Microbiological analysis: Leaf samples were collected immediately after treatment for the enumeration of surviving *Salmonella*. Leaf samples (10 g) were pummeled in the stomacher at normal speed (230 rpm) in 90 ml BPW for 1 min. Enumeration of survivors following treatment was carried out by spread plating the serially diluted abovementioned suspensions on XLD agar containing 100µg/ml ampicillin, and 25µg/ml streptomycin The plates were incubated at 37°C for 24 h and the *Salmonella* colonies were counted. The experiments were repeated at least three times.

Results

Iceberg lettuce leaf treatment with ozone- oregano oil combinations

Ozone was used in combination with oregano oil to wash iceberg lettuce. The concentrations of oregano oil used were 0.1 and 0.5% and the duration of washing was 60, 90 and 120 min. The use of 0.1% oregano oil in combination with ozone resulted in a decrease in population from 6.4±0.20 to 2.3±2.4, 3.0±1.77 and 3.0±1.2 log CFU/g reduction of *S*. Newport cells on iceberg lettuce after 60, 90 and 120 min respectively (Figure 2). Use of ozone- 0.5% oregano oil combination resulted in reduction of *S*. Newport population to below detection limits from an initial population of 6.39±0.5 log CFU/g (Figure 2).

Iceberg lettuce leaf treatment with ozone- olive extract combinations

Treatment of iceberg lettuce with ozone and olive extract combinations was performed for a duration of 60, 90 and 130 min. The concentration of olive extract used for the combination wash with ozone was 1% and 5%. The use of 1% olive extract resulted in a decrease in *S*. Newport population from 6.05±0.89 to 5.21±0.55, 3.67±0.57 and 3.7±0.76 log CFU/g reduction of *S*. Newport cells on iceberg lettuce after 60, 90 and 120 min respectively (Figure 2). The use of 5%

olive extract for 60, 90 and 120 min resulted in a 3.52±1.00, 2.92±0.93, 2.60±1.8 log CFU/g reduction of *S.* Newport cells on iceberg lettuce respectively, from an initial population of 6.47±0.41 log CFU/g (Figure 2).

Iceberg lettuce leaf treatment with ozone- carvacrol combinations

Combination treatments of ozone with carvacrol were performed for 60 and 90 minutes. The concentrations of carvacrol used were 0.1%, 0.3% and 0.5%. The use of carvacrol in combination with ozone at all three concentrations and both treatment times resulted in reduction of *S.* Newport populations to levels below detection (Table 1). The use of 0.1, 0.3 and 0.5% carvacrol in combination with ozone for 60 min resulted in reductions of 5.11±0.38, 5.99±0.52, 7.29±0.32 log CFU/g of *S.* Newport cells on iceberg lettuce, respectively. The use of 0.1, 0.3 and 0.5% carvacrol in combination with ozone for 90 min resulted in reductions of 6.34±1.69, 6.55±1.3, 7.54 ±0.21 CFU/g *S.* Newport cells on iceberg lettuce, respectively.

Iceberg lettuce leaf treatment with ozone- Saponin and plant antimicrobial combinations

To determine the efficacy of ozone washes in combination with the tensioactive agent, saponin and plant antimicrobials, the lowest concentrations and exposure times were used. The concentrations of saponin, olive extract and oregano oil used were 0.0001%, 1% and 0.1%, respectively. All treatments were performed for 20 min. The use of ozone and saponin combination resulted in a reduction from 7.48±0.23 log CFU/g to 6.26±0.29 CFU/g *S.* Newport cells on iceberg lettuce (Figure 1). The use of ozone in combination with saponin and oregano oil resulted in a reduction of 4.9±0.3 log CFU/g from 7.48±0.23 log CFU/g (Figure 1). Combination of ozone, saponin and olive extract resulted in a reduction of 4.19±0.3 log CFU/g from 7.48±0.23 log CFU/g (Figure 1). The highest reduction of *S.* Newport population on iceberg lettuce leaf resulted from a

combination of ozone, saponin, oregano oil and olive extract resulting in a reduction of 3.4 log CFU/g. The treatment resulted in a population of 4.08±0.56 log CFU/g of *S.* Newport cell from a population of 7.48±0.23 log CFU/g (Figure 1).

Discussion & Conclusion

The use of plant based antimicrobials in combination with aqueous ozone is a novel approach toward postharvest washing of produce. The study involved the use of ozonated water in combination with oregano oil, olive extract, carvacrol and saponins. Previous research with aqueous ozone wash of leafy greens have indicated mixed outcomes. The washing of cilantro leaves with ozonated water did not result in a decrease in total plate counts after treatment (Wang, Feng, & Luo, 2004). Exposure to aqueous ozone for 3 min of a mixture of shredded lettuce and water resulted in decreased counts of mesophilic and psychrotrophic bacteria by 1.4 and 1.8 log cfu/g, respectively (Kim, Yousef, & Chism, 1999).

The combination of ozone and plant antimicrobials resulted in a significant decrease in *S.* Newport population from iceberg lettuce. The use of ozone with carvacrol (0.1%, 0.3% and 0.5%) and 0.5% oregano oil exhibited a reduction of the pathogen to below levels of detection from initial populations exceeding 6 logs CFU/g. The duration of treatment was 60, 90 and 120 min. Ozone is an allotropic modification of oxygen with a pungent characteristic odor. Ozone is a powerful antimicrobial because of its progressive oxidation of vital cellular components. Ozone may cause damage to the unsaturated lipids and peptidoglycan in cell envelopes, respiratory enzymes, lipopolysaccharide layer of Gram-negative bacteria, intracellular enzymes and nucleic acids present in the cytoplasm. It may act as a protoplasmic oxidant leading to cell lysis and leakage (Kim, Yousef, & Khadre, 2003; White, 2010). Essential oils such as oregano oil and its components such as carvacrol possess antimicrobial activity and can result in increased cell

permeability, leakage of inorganic ions and dissipated pH gradients (Lambert, Skandamis, Coote, & Nychas, 2001).

While the antimicrobial activity of ozone was determined through microbiological analysis, aqueous ozone was not detected in water containing olive extract and saponins. Ozone is relatively unstable in aqueous solutions decomposing continuously to oxygen. Various parameters affect the disassociation of ozone in water. Solubility of ozone in water increases with a decrease in temperature and smaller bubble size. Purity and pH also greatly affect the solubility of ozone. Organic matter consumes ozone and may compete with microorganisms, reducing the efficacy of ozone (Guzel-Seydim et al., 2004; Khadre et al., 2001; Kim, Yousef, & Dave, 1999). The presence of olive extract and saponin in wash water could have resulted in the quenching of ozone and absence of residual ozone in the aqueous phase.

The efficacy of saponin-ozone-plant antimicrobial combinations was also determined. Saponins obtained from the quillaja bark and olive extracts caused foaming of the wash water. The concentration of 0.0001% saponin, 1% olive extract and 0.1% oregano oil resulted in excess of 4 log CFU/g reduction of *S.* Newport within 20 min. While chlorination could result in pitting or corrosion of equipment and presence of carcinogenic byproducts (Pao, Kelsey, Khalid, & Ettinger, 2007), the use of quillaja saponin could be advantageous because of its detergent like nature which could result in reduced attachment strength of pathogenic bacteria to equipment surfaces. Hence, use of ozone in combination with plant antimicrobials could be an effective post-harvest processing step for the iceberg lettuce industry. Parameters such as equipment, water temperature, organic load of water, plant antimicrobial and ozone dispersion play an important role in the efficacy of the treatments. Both ozone and plant essential oils have GRAS status and the ability of

ozone to neutralize off odors and taste could also result in an organoleptic synergy for essential oil use.

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Tables & Figures

Table 1. Survival of *S.* Newport (Log CFU/g) on iceberg lettuce after ozone and carvacrol combined treatment

Sample	Ozone+0.1% Carvacrol	Ozone+0.3% Carvacrol	Ozone+0.5% Carvacrol
Inoculum	6.58±0.13	6.77±0.59	7.49±0.32
Control 1	5.66±0.37	5.63±0.07	5.48±0.48
Control 2	5.69±0.20	5.86±0.15	5.45±0.44
60 min	0.00±0.00	0.00±0.00	0.00±0.00
90 min	0.00±0.00	0.00±0.00	0.00±0.00

Figure 1: *S.* Newport population (log CFU/g) on iceberg lettuce leaves after washing for 20 min in ozone-saponin, ozone-saponin-oregano oil, ozone-saponin-olive extract, ozone-saponin-olive extract-oregano oil combinations. Concentrations of saponin, olive extract and oregano oil were 0.0001%, 1% and 0.1%, respectively.

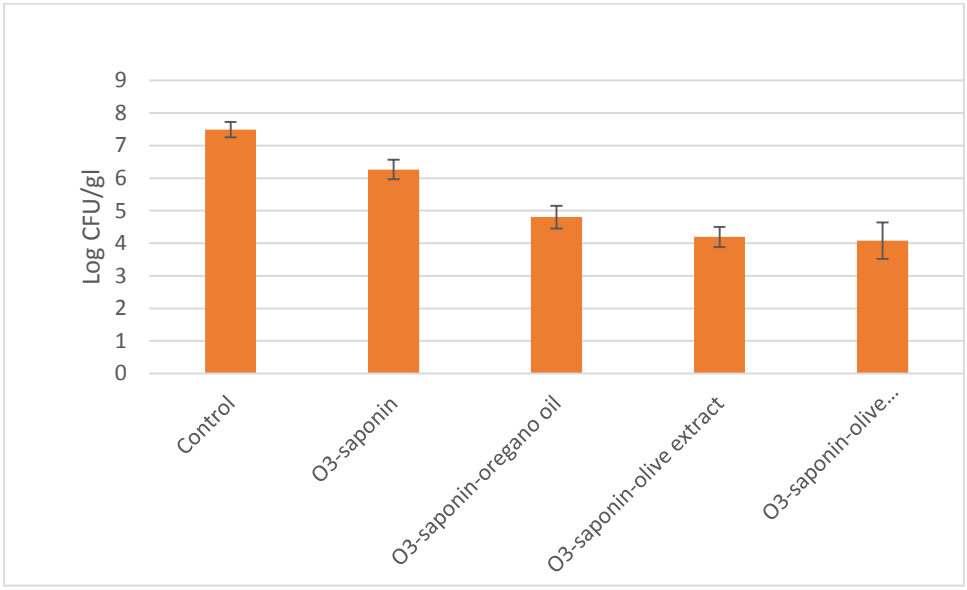

Figure 2: Comparative evaluation of antimicrobial efficacy of ozone and combinations of ozone-olive extract, and ozone-oregano oil against *S.* Newport (reductions shown in log CFU/g) on iceberg lettuce leaves. Ozone, air and air-antimicrobial combinations were used as controls.