CENTER *for* PRODUCE SAFETY

CPS 2020 RFP
FINAL PROJECT REPORT

Project Title
Application of ultra-fine bubble technology to reduce *Listeria monocytogenes* contamination of fresh produce

Project Period
January 1, 2021 – December 31, 2021 (extended to February 28, 2022)

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Objectives

1. *Investigate the stability of ultra-fine ozone (UFO) bubbles in a simulated single pass or circulating hydrocooling system.*
2. *Investigate the efficacy of UFO bubble water wash (either alone or in combination with commercial sanitizers) in inactivating Listeria monocytogenes on Gala apples, romaine lettuce and celery, and test the survival of the pathogen in the wash water.*
3. *Evaluate the effect of UFO bubble wash (with or without commercial sanitizers) on shelf life and color of Gala apples, romaine lettuce and celery.*

Funding for this project provided by the Center for Produce Safety through:
CPS Campaign for Research

FINAL REPORT

Abstract

The widespread distribution of *Listeria monocytogenes* in agricultural environments, such as soil, manure and water, results in frequent contamination of food processing areas. Water used for washing or hydrocooling can act as a source of equipment and produce contamination with *L. monocytogenes.* Since this could lead to human infections, controlling *L. monocytogenes* in hydrocooling water and on the surface of fresh produce is critical for food safety. Currently used commercial disinfectants are not completely effective in killing *L. monocytogenes* in wash water or on the surface of produce, especially in the presence of organic load. Therefore, there is a need for developing novel strategies that could be employed to control *L. monocytogenes* in wash water and on surface of fresh produce. The overall goal of this proof-of-concept proposal was to develop novel washing treatments using ultra-fine bubble technology in combination with ozone to reduce the survival of *L. monocytogenes* on fresh produce and in wash water. Washing of apples, celery and lettuce with ultra-fine ozone-bubble water significantly reduced *L. monocytogenes* load by ~1 log CFU/sample with 1 min of treatment time ($P<0.05$). Ultra-fine ozone bubbles were effective in reducing *L. monocytogenes* on celery, apples and lettuce at both 25 and 4°C ($P<0.05$) without affecting color parameters of the food products ($P>0.05$).

Background

Listeria monocytogenes is a major foodborne pathogen that has been responsible for multiple multistate outbreaks in the United States (Scallan et al., 2011). The ubiquitous distribution of *L. monocytogenes* in the environment results in frequent contamination of food processing facilities, and food products (Ferreira et al., 2014). Although listeriosis outbreaks have been traditionally associated with consumption of ready-to-eat (RTE) and cold-stored meat and dairy products, an increasing number of reports in the past two decades show increasing contamination and prevalence of *L. monocytogenes* in fresh produce. Produce such as lettuce (Thunberg et al., 2002; Ding et al., 2013; Althaus et al., 2012; Self et al., 2019), celery (Gaul et al., 2013), melons (CDC, 2012) and caramel apples (CDC, 2015) have been linked to listeriosis outbreaks in the recent past. Water used for cleaning (e.g., in dump tanks) or hydrocooling (single pass or recirculated systems) produce can act as a source of product contamination with *L. monocytogenes.* Moreover, since *L. monocytogenes* is able to form sanitizer tolerant biofilms (Kostaki et al., 2012; Upadhyay et al., 2013) that can survive at refrigeration temperature, there is a high risk of persistence of the pathogen in equipment and hydrocoolers, resulting in recurring contamination of produce. Therefore, decontamination of dump tank wash water and water used for hydrocooling is critical for maintaining the microbiological safety of fresh produce. Scientific literature suggests that commonly employed disinfectants, including chlorine, peracetic acid and quaternary ammonium compounds, are not completely effective in killing *L. monocytogenes* on food products (Laird et al., 1991; Gil et al., 2009; Pezzuto et al., 2016). Moreover, the presence of chemical residues and the formation of harmful organochlorine compounds is of concern due to associated health risks, including cancer (Zhang and Farber, 1996; Richardson et al., 1998; Parish et al., 2003; Donato and Zani, 2010). Therefore, there is a need for developing a novel antimicrobial strategy for reducing *Listeria* contamination on fresh produce and survival in wash water and water used for hydrocooling.

Ultra-fine bubble technology: In recent years, ultra-fine bubble technology has drawn tremendous attention, due to its application in a wide variety of fields, such as water/sewage

treatment (Liu et al., 2010; Agarwal et al., 2011), biomedical engineering (Suzuki et al., 2011), pesticide removal (Ikeura et al., 2011), dental hygiene (Lin et al., 2015) and drug delivery (Thakur et al., 2017). The unique characteristics of ultra-fine bubbles, including increased solubility of gases in liquids, reduced friction, and capacity to generate free radicals, provide efficacy in the aforementioned applications (Takahashi et al., 2003; Chu et al., 2008). Ultra-fine bubbles are defined as small, spherical, gas-filled cavities within liquids, with a diameter between 50 to 200 µm (Takahashi, 2005; Hideki, 2014). The electrostatic interactions between nanobubbles (due to their surface negative charge) in liquid prevent coalescence and facilitate their uniform distribution in liquid. When combined with a gas of choice, the technology has the potential to influence several sectors of agriculture. Ozone (triatomic form of oxygen) is a strong oxidizing agent that is widely used in the industry for sterilization, virus inactivation, deodorization, and organic matter decomposition (Takahashi et al., 2007). Aqueous ozone has strong antimicrobial activity against bacteria and does not induce microbial resistance (Kim et al., 1999; Unal et al., 2001; Paraskeva and Graham, 2002; Nagayoshi et al., 2004; Huth et al., 2009). Moreover, ozone readily decomposes to oxygen without generating harmful residues, making it safe for food washing applications. However, ozone has low water solubility and a half-life of about 20 min before it degrades back to oxygen (Ikeura et al., 2011; Hayakumo et al., 2014). Therefore, aqueous ozone should be used within the first 5 to 10 min after production to ensure antimicrobial potency. This requirement significantly reduces its application for produce decontamination. The application problem due to short half-life of ozonated water could be overcome by developing ozone nanobubbles in water for washing fresh produce. Previous research conducted by Chiba and Takahashi (2008) has shown the successful development of ozone micro and nanobubbles. Since then, several applications of ozone micro/nanobubbles have been published. For example, ozone microbubbles were found to be highly effective in removing agricultural chemical residues from leafy vegetables (lettuce) and fruits such as tomatoes and strawberries (Ikeura et al., 2011). In another study, Hayakumo and coworkers (2014) inactivated periodontopathic bacteria (causing oral/gum disease) using ozone nanobubble water application. In addition, ozone nanobubble water can be stored without losing antimicrobial efficacy thereby further improving its application potential. In a recent study, Seki and coworkers (2017) showed that ozone nanobubble water, when stored frozen, maintained its antimicrobial efficacy against *Mycobacterium megmatis*, one of the most disinfectant-resistant bacteria. Application of ozone nanobubble water after 1 year of frozen storage killed the bacteria within 15 min, rendering it as an attractive sanitizer for small, medium and large farm operations. With an aim to target the industry's need of a microbiologically safe fresh produce supply chain, the overall goal of this proof-of-concept proposal was to develop novel washing treatments using ultra-fine bubble technology in combination with ozone to reduce the survival of *L. monocytogenes* on fresh produce and in water used for washing (dump tank, hydrocooling water).

Research Methods and Results

Objective 1: Investigate the stability of ultra-fine ozone (UFO) bubbles in a simulated single pass or circulating hydrocooling system.

Study 1: Effect of water temperature on ozone solubility and stability.

Rationale: Since ozone is generated on-site, it is critical to study the impact of type of machine, liquid temperature, and bubble time on ozone solubility. Also, since ozone naturally disintegrates to oxygen, understanding the disintegration kinetics of ozone in liquid medium and the various factors that impact the rate of disintegration is important for designing appropriate food safety experiments and develop recommendations for the produce industry.

Method: The solubility and stability of ozone in water maintained at 25 and 4°C was measured at different bubbling times (0, 5, 10, and 15 min). The ozone was generated using an ozone generator (Ivation, USA; ozone generation rate of 600 mg/h) and bubbled in 500 ml of DI water using a micro bubble diffuser. The concentration of ozone was measured post-bubble time at 0, 5, 10, 15, 20, 40 and 60 min using the Vacu-vials kit (SAM, OZONE, CHEMetrics, I-2019).

Results and discussion: The temperature of the liquid had a significant effect on ozone solubility. Approximately 1 ppm of dissolved ozone was observed in the water maintained at 4°C after 15 min of bubbling time. However, when the water was maintained at 25°C, the dissolved ozone level obtained after 15 min of bubbling time was ~0.5 ppm (~50% less solubility; $P<0.05$). The ozone disintegration time was also higher in water maintained at 25°C than at 4°C. After 60 min post-bubble time, the ozone level reduced by ~90% in water maintained at 25°C, whereas the ozone level reduced by ~30% in water maintained at 4°C ($P<0.05$), suggesting that ozone has a higher solubility and stability at lower temperature.

Study 2: Effect of oxygen concentrator and corona discharge tubes on ozone generation.

Rationale: The on-site production of ozone is modulated by the strength of the machine (number of corona discharge tubes) and the supply of oxygen. In order to investigate the effect of aforementioned parameters, study 2 was performed.

Method: The generation of ozone was investigated using the VMUS-4 ozone generator (Oxidation Technologies, USA) with or without an oxygen concentrator, as described above.

Results and discussion: VMUS-4 produces ozone at the rate of 4 g/h from dry air and 10 g/h from oxygen. This ozone generation rate is significantly higher than with the Ivation generator due to double corona discharge tubes present in the VMUS-4. Use of an oxygen concentrator facilitated the production of ~5 ppm of ozone in DI water (1500 ml) maintained at 25°C in 5 min. Without the oxygen concentrator, a maximum ozone concentration of ~1 ppm was observed in 5 min. These results suggest that in order to generate a higher ozone concentration in water, an ozone generator with a higher ozone output (g/h) is required. Moreover, an oxygen concentrator facilitates in generating higher levels of dissolved ozone in solution.

Study 3: Effect of water turbulence on degradation rate of dissolved ozone.

Rationale: Since washing of produce, especially in hydrocooling systems, involves circulating water, we investigated the effect of water turbulence on the stability of dissolved ozone.

Method: Ozone gas was produced using the VMUS-4 generator and oxygen concentrator set up and bubbled in 750 ml of sterilized, deionized water maintained at 4°C for 15 min. Initial ozone concentration was measured and the ozonated water was divided into three groups (control, treatment 1 and treatment 2). The water in treatment 1 was stirred with a magnetic stirrer for 15 min at 80 rpm while the water in treatment 2 was stirred for 15 min at 1150 rpm. Dissolved ozone levels were measured at 1, 5, 10, and 15 min of stirring.

Results and discussion: Stirring the ozonated water at 80 rpm for 10 and 15 min led to ~8% and 17% degradation of dissolved ozone, respectively ($P<0.05$). A higher stirring speed of 1150 rpm increased the degradation rate as compared to control. Approximately 31% and 69% of dissolved ozone degradation was observed when the ozonated water was stirred at 1150 rpm for 10 and 15 min, respectively ($P<0.05$). These results suggest that circulation or turbulence of water accelerates the degradation rate of ozone.

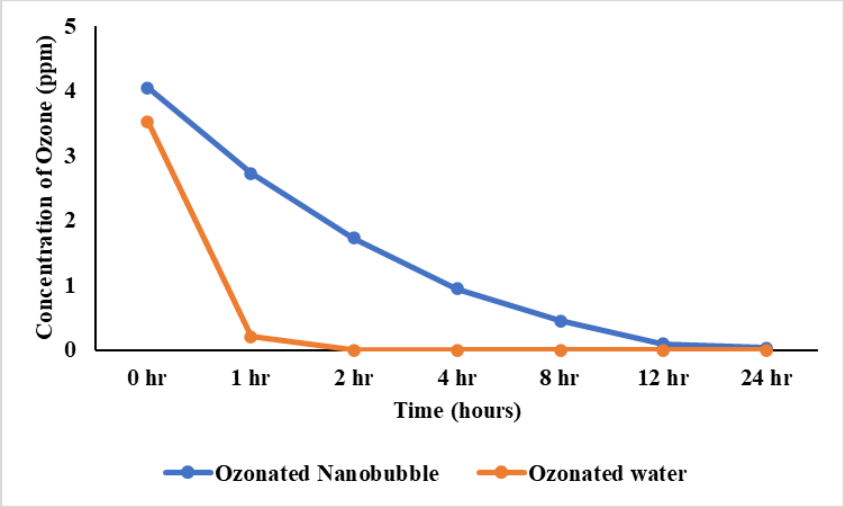
Study 4: Degradation kinetics of ozone in UFO bubbles in water.

Rationale: Once UFO bubbles are generated, it is critical to understand the degradation behavior of ozone when present in ultra-fine bubbles vs normal ozone dissolved in water (control).

Method: UFO bubbles were generated using an oxygen concentrator (Oxidation Tech)–ozone generator (Oxidation Tech)–nanobubble generator (Acniti, Japan) combination. The run time was 15 min, with an oxygen pressure of 15 psi, oxygen flow of 3 liters per min, water temperature of 25°C, and nanobubble generator spin speed of 27 Hz. For the control a similar set up was used, except for the nanobubble generator. Dissolved ozone was measured immediately after generation (time zero) and at regular intervals up to 24 h.

Results and discussion: **Figure 1** shows the degradation kinetics of ozone in UFO bubbles in water at 25°C. We observed that a dissolved ozone level of ~3.5–3.8 ppm was obtained after 15 min of run time in both control (without nanobubble generator) and treatment (with nanobubble generator). At 1, 2 and 4 h of storage at room temperature, the dissolved ozone level in ultra-fine ozone bubble water (blue line) was significantly higher as compared to normal ozone water (orange line), indicating that ultra-fine ozone bubbles might be facilitating higher dissolved ozone levels in the water ($P<0.05$).

Figure 1: Degradation kinetics of ozone in ozone nanobubbles present in water at 25°C.



Objective 2: Investigate the efficacy of UFO bubble water wash (either alone or in combination with commercial sanitizers) in inactivating *L. monocytogenes* on Gala apples, romaine lettuce and celery, and test the survival of the pathogen in the wash water.

Rationale: Once the UFO bubbles were characterized (objective 1), the next step in the project was to investigate their antimicrobial efficacy against *L. monocytogenes* on produce.

Methods:
Inoculum preparation: Five strains of *L. monocytogenes* (Scott A, AT19115, LM1, LM2 and LM3) were used in this study. Equal portions of the washed cultures were mixed together and diluted appropriately to yield a final inoculum concentration of 6 log CFU/ml. The average inoculum on apples or celery or lettuce were ~5–5.5 log CFU/sample.

Preparation of apples, celery or lettuce: Circular apple and lettuce pieces were prepared using a steel corer. For celery, the celery stalk was cut uniformly measuring ~1.5 inches in length. The cocktail of *L. monocytogenes* strains was spot inoculated (200 µl volume with 20 spots of 10 µl each; ~5.5 log CFU/sample) on the prepared fresh produce samples, followed by incubation for 2 h at 25°C in the biosafety cabinet, to facilitate bacterial attachment. The uninoculated rind plug samples were used as the negative control.

Preparation of ultra-fine ozone (UFO) bubble water: UFO bubbles were generated by using an ozone generator (Oxidation Technologies) connected to a nanobubble generator (Acniti, Japan) by running the machine for 30 min with 15 Psi of oxygen pressure. The dissolved ozone was measured post-bubble using the Vacu-vials kit (SAM, OZONE, CHEMetrics, I-2019).

Ultra-fine ozone bubble water as a wash treatment: For the control, sterile DI water was used for washing. For treatment, glass containers were filled with 500 ml of DI water for the control and 500 ml of UFO bubble water for the treatment group. The experiment had three replicates with two sub samples and repeated two times. The inoculated apples, celery or lettuce pieces were added (separately) to the containers containing respective treatments and treated for 1, 3 or 5 min at 25 or 4°C. After treatment the samples were transferred to separate Whirl-Pak™ bags (Nasco, Fisher Scientific) containing 10 ml of Dey-Engley neutralizing broth. The samples were stomached for 1 min at 300 rpm followed by dilution and plating on Oxford agar plates. The plates were incubated at 37°C for 24-48 h for microbial enumeration.

Survival of *L. monocytogenes* on apples and lettuce after UFO bubble water wash treatment: The inoculated samples were treated with the UFO bubble water at 4°C for 5 min. The control and treated samples were stored in sterile petri plates at 25°C for 3 days. The survival of the pathogen was enumerated on days 1 and 3, followed by plating onto Oxford agar plates. (*Note:* Experiments investigating the survival of *L. monocytogenes* on celery are currently underway and will be included in an addendum upon completion).

Results and discussion: The effects of ozone nanobubbles in inactivating *L. monocytogenes* on apples, celery and lettuce are presented in **Figures 2** and **3**. The level of dissolved ozone was ~8 ppm at 4°C and ~5 ppm at 25°C. At 4°C, UFO bubbles were effective in reducing *L. monocytogenes* populations on apples and lettuce by at least 1 log CFU/sample, with 1 min of wash time (Fig. 3; $P<0.05$). Increasing the treatment from 1 to 5 min improved the anti-listerial efficacy on apples, and by 5 min, *L. monocytogenes* populations were reduced by ~1.5 log CFU/sample as compared to the control ($P<0.05$). No significant increase in efficacy against *L. monocytogenes* on lettuce was observed by increasing the wash time to 5 min ($P>0.05$). In the case of celery, washing with ozone nanobubbles for 3 min at 4°C reduced *L. monocytogenes* by ~1 log CFU/sample ($P<0.05$), and increasing the wash time to 5 min did not increase the antimicrobial efficacy of UFO bubbles ($P>0.05$). At 25°C, washing of celery and lettuce for 1 min with UFO bubble water reduced *L. monocytogenes* by ~1.5 log CFU/sample (Fig. 2; $P<0.05$). UFO bubble wash at 25°C did not reduce *L. monocytogenes* on apples (Fig. 2; $P>0.05$).

Objective 3: Evaluate the effect of UFO bubble wash on shelf life and color of Gala apples, romaine lettuce and celery.

Method: The effect of UFO bubble wash on the color of apples, celery and lettuce was investigated in this study. HunterLab MiniScan XE Plus colorimeter (HunterLab Associates, Reston, VA, USA) with illuminant A, 2.54-cm diameter aperture, and 10° standard observer was used, and a* and

b* values were recorded each day for 7 days at 4°C. The a* values indicate redness of the surface and b* values indicate the yellowness of the samples.

Results and discussion: The effect of ozone nanobubble wash on produce color is presented in **Figure 4**. Washing with water containing ozone nanobubbles did not change lightness, redness or yellowness of celery, lettuce or apples ($P>0.05$), indicating that the ozone nanobubble treatment does not modulate produce color at the tested dose and time. Experiments on the effect of ozone nanobubble wash on shelf-life parameters are currently underway and results will be included in an addendum.

Statistical analysis: All experiments had duplicate samples and were repeated at least three times**.** Bacterial counts were $\log_{10}$ transformed ($\log_{10}$ CFU/sample) for analysis to achieve homogeneity of variance. Data were pooled and were analyzed using ANOVA on R statistical software. Means were partitioned by LSMEANS analysis, and a $P<0.05$ was considered statistical significance.

Outcomes and Accomplishments

The major accomplishment from this proof-of-concept project was that we were able to successfully develop ultra-fine bubbles of ozone in water and then test their efficacy in reducing *L. monocytogenes* contamination of selected fresh produce (apples, celery, lettuce)**.** Ultra-fine ozone bubbles were effective in reducing *L. monocytogenes* on fresh-cut samples of apples, celery and lettuce, without affecting their color profiles.

Summary of Findings and Recommendations

The overall aim of this proof-of-concept proposal was to investigate the efficacy of a new, nanobubble-based technology in improving the microbiological safety of apples, celery and lettuce. We prepared the antimicrobial solution containing ultra-fine bubbles of ozone, followed by quantification and characterization. The first major finding from this project was that the presence of ozone as ultra-fine bubbles modulates its degradation kinetics in the liquid (Figure 1). This discovery could have major applications in areas where extended contact time is recommended with antimicrobials. Second, we observed that the presence of ultra-fine bubbles of ozone in water was effective in inactivating *L. monocytogenes* on produce types with different surface texture. For example, washing of apples, celery and lettuce with ultra-fine ozone bubble water significantly reduced the *L. monocytogenes* load by ~1 log CFU/sample with 1 min of treatment time at 4°C ($P<0.05$; Figure 3). Ultra-fine ozone bubbles were effective in reducing *L. monocytogenes* on celery and lettuce at both 25 and 4°C ($P<0.05$; Figures 2 and 3) without affecting color parameters of the food products ($P>0.05$; Figure 4).

Based on the findings, ultra-fine ozone bubble water could be used for produce decontamination. Considering that the dissolved ozone concentration used in the experiments was between 5 to 10 ppm, higher concentrations of ozone could be tested for improving antimicrobial efficacy. In addition, combination treatments with currently used disinfectants could also be tested to improve antimicrobial efficacy against *L. monocytogenes.*

APPENDICES

Publications and Presentations

No publications have been completed; however, the research team anticipates that one conference presentation and one peer-reviewed paper will be submitted based on the research described in the report.

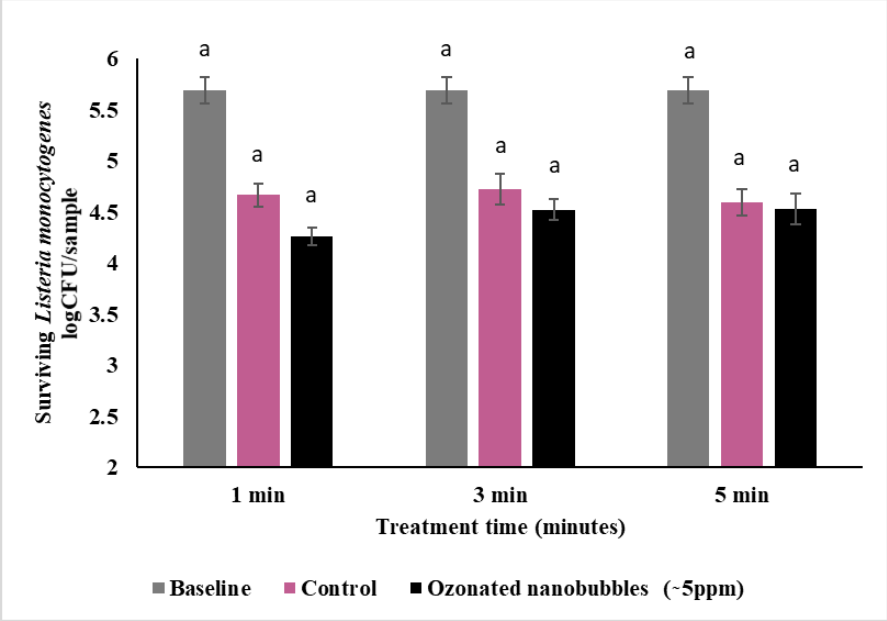
Budget Summary

A total of $49,817 was awarded to this project and the majority of the funds have been spent, pending final reconciliation.

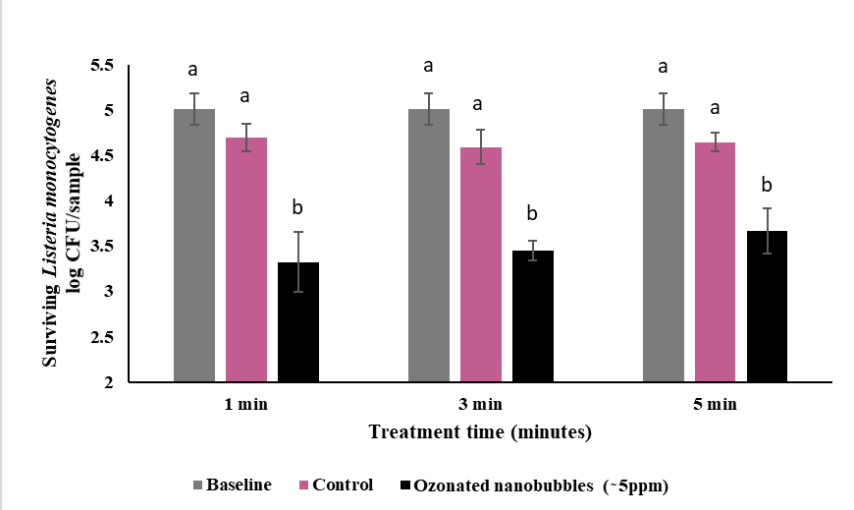
Figures 2–4 (see below)

Figure 2: Inactivation of *Listeria monocytogenes* on (a) apples (b) celery (c) lettuce at 25°C by ozone nanobubbles. Within each treatment time, bars with different letters represent significant difference between treatments.

(a) Apples



(b) Celery



(c) Lettuce

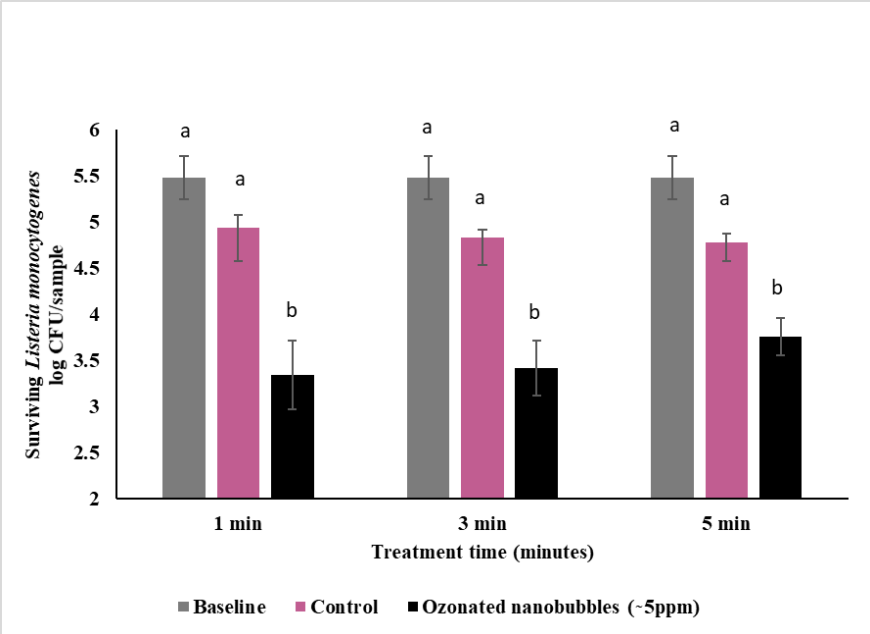
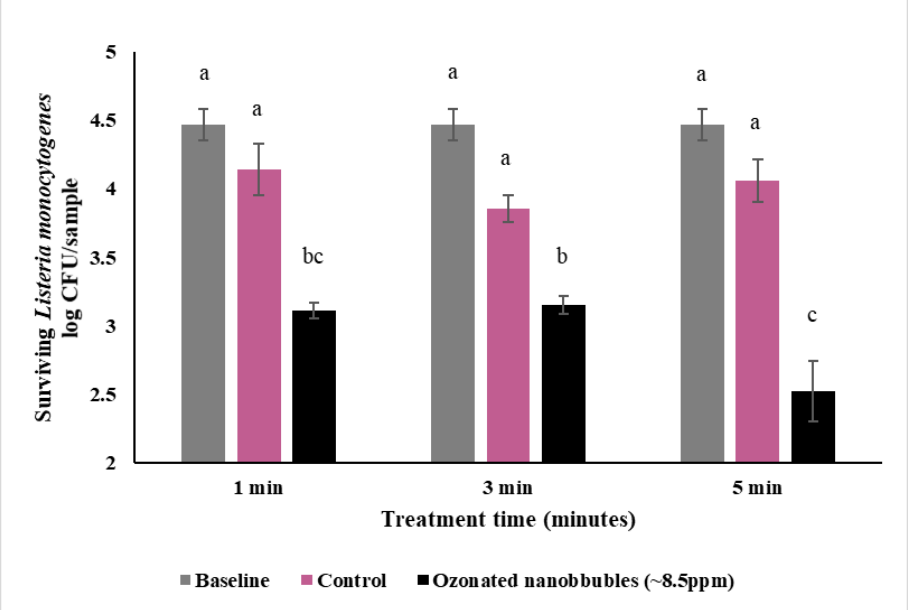
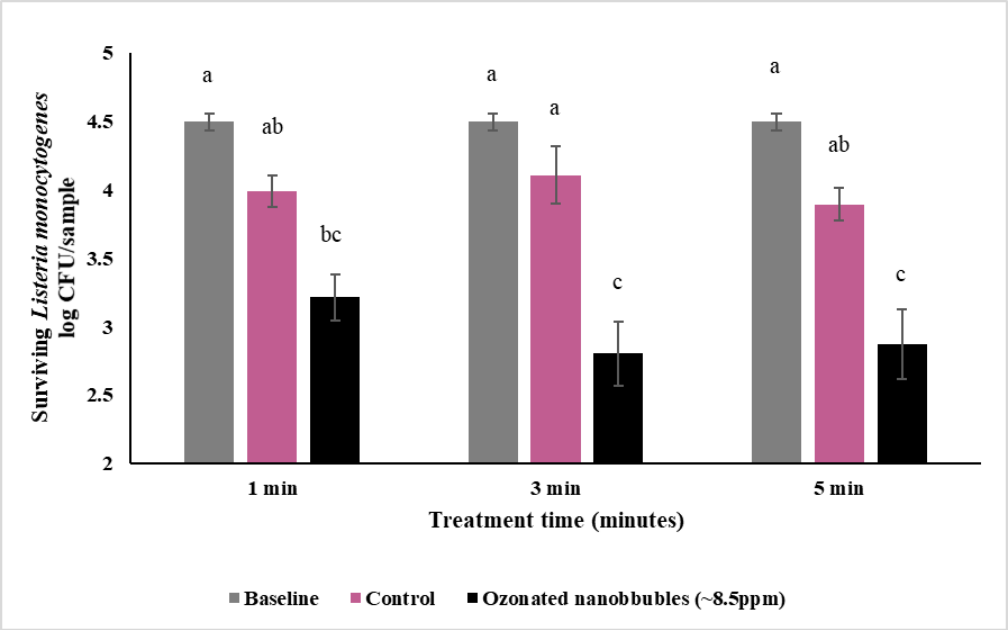


Figure 3: Inactivation of *Listeria monocytogenes* on (a) apples (b) celery (c) lettuce at 4°C by ozone nanobubbles. Within each treatment time, bars with different letters represent significant difference between treatments.

(a) Apples



(b) Celery



(c) Lettuce

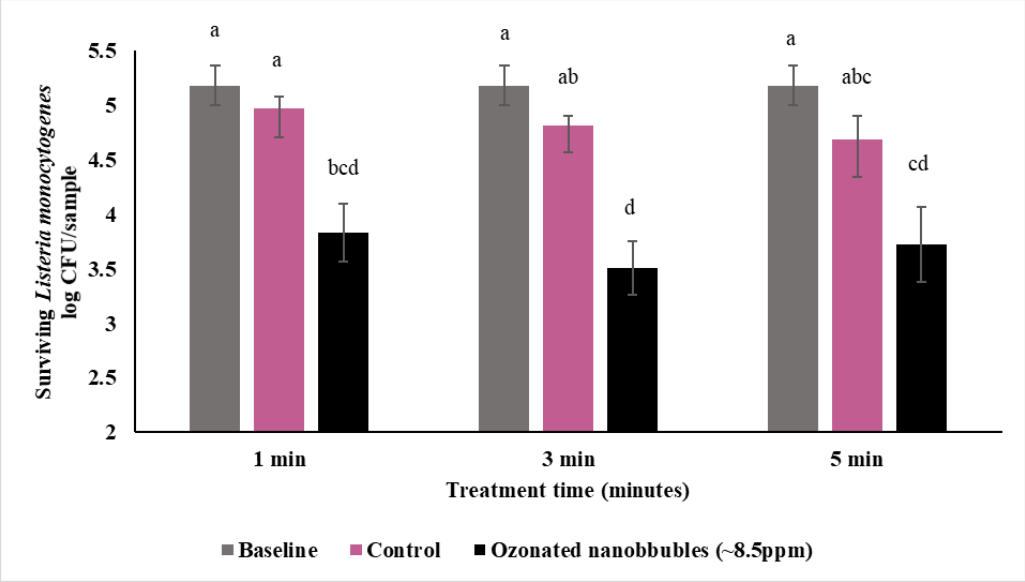
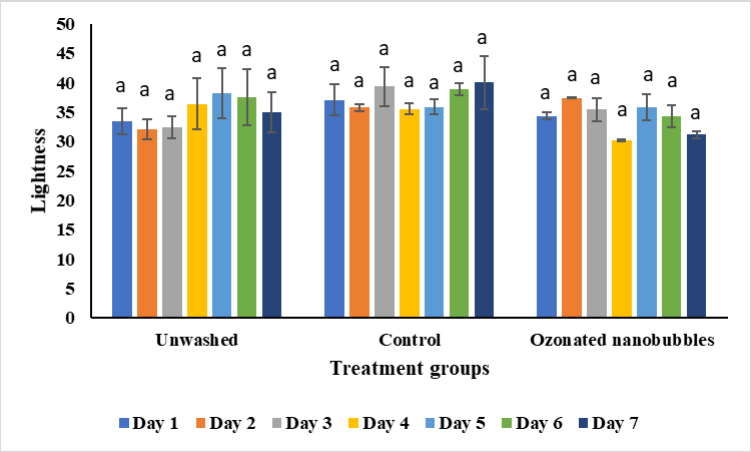
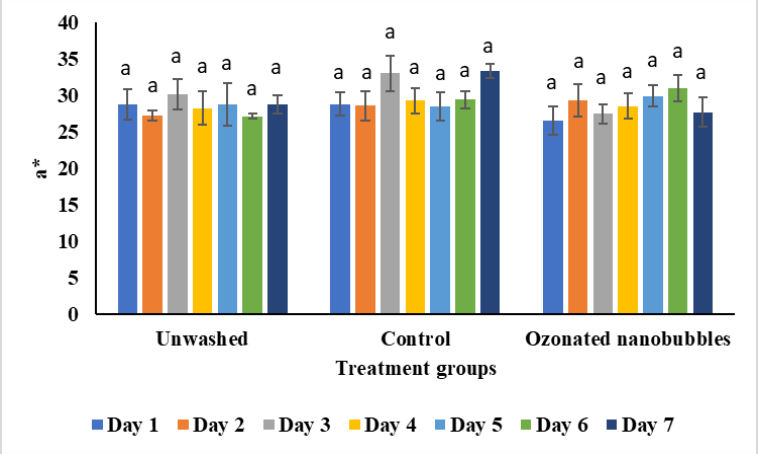
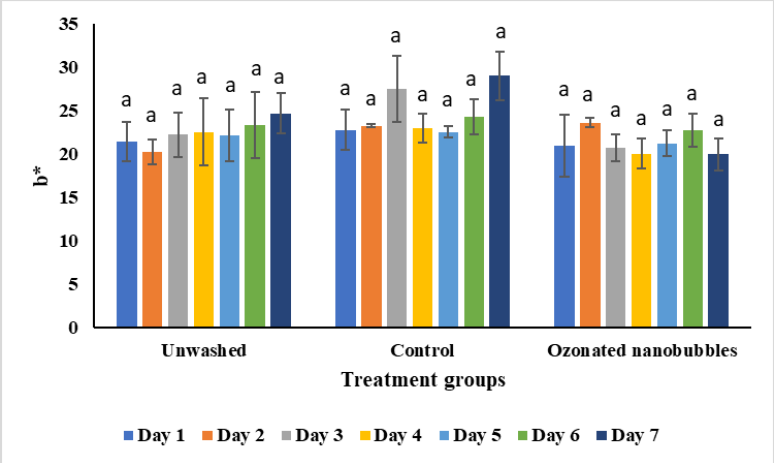


Figure 4. Effect of ozone nanobubble wash on color of (a) apples, (b) celery and (c) lettuce. Within each day, bars with different letters represent significant difference between treatments.

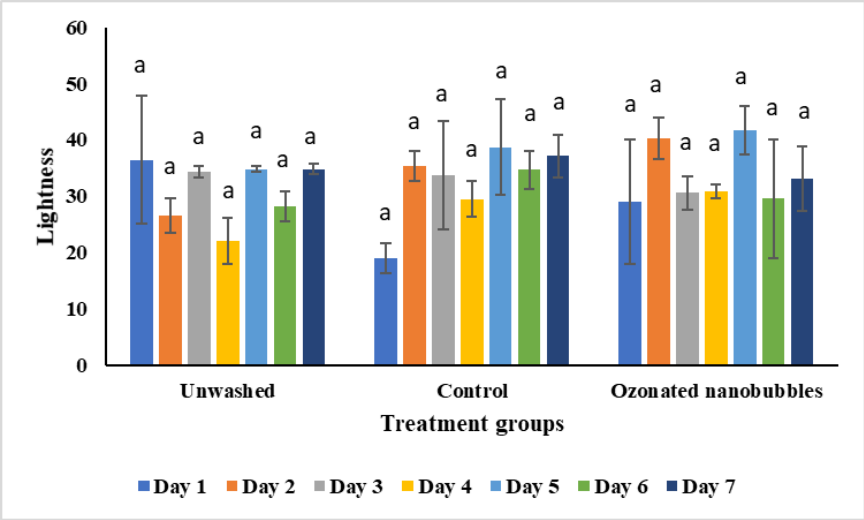
(a) Apples







(b) Celery


Lightness
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Treatment groups
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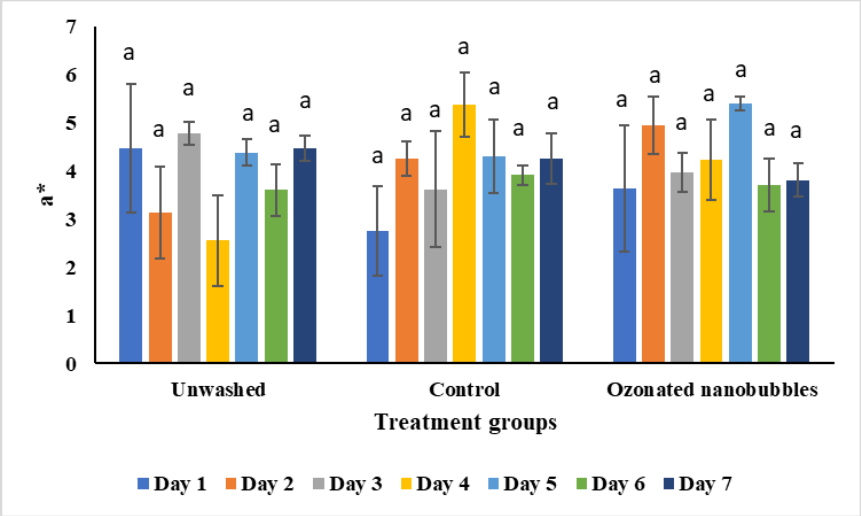
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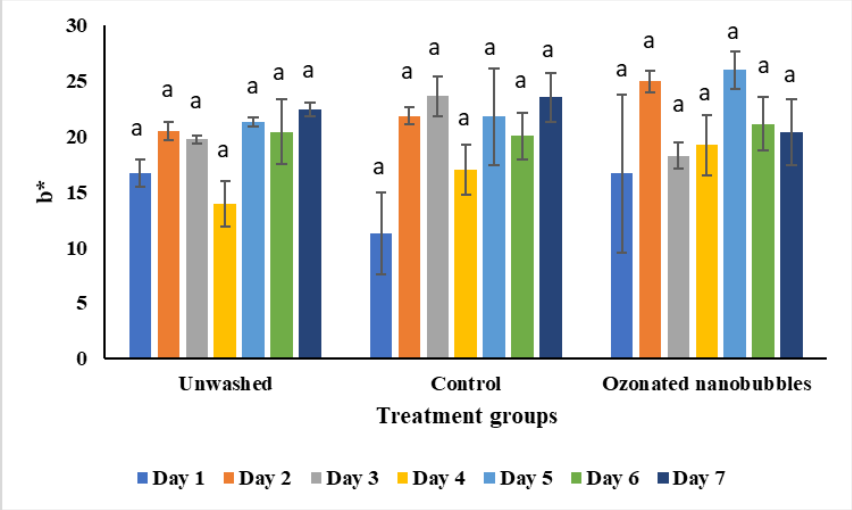
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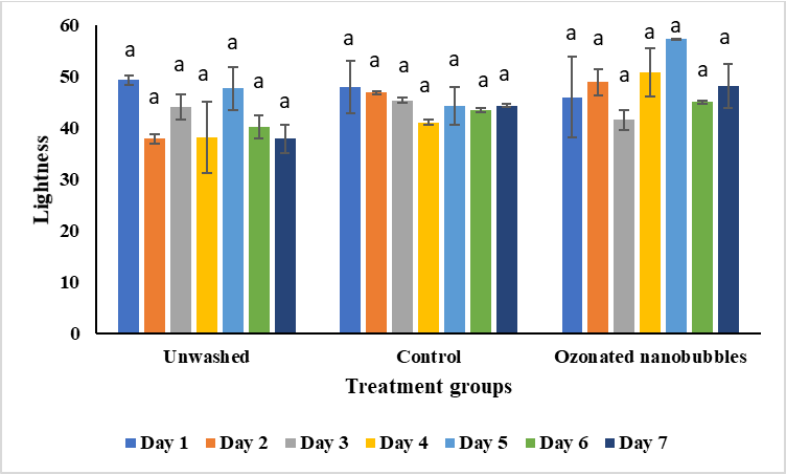
(c) Lettuce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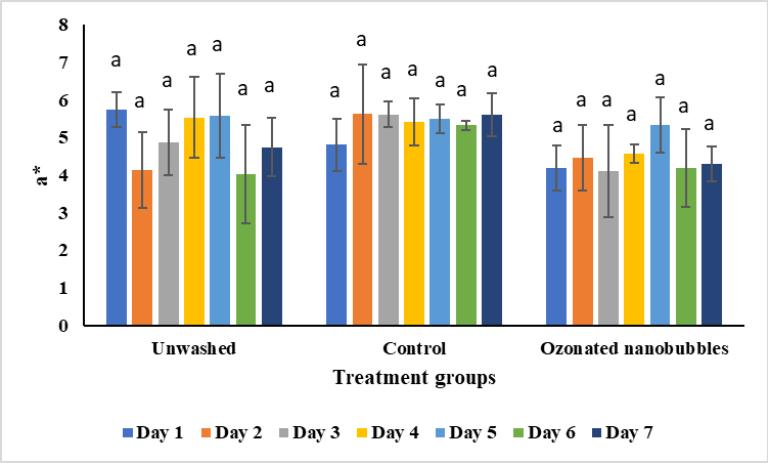
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Treatment groups
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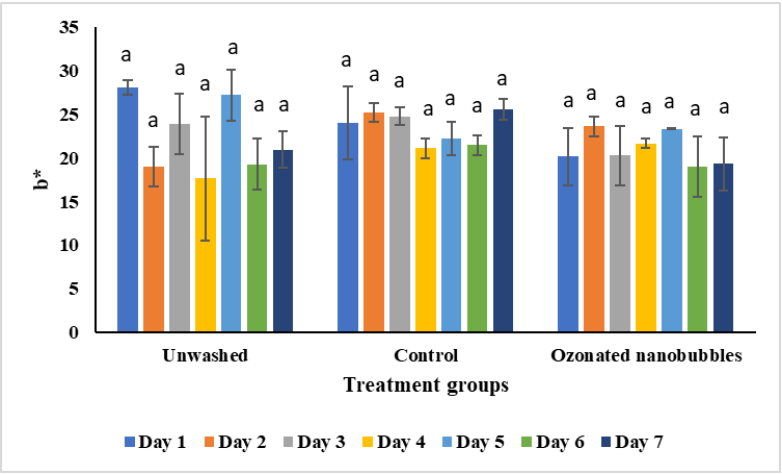
b*
Unwashed
Control
Ozonated nanobubbles
Treatment groups
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7

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