



Cold plasma enhances the efficacy of aerosolized hydrogen peroxide in reducing populations of *Salmonella* Typhimurium and *Listeria innocua* on grape tomatoes, apples, cantaloupe and romaine lettuce

Yuanyuan Song, Xueting Fan*

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA, 19038, USA

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ABSTRACT

In the present study, we investigated whether cold plasma activation affected the efficacy of aerosolized hydrogen peroxide against *S. Typhimurium* and *L. innocua*. Stem scars and smooth surfaces of grape tomatoes, surfaces of Granny Smith apples and Romaine lettuce (both midrib and upper leaves) and cantaloupe rinds were inoculated with two-strain cocktails of *S. Typhimurium* and 3-strain cocktails of *L. innocua*. The inoculated samples were treated with 7.8% aerosolized H_2O_2 with and without cold plasma for various times. For all fresh produce items and surfaces, cold plasma significantly ($P < 0.05$) improved the efficacy of aerosolized H_2O_2 against *Salmonella* and *L. innocua*. Without cold plasma activation, H_2O_2 aerosols only reduced populations of *Salmonella* by 1.54–3.17 log CFU/piece while H_2O_2 with cold plasma achieved 2.35–5.50 log CFU/piece reductions of *Salmonella*. *L. innocua* was more sensitive to the cold plasma-activated H_2O_2 than *Salmonella*. Cold plasma activated H_2O_2 aerosols reduced *Listeria* populations by more than 5 log CFU/piece on all types and surfaces of fresh produce except for the tomato stem scar area. Without cold plasma, the reductions by H_2O_2 were only 1.35–3.77 log CFU/piece. Overall, our results demonstrated that cold plasma activation significantly enhanced the efficacy of H_2O_2 mist against bacteria on fresh produce.

1. Introduction

Consumers are becoming more conscious about the benefits of eating fresh fruits and vegetables with high nutritional values that are free from additives as global incomes rise and nutritional information is broadly spread by producers and governmental health agencies. However, the microbial safety of fresh produce remains a worldwide concern (Sivapalasingam et al., 2004; U.S.FDA, 2008). Fresh fruits and vegetables such as leafy greens, tomatoes and melons (Doyle and Erickson, 2008; Elviss et al., 2009; Gravani, 2009; Hanning et al., 2009; Berger et al., 2010; Callejón et al., 2015) are increasingly implicated in outbreaks of foodborne illnesses. Among the human pathogens of concern are *Salmonella* spp. *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Beuchat, 1996; Jablasone et al., 2005). Controlling and destroying these pathogenic microorganisms in foods remains a formidable challenge.

Hydrogen peroxide (H_2O_2) has been widely tested to reduce microorganisms in fresh produce as a sanitizer agent (Lin et al., 2002; Moore et al., 2011; Ukuku et al., 2016). H_2O_2 possesses broad bactericidal and inhibitory activity due to its properties as an oxidant and

potential to generate cytotoxic species such as hydroxyl radicals, which attack essential cell components including lipids, proteins, and DNA (McDonnell and Russell, 1999; Khadre and Yousef, 2001; Ölmez and Kretschmar, 2009). H_2O_2 exhibits biocidal activity against a wide range of organisms including vegetative bacteria, bacterial spores, yeasts, fungi, and viruses (McDonnell and Russell, 1999). H_2O_2 improves the sensory quality and shelf life of some fresh produce (Lin et al., 2002; McWatters et al., 2002) and residue in fresh produce after treatment can be removed by endogenous catalase or by rinsing immediately with water (Sapers, 2001). Aqueous chemical sanitizers have very limited effectiveness in reducing pathogen levels (Fan et al., 2012; Back et al., 2014). Pathogens on the surfaces of fresh fruits and vegetables may reside in protected sites such as crevices, stomata, or cracks that aqueous sanitizers cannot reach (Oh et al., 2005a, 2005b). Therefore, more effective treatment methods are needed to meet consumer demand for fresh produce free from contaminants.

Aerosolization is defined as the dispersion of a liquid material into the air or a solution in the form of fine mist, usually for therapeutic and sanitary purposes (Hiom et al., 2003). A few studies have demonstrated the efficacy of aerosolized sanitizers for inhibiting pathogens (*Bacillus*

* Corresponding author.

E-mail address: xueting.fan@usda.gov (X. Fan).

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cerus, *L. innocua*, *Staphylococcus aureus*, and *Salmonella* Typhimurium) in foods and related environments (Oh et al., 2005a, 2015b; Cho et al., 2017; Ng et al., 2018). It is reported that using aerosolized peroxyacetic acid as a sanitizer for lettuce leaves reduced populations of *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* by 3.4, 4.5 and 3.8 log, respectively after a 60 min treatment. Due to its high penetration ability, aerosolization technology has the potential to be an alternative sanitizer delivery system for fresh produce.

Cold plasma is a relatively new sanitizing technology in the field of food processing with many technological hurdles to be addressed (Gilmore et al., 2018; Sarangapani et al., 2018). Although plasma has many gas-like qualities, it is technically a distinct state of matter. As additional energy is added, the intra-atomic structures of the components of the gas break down, yielding plasmas-concentrated collections of ions, radical species, and free electrons (Birmingham and Hammerstrom, 2000). The generation of plasma produces active species which can cause alterations in lipids, proteins, and nucleic acids and exert the antimicrobial effects (Fridovich, 1995). Studies have shown that the plasma is effective in reducing microbial populations on food and non-food surfaces (Montie et al., 2000; Kayes et al., 2007; Niemira, 2012; Thirumdas et al., 2015). However, the effectiveness of cold plasma is rather limited, frequently achieving 1–2 log reductions of pertinent human pathogens on fresh produce (Bermúdez-Aguirre et al., 2013; Min et al., 2016, 2017, 2018).

Considerable attention has been paid to advanced oxidation processes (AOPs) which are defined as processes that involve generation of highly reactive radical intermediates, particularly the hydroxyl radical (OH $\cdot$). The hydroxyl radical is among the strongest oxidizers and is more potent than ozone, H $_2$ O $_2$, chlorine dioxide or chlorine. In our earlier study (Jiang et al., 2017), we applied cold plasma-activated H $_2$ O $_2$ aerosols (called ionized hydrogen peroxide, or iHP) onto tomato, cantaloupe and spinach in a treatment chamber. The treatment reduced populations of *E. coli* O157:H7, *S. Typhimurium*, and *L. innocua* by up to 5.0 log CFU/piece. The initial study only tested one condition and the process was not optimized. By treating aerosolized H $_2$ O $_2$ with cold plasma to create iHP, it is assumed that highly reactive species such as hydroxyl radicals are produced. However, hydroxyl radicals are not stable with an estimated half-life of approximately 10 $^{-9}$ s (Pryor, 1986). While the initial study proved effective reduction of pathogens on produce, it was not clear to what degree the cold plasma application contributed to the effectiveness of aerosolized H $_2$ O $_2$.

Therefore, the objectives of the present study were to optimize treatment conditions of the cold plasma-activated aerosolized H $_2$ O $_2$ process for grape tomatoes, Granny Smith apples, Romaine lettuce and cantaloupes, and to evaluate whether cold plasma activation affected the efficacy of aerosolized H $_2$ O $_2$ against *S. Typhimurium* and *L. innocua* on the produce items.

2. Materials and methods

2.1. Bacterial strains

Two strains of non-pathogenic *S. Typhimurium* (ATCC 53647 and 53648) and three strains of *L. innocua* (ATCC 33090, ATCC 51742 and ATCC BAA680) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and were used in this study. The bacteria were made to be resistant to nalidixic acid by successive growth in Tryptic Soy Broth (TSB) (Difco, Sparks, MD, USA) with increasing nalidixic acid concentrations. All strains were stored at -80°C in 1 mL of TSB containing 10% glycerol. Working cultures of *S. Typhimurium* and *L. innocua* were maintained in 9 mL TSB (supplemented with 100 μg /mL nalidixic acid for *S. Typhimurium*) at 4°C after incubating at 37°C for 40 and 48 h, respectively, and sub-cultured each time.

2.2. Preparation of inocula

Each strain of *S. Typhimurium* and *L. innocua* was cultured in 9 mL TSB (supplemented with 100 μg /mL nalidixic acid for *S. Typhimurium*) and incubated at 37°C for 40 h and 48 h, respectively, harvested by centrifugation at 4000 rpm for 10 min at 4°C , and resuspended in 9 mL sterile Peptone Water (PW; Difco). The pellets were resuspended in PW to form cell suspensions. The populations of *S. Typhimurium* ATCC 53747 and 53648 were 8.76 ± 0.30 and 8.82 ± 0.27 log CFU/mL, respectively while populations of *L. innocua* ATCC 33090, 51742, and BAA680 were 8.45 ± 0.23 , 8.25 ± 0.32 , 8.05 ± 0.43 log CFU/mL, respectively. Subsequently, suspended pellets of two strains of *Salmonella* and three strains of *L. innocua* were combined to obtain two separate culture cocktails.

2.3. Sample preparation and inoculation

Grape tomatoes, Granny Smith apples, Romaine lettuce and cantaloupes were purchased from local markets (Philadelphia, PA, USA) and kept at 10°C prior to use. Fruits and vegetables were removed from 10°C refrigeration and equilibrated to ambient temperature before being inoculated. The produce items were not sanitized prior to inoculation to take account of possible interaction of native microflora and the inoculated bacteria. Romaine lettuce was divided into upper green leaf and midrib tissues. Six tomatoes, six pieces (2×2 cm) of apple skins, five pieces (3×3 cm) of Romaine lettuce upper leaves, five pieces (3 cm length) of Romaine lettuce midrib area and six pieces (2×2 cm) of cantaloupe rinds with 2–3 mm thickness of flesh were used for each treatment per replicate. Each piece was spot inoculated with 10 μL of *Salmonella* and *L. innocua* by depositing droplet with a micropipette. The inoculated samples were dried in bio-hood for 1 h (for tomato surfaces, romaine lettuce, apple skins and cantaloupe rinds) or 2 h (for tomato stem scars) at ambient temperature. It took a longer time for the bacteria inoculum to dry on the stem scar area of tomatoes.

2.4. Treatments

Produce items were placed onto a sterile test tube rack with the inoculated area facing up and the rack with produce pieces were placed inside of a treatment chamber ($12 \times 12 \times 24$ inch). H $_2$ O $_2$ (7.8%) (TOMI $^{\text{TM}}$ Environmental Solutions, Inc., MD, USA) was aerosolized with pressurized air into a treatment chamber using the SteraMist Select Machine (TOMI $^{\text{TM}}$ Environmental Solutions). The aerosolized H $_2$ O $_2$ was activated by cold plasma generated between two pin electrodes in the TOMI $^{\text{TM}}$ SteraMist Select applicator (TOMI $^{\text{TM}}$ Environmental Solutions, Inc) to create ionized hydrogen peroxide (iHP) which was applied to the produce. The distance and voltage between the two electrodes were 9 mm and 17 kV, respectively. The flow rate for H $_2$ O $_2$ used in each trial was 5.0 mL/min with an air pressure of 7 psi.

2.4.1. Effect of treatment time against *S. Typhimurium* on fresh produce

In preliminary experiments, we evaluated the effects of treatment time and dwell time, and found that for bacteria on smooth surface, a very short treatment time (a few seconds) was enough to inactivate bacteria to an undetectable level. Therefore, longer treatments were not attempted. However, for bacteria on rough surface, the short treatment times had very limited effectiveness on bacteria populations. Therefore, we increased the treatment time and the number of cycles to improve the effectiveness of the treatments.

For tomato surfaces and apple skins, the treatment times (spray times) were set as 5, 8 and 10 s followed by 30 min dwell time. For upper leaves and midrib tissues of Romaine lettuce, the treatment times were 10, 20, 30 and 60 s followed by 30 min dwell time. For cantaloupe rinds, the treatment times were set as 10, 30 and 60 s followed by 30 min dwell time. For tomato stem scars, the treatment procedure included 5, 8, 10, 20, 30, 60 s followed by 30 min dwell time, 6 cycles

composed of 10 s treatment time followed by 10 min dwell time per cycle, and 3 cycles composed of 20 s treatment time followed by 20 min dwell time per cycle. A fan (E.Z.FAN, FP-108-1, Common Wealth Industrial Corp., Taiwan) was used in the chamber to facilitate the uniform distribution of mist during dwell time for treating stem scars of tomatoes.

2.4.2. Effect of cold plasma activation on the efficacy of aerosolized H_2O_2 against *S. Typhimurium* and *L. innocua*

For treatments without cold plasma, the two pin electrodes were removed resulting in no cold plasma generation/application to the H_2O_2 aerosol. Therefore, each kind of produce was treated with and without cold plasma activated under the same optimized conditions.

2.5. Enumeration

After each treatment, the tomato stem scars and surfaces with the inoculated bacteria were removed using a pair of sterile scissors. Each type of produce item from the same treatment was combined (total weight: 1.51 ± 0.29 g for tomato stem scars; 2.07 ± 0.15 g for tomato surfaces; 6.37 ± 0.92 g for apple skins; 1.62 ± 0.34 g for Romaine lettuce upper leaves; 13.30 ± 2.97 g for Romaine lettuce midrib tissues; 9.58 ± 1.26 g for cantaloupe rinds) and transferred into an 80 mL filtered stomacher bag. After addition of 30 mL BBL buffered peptone water (BPW; Difco) into the bag, the sample in the bag was homogenized using a mini blender (Mini Mix CC, Interscience Laboratories Inc., Wobum, MA, USA) for 2 min at a setting of 4. The homogenate (after build-in filtration) was serially diluted (if needed) and plated (0.1 mL or 1 mL depending on the treatments) onto selective media. Tryptic Soy Agar with 200 μ g/mL pyruvate acid and 100 μ g/mL nalidixic acid (TSA-PN) and PALCAM Agars (Difco) were used for the enumeration of *S. Typhimurium* and *L. innocua*, respectively. Nalidixic acid and selective media were used to suppress the growth of native microflora from produce items while pyruvate was added to enhance the recovery of the injured cells (Gurtler and Kornacki, 2009). The plates were incubated at 37 °C for 40 and 48 h, respectively, and counted after incubation.

2.6. Statistical analysis

All experiments were repeated three times, and each experiment were conducted on a different day. Colony counts were converted to log CFU/piece. When no colony was present on a plate, the limit of detection was used to calculate the log reductions. The results were expressed as mean $\pm$ standard deviation and data were analyzed by SPSS Statistics 22 software (IBM, Amok, USA) through one-way analysis of variance (ANOVA). The $P < 0.05$ (Duncan Multiple Range test) was used to determine statistical significance.

3. Results and discussion

The effects of treatment time of ionized hydrogen peroxide (aerosolized H_2O_2 treated with cold plasma) against *S. Typhimurium* on grape tomatoes, Granny Smith apples, Romaine lettuce and cantaloupes are shown in Tables 1–6. Based on these results, it can be concluded that ionized hydrogen peroxide is very effective in inactivating bacteria on the smooth surfaces of tomato and apple fruits (Tables 1 and 2). After an 8 s treatment time, the population of inoculated *S. Typhimurium* was reduced to a level below the detection limit (0.70 log CFU/piece). There was no significant difference between 8 s and 10 s treatment times on the reductions of *S. Typhimurium*. Therefore, the 8 s treatment time followed by 30 min dwell time was chosen as the optimal treatment condition for smooth surfaces of tomatoes and apples.

S. Typhimurium on stem scars of grape tomato was inactivated at lower rates than those on the smooth surface of tomatoes (Table 3). A 5 s treatment only reduced *Salmonella* populations by 1.06 log CFU/

Table 1

Effects of treatment time on populations (log CFU/piece) of *S. Typhimurium* inoculated on grape tomato surfaces with cold plasma activated H_2O_2 aerosol.

Treatment time (s)	Populations (log CFU/piece)		Reductions (log CFU/piece)
	Control	treated	
5	6.28 ± 0.19^a	4.44 ± 0.25^a	1.84 ± 0.43^b
8	5.91 ± 0.49^a	ND ^b	5.21 ± 0.49^a
10	6.35 ± 0.21^a	ND ^b	5.65 ± 0.21^a

ND: not detectable (detection limit: 0.70 log CFU/piece).

Means followed by the same letters in the same column are not significantly different (Duncan Multiple Range test, $P = 0.05$). Numbers are averages $\pm$ standard deviations ($n = 3$).

Table 2

Effects of treatment time on populations (log CFU/piece) of *S. Typhimurium* inoculated on Granny Smith apple skins with cold plasma activated H_2O_2 aerosol.

Treatment time (s)	Populations (log CFU/piece)		Reductions (log CFU/piece)
	Control	treated	
5	5.72 ± 0.48^a	4.09 ± 0.13^a	1.63 ± 0.35^b
8	5.88 ± 0.19^a	ND ^b	5.18 ± 0.19^a
10	5.56 ± 0.58^a	ND ^b	4.86 ± 0.58^a

ND: not detectable (detection limit: 0.70 log CFU/piece).

Means followed by the same letters in the same column are not significantly different (Duncan Multiple Range test, $P = 0.05$). Numbers are averages $\pm$ standard deviations ($n = 3$).

Table 3

Effects of treatment time on populations (log CFU/piece) of *S. Typhimurium* inoculated on grape tomato stem scars with cold plasma activated H_2O_2 aerosol. The 10sx6 refers to 6 cycles of 10 s spray time followed by 10 min dwell time while 20sx3 refers 3 cycles of 20s spray time with 20 min dwell time.

Treatment time (s)	Populations (log CFU/piece)		Reductions (log CFU/piece)
	Control	treated	
5	5.32 ± 0.71^c	4.26 ± 0.89^{ab}	1.06 ± 0.35^d
8	5.45 ± 0.58^{bc}	4.23 ± 0.31^{ab}	1.22 ± 0.28^d
10	5.46 ± 0.57^{bc}	4.26 ± 0.71^{ab}	1.20 ± 0.14^d
20	6.22 ± 0.30^{ab}	4.71 ± 0.26^{ab}	1.51 ± 0.34^{cd}
30	6.17 ± 0.22^{ab}	4.31 ± 0.20^a	1.85 ± 0.11^{bc}
60	6.01 ± 0.24^{abc}	3.85 ± 0.66^{ab}	2.16 ± 0.43^b
10s $\times$ 6	6.08 ± 0.32^{abc}	3.89 ± 0.39^{ab}	2.19 ± 0.41^b
20s $\times$ 3	6.33 ± 0.15^a	3.60 ± 0.42^b	2.73 ± 0.28^a

Means followed by the same letters in the same column are not significantly different (Duncan Multiple Range test, $P = 0.05$). Numbers are averages $\pm$ standard deviations ($n = 3$).

piece. As the treatment time increased, the greater reductions were generally achieved. However, even after 60 s treatment, the reduction was only 2.16 log CFU/g. Therefore, ionized hydrogen peroxide was repeatedly applied in bursts for a total of 60 s treatment time to optimize efficacy. Three cycles of 20 s spray time plus 20 min dwell time proved to be the most effective application to reduce *S. Typhimurium* populations on the stem scar, achieving a 2.73 log CFU/piece reduction. Therefore, 20 s spray time followed by 20 min dwell time was chosen as the optimum condition to reduce the bacteria on tomato stem scars.

For the two tissues of Romaine lettuce, *Salmonella* on midrib tissues proved easier to inactivate by ionized hydrogen peroxide compared to those on the upper leaf, with reductions increasing with longer treatment times on both types of tissues (Tables 4 and 5). The 20 s treatment time significantly reduced the populations of inoculated *S. Typhimurium* on both tissues. With spray time extended to 30 s, the reductions were 2.86 and 3.63 log CFU/piece on the upper leaf and midrib

Table 4

Effects of treatment time on populations (log CFU/piece) of *S. Typhimurium* inoculated on Romaine lettuce midrib area with cold plasma activated H₂O₂ aerosol.

Treatment time (s)	Populations (log CFU/piece)		Reductions (log CFU/piece)
	Control	treated	
10	6.34 ± 0.37 ^a	4.81 ± 0.22 ^a	1.53 ± 0.23 ^c
20	6.12 ± 0.30 ^a	4.09 ± 0.25 ^b	2.04 ± 0.33 ^b
30	6.17 ± 0.41 ^a	2.54 ± 0.32 ^c	3.63 ± 0.21 ^a
60	6.07 ± 0.59 ^a	2.94 ± 0.18 ^c	3.13 ± 0.48 ^a

Means followed by the same letters in the same column are not significantly different (Duncan Multiple Range test, *P* = 0.05). Numbers are averages ± standard deviations (*n* = 3).

Table 5

Effects of treatment time on populations (log CFU/piece) of *S. Typhimurium* inoculated on Romaine lettuce leaves with cold plasma activated H₂O₂ aerosol.

Treatment time (s)	Populations (log CFU/piece)		Reductions (log CFU/piece)
	Control	treated	
10	6.24 ± 0.23 ^a	5.45 ± 0.34 ^a	0.79 ± 0.16 ^c
20	5.95 ± 0.39 ^a	3.98 ± 0.37 ^b	1.98 ± 0.24 ^b
30	6.40 ± 0.16 ^a	3.54 ± 0.11 ^b	2.86 ± 0.17 ^a
60	6.36 ± 0.38 ^a	3.57 ± 0.25 ^b	2.79 ± 0.20 ^a

Means followed by the same letters in the same column are not significantly different (Duncan Multiple Range test, *P* = 0.05). Numbers are averages ± standard deviations (*n* = 3).

tissue, respectively. No additional significant increases in inactivation were achieved when the spray time was increased to 60 s. Considering the efficiency of the operation, 30 s treatment time followed by 30 min dwell time was regarded as the optimized condition for Romaine lettuce.

For *Salmonella* on the rind of the cantaloupe, after 30 s of treatment, the population of the bacterium was reduced by 2.48 log CFU/piece. The 60 s treatment failed to achieve greater reductions compared to the 30 s treatment. Therefore, 30 s treatment time followed by 30 min dwell time was the best condition for reducing the bacteria inoculated onto cantaloupe rinds (Table 6).

In our previous study (Jiang et al., 2017), the cold plasma-activated H₂O₂ aerosol (iHP) was applied on various fresh produce items for 45 s followed by an additional 30 min dwell time without optimization. In our current study, we optimized the conditions to achieve maximum reductions of *Salmonella* on apple, tomatoes, Romaine lettuce and cantaloupe. Results indicated that a very short treatment time (8 s) was enough to reduce the bacterial populations by more than 5 logs on the smooth surface of apples and tomatoes. *Salmonella* on the stem scar area of tomatoes were most difficult to inactivate, and even after several repeat treatments, the reductions were less than 3 logs.

The stem scar area of tomatoes and the rinds of cantaloupes have

Table 6

Effects of treatment time on populations (log CFU/piece) of *S. Typhimurium* inoculated on cantaloupe rinds with cold plasma activated H₂O₂ aerosol.

Treatment time (s)	Populations (log CFU/piece)		Reductions (log CFU/piece)
	Control	treated	
10	5.89 ± 0.13 ^b	4.39 ± 0.32 ^a	1.50 ± 0.21 ^b
30	6.57 ± 0.56 ^a	4.09 ± 0.24 ^a	2.48 ± 0.68 ^a
60	5.68 ± 0.07 ^b	3.23 ± 0.28 ^b	2.44 ± 0.32 ^a

Means followed by the same letters in the same column are not significantly different (Duncan Multiple Range test, *P* = 0.05). Numbers are averages ± standard deviations (*n* = 3).

been identified as important sources of enteric pathogen contamination due to the roughness and porosity as well as the inability of sanitizers to effectively penetrate into the areas (Bowen et al., 2006; Fan et al., 2012; Guo et al., 2002). Furthermore, bacteria inoculated into stem scar of tomatoes and cantaloupe rinds appear to have a great capacity for survival and/or growth (Ukuku and Fett, 2002; Yuk et al., 2005).

This is the first report demonstrating H₂O₂ aerosol run through a cold plasma arc (iHP) provides enhanced efficacy against bacteria on various fresh produce. In previous research regarding advanced oxidation processes, the plasma arc was applied in aqueous phase to treat water and wastewater, and to degrade organic pollutants in water (Oturán and Aaron, 2014; Iqbal et al., 2015; Kanakaraju et al., 2018). In the present study, we applied the advanced oxidation process in the aerosolized phase, and showed that the aerosolized H₂O₂ activated by cold plasma was also more effective on smooth surface than the rough surface. Future studies may be conducted to evaluate if the droplet size of H₂O₂ mist can be reduced to facilitate the diffusion of the aerosolized H₂O₂ to rough surface.

After establishing the optimum conditions for the inactivation of *Salmonella*, and demonstrating that cold plasma-activated H₂O₂ reduced populations of *Salmonella* by 2.48 to > 5 log CFU/piece (depending on type and nature of produce), we studied whether cold plasma played any role in the H₂O₂ inactivation of *Salmonella* and *Listeria*. Results revealed that cold plasma significantly enhanced the efficacy of aerosolized H₂O₂ on all produce items (Table 7). For example, without cold plasma activation, the reduction of *Salmonella* populations were only 3.17 and 2.08 log CFU/piece on smooth surface of tomatoes and apples, respectively, while the reductions were more than 5 logs when the aerosolized H₂O₂ passed through a cold plasma arc (iHP process).

We then used the same treatment conditions with and without cold plasma to inactivate *L. innocua* on the same type of produce items. Results demonstrated that, without cold plasma, reductions of *L. innocua* populations ranged from 1.35 on the stem scar of tomatoes to 3.77 log CFU/piece on Romaine lettuce midribs. When the iHP process of running the aerosolized H₂O₂ aerosols through the plasma arc was applied, *Listeria* populations were reduced by more than 5 logs for all types of fresh produce and surfaces except for the stem scar area of tomatoes, on which only 2.36 log CFU/piece reduction (Table 8). Significantly higher reductions of *Listeria* were achieved by the plasma activated H₂O₂ process on Romaine lettuce and cantaloupes than *Salmonella*.

There is a synergistic effect between cold plasma and aerosolized H₂O₂ in the iHP process that maximizes pathogen reduction. We evaluated the efficacy of cold plasma-activated water in inactivating *Salmonella* and *Listeria* on various fresh produce items. The reductions of *Salmonella* by the cold plasma-activated water were 0.93 ± 0.45, 0.85 ± 0.16, 0.53 ± 0.08, 0.23 ± 0.35, 1.03 ± 0.04, 0.37 ± 0.06 log CFU/piece, and the reductions of *Listeria* were 0.37 ± 0.02, −0.01 ± 0.18, 0.22 ± 0.06, 0.28 ± 0.16, 0.40 ± 0.20, 0.12 ± 0.15 log CFU/piece on Granny Smith apple surface, Romaine lettuce upper leaf, Romaine lettuce lower midrib, cantaloupe rind, tomato smooth surface and tomato stem scar, respectively. The results suggest that cold-plasma treated water had very limited effectiveness in reducing populations of *Salmonella* and *Listeria* on fresh produce. H₂O₂ aerosols without cold plasma reduced the populations of *Salmonella* by 1.53–3.17 log CFU/piece and *Listeria* by 1.35–3.77 log CFU/piece on various fresh produce items. However, when cold plasma was applied to aerosolized H₂O₂ in the iHP process, the reductions of *Salmonella* were 2.35–5.50 log CFU/piece and *Listeria* reductions were more than 5 log CFU/piece on all produce items except tomato stem scar (Tables 7 and 8).

Our results demonstrated that cold plasma enhanced the effectiveness of aerosolized H₂O₂ against the inoculated bacteria on all tested produce items and surfaces. An earlier study (Nam et al., 2013) demonstrated that cold plasma enhanced the efficiency of H₂O₂ gel in

Table 7

Cold plasma activation on the efficacy of aerosolized H₂O₂ in inactivating *Salmonella* Typhimurium on various fresh produce surfaces. Treatment conditions: 8 s spray time followed by 30 min dwell time for Granny Smith apple and tomato smooth surface; 30 s spray time followed by 30 min dwell time for upper leaf and midrib tissues of Romaine lettuce and cantaloupe rind; three cycles of 20 s spray time plus 20 min dwell time for tomato stem scar.

Type of produce	Populations (log CFU/piece)			Reductions (log CFU/piece)	
	Control	H ₂ O ₂ without cold plasma	H ₂ O ₂ with cold plasma	H ₂ O ₂ without cold plasma	H ₂ O ₂ with cold plasma
Granny Smith apple	5.85 ± 0.15 ^a	3.77 ± 0.17 ^b	ND ^c	2.08 ± 0.17 ^y	5.50 ± 0.19 ^x
Romaine lettuce-upper leaf	6.42 ± 0.20 ^a	4.70 ± 0.23 ^b	3.54 ± 0.11 ^c	1.72 ± 0.23 ^y	2.88 ± 0.11 ^x
Romaine lettuce-midrib	5.94 ± 0.41 ^a	4.16 ± 0.28 ^b	2.54 ± 0.32 ^c	1.78 ± 0.28 ^y	3.40 ± 0.32 ^x
Cantaloupe rind	6.37 ± 0.43 ^a	4.83 ± 0.36 ^b	3.76 ± 0.53 ^c	1.54 ± 0.36 ^y	2.61 ± 0.53 ^x
Tomato-surface	5.63 ± 0.46 ^a	2.46 ± 0.33 ^b	ND ^c	3.17 ± 0.33 ^y	5.28 ± 0.49 ^x
Tomato-stem scar	5.95 ± 0.40 ^a	4.40 ± 0.28 ^b	3.60 ± 0.70 ^c	1.54 ± 0.28 ^y	2.35 ± 0.30 ^x

ND: not detectable (detection limit: 0.70 log CFU/piece).

Means followed by the same letters in the same row for population (a, b, c) or reduction (x, y) are not significantly different (Duncan Multiple Range test, P = 0.05). Numbers are averages ± standard deviations (n = 3).

whitening teeth. It is assumed that the combination of cold plasma and H₂O₂ initiates an advanced oxidation process, producing hydroxyl radicals. However, the production of hydroxyl radicals was not able to be quantified in the present study at this time. The exact reactive species produced by the combinations of H₂O₂ mist and cold plasma warrant further investigations. It is known that cold plasma accelerates the degradation of H₂O₂ and production of hydroxyl radicals (Joshi et al., 2011). In addition, it is possible that cold plasma produced a small amount of ozone, which in turn, reacts with H₂O₂ and produce hydroxyl radicals. Still, hydroxyl radicals if produced in the cold plasma field may not have sufficient life span to be able to reach the fresh produce items in the treatment chamber. Perhaps, as the aerosolized H₂O₂ microdroplets go through the cold plasma field, the droplets are electrically charged in addition to the production of free radicals. Electrically charging increases the stability of free radicals in the nanometer sized droplets (Pyrgiotakis et al., 2015). In our earlier report (Jiang et al., 2017), we found that there were two ranges of H₂O₂ droplets produced by the device: one in nanometer range (with average diameter of 40 nm) and another in micrometer range (with average of 3.4 µm). Nanometer sized droplets were more stable than micrometer sized ones. Earlier studies demonstrated that the antibacterial activity of essential oils was enhanced by cold plasma due to increased availability of essential oils to food (Cui et al., 2016a, 2016b).

Our results demonstrated that *L. innocua*, a Gram-positive bacterium, was more sensitive to the cold plasma-activated H₂O₂. More than a 5 log reduction of *Listeria* was observed on all fresh produce items except the stem scar area of tomatoes, while the *Salmonella* reductions were 2.61–3.40 log CFU/piece for cantaloupes and two locations on lettuces (Table 8). The cell envelope of Gram-negative bacteria is composed of a multilayer system with an inner cytoplasmic membrane made of phospholipids and proteins, a peptidoglycan layer, and an outer membrane of polymers such as polysaccharides. Gram-positive

bacteria have a thicker peptidoglycan layer and only one layer of cytoplasmic membrane. Reactive oxygen species play a crucial role in the inactivation of bacteria by cold plasma. Bacteria cells can be damaged by the strong oxidative stress via lipid peroxidation, enzyme inactivation, and/or DNA cleavage. Some earlier studies showed that Gram-positive bacteria were more resistant to direct cold plasma treatments than Gram-negative bacteria (Lee et al., 2016). Our results showed that the Gram-positive bacteria were more sensitive to the aerosolized H₂O₂ activated by cold plasma. It has been suggested that reactive species either react with the cell envelope or damage intracellular components. Gram-negative bacteria such as *E. coli* were inactivated by cell envelope damage-induced leakage, while Gram-positive bacteria were mainly inactivated by intracellular damage (Han et al., 2016). Perhaps the reactive species generated from the advanced oxidation process penetrate better into the single membrane of Gram-positive bacteria. Bactericidal effects of reactive species such as ozone and H₂O₂ were due to peroxidation of phospholipids and lipoproteins found in the inner membrane of both Gram-negative and Gram-positive cells (Joshi et al., 2011). It is likely that different mechanisms are involved in the inactivation of the bacteria between the present study and previous reports. Exposure of water to cold plasma resulted in the generation of secondary reactive species, such as H₂O₂, nitrites, nitrates, and others (Tsoukou et al., 2018). In our present study, we exposed H₂O₂ mist to cold plasma, and H₂O₂ was probably ionized to become more reactive species such as hydrogel radicals. Whether other species such as nitrate or nitrites are formed needs further investigation.

4. Conclusion

We demonstrated that bacteria on the smooth surface of fresh produce items such as apples and tomatoes were easier to inactivate than those on rough surfaces such as tomato stem scars and cantaloupe rinds

Table 8

Cold plasma activation on the efficacy of aerosolized H₂O₂ in inactivating *Listeria innocua* on various fresh produce surfaces. Treatment conditions: 8 s spray time followed by 30 min dwell time for Granny Smith apple and tomato smooth surface; 30 s spray time followed by 30 min dwell time for upper leaf and midrib tissues of Romaine lettuce and cantaloupe rind; three cycles of 20 s spray time plus 20 min dwell time for tomato stem scar.

Type of produce	Populations (log CFU/piece)			Reductions (log CFU/piece)	
	Control	H ₂ O ₂ without cold plasma	H ₂ O ₂ with cold plasma	H ₂ O ₂ without cold plasma	H ₂ O ₂ with cold plasma
Granny Smith apple	5.59 ± 0.25 ^a	2.90 ± 0.20 ^b	ND1 ^c	2.69 ± 0.20 ^y	5.24 ± 0.22 ^x
Romaine lettuce-upper leaf	5.57 ± 0.23 ^a	2.80 ± 0.34 ^b	ND2 ^c	2.77 ± 0.34 ^y	5.19 ± 0.08 ^x
Romaine lettuce-midrib	5.59 ± 0.23 ^a	1.82 ± 0.35 ^b	ND2 ^c	3.77 ± 0.35 ^y	5.21 ± 0.11 ^x
Cantaloupe rind	5.45 ± 0.26 ^a	3.88 ± 0.37 ^b	ND1 ^c	1.57 ± 0.37 ^y	5.10 ± 0.30 ^x
Tomato-surface	5.51 ± 0.28 ^a	2.77 ± 0.21 ^b	ND1 ^c	2.74 ± 0.21 ^y	5.16 ± 0.20 ^x
Tomato-stem scar	5.57 ± 0.25 ^a	4.21 ± 0.60 ^b	2.94 ± 0.89 ^c	1.35 ± 0.60 ^y	2.62 ± 0.89 ^x

ND: Not detectable (detection limit: 0.70 log CFU/piece ND1, 0.76 log CFU/piece ND2).

Means followed by the same letters in the same row for population (a, b, c) or reduction (x, y) are not significantly different (Duncan Multiple Range test, P = 0.05). Numbers are averages ± standard deviations (n = 3).

through the application of ionized hydrogen peroxide (IHP). On smooth surfaces, greater than 5 log reductions of *Salmonella* were achieved with a short (8–10 s) treatment time, following by a 30 min dwell time. On rough surfaces, similar treatment times resulted in 2.8–3.6 log reductions of *Salmonella*. *Listeria* appears to be more sensitive to this treatment than is *Salmonella*. Cold plasma and aerosolized H₂O₂ had synergistic effects to quickly reduce the populations of *Salmonella* and *Listeria* inoculated onto different types and surfaces of fresh produce items. Greater reductions were documented when the aerosolized hydrogen peroxide was passed through the plasma arc, confirming cold plasma enhances the activity of H₂O₂ mist. Further research is needed to study the mechanism(s) of cold plasma enhancement of H₂O₂ efficacy.

Declaration of competing interest

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA). The USDA is an equal opportunity provider and employer. This work was partially funded through a collaborative research and development agreement with TOMI Environmental Solutions Inc.

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