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Microbial Inactivation and Quality Changes in Orange Juice Treated by High Voltage Atmospheric Cold Plasma

Lei Xu[1] · **Allen L. Garner**[2] · **Bernard Tao**[3] · **Kevin M. Keener**[4,5]

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Abstract Although atmospheric cold plasma is well known for nonthermal inactivation of microorganisms on surfaces, few studies examine its application to liquid food within a package. This study explores the decontamination efficiency of high voltage atmospheric cold plasma (HVACP) on *Salmonella enterica* serovar Typhimurium (*S. enterica*) in orange juice (OJ). Both direct and indirect HVACP treatments of 25-mL OJ induce greater than a 5-log reduction in *S. enterica* following 30 s of treatment with air and MA65 gas with no storage. For 50-mL OJ, 120 s of direct HVACP treatment followed by 24-h storage induced a 2.9-log reduction of *S. enterica* in air and a 4.7-log reduction in MA65 gas; 120 s of indirect HVACP treatment followed by 24-h storage resulted in a 2.2-log reduction in air and a 3.8-log reduction in MA65. No significant ($P < 0.05$) Brix or pH change occurred following 120-s HVACP treatment. Applying 120-s HVACP direct treatment reduced vitamin C by 22% in air (compared to 50% for heat pasteurization) and pectin methylesterase activity by 74% in air and 82% in MA65. These results demonstrate that HVACP can effectively inactivate *Salmonella* in OJ with minimal quality degradation.

Keywords Cold atmospheric pressure plasmas · Nonthermal treatment · Orange juice · Microorganism inactivation

✉ Allen L. Garner
algarner@purdue.edu

✉ Bernard Tao
tao@purdue.edu

[1] Department of Food Science, Purdue University, West Lafayette, IN 47907, USA

[2] School of Nuclear Engineering, Purdue University, West Lafayette, IN 47907, USA

[3] School of Agricultural and Biological Engineering, Purdue University, West Lafayette, IN 47907, USA

[4] Center for Crops Utilization Research, BioCentury Research Farm, Boone, IA 50036, USA

[5] Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011-1061, USA

Introduction

Foodborne pathogens are an increasing concern worldwide. The Center for Disease Control and Prevention (CDC) reports that 1.2 million illnesses and 450 deaths (31% of food-related deaths) occur in the USA annually due to *Salmonella* infections (Scallan et al. 2011). The 2013 *Salmonella* annual summary stated that *Salmonella enterica* serovar Typhimurium (*S. enterica*) was one of the most commonly isolated serotypes, causing 12.8% of all human salmonellosis illnesses (CDC 2013), mostly in ready-to-drink (RTD) food.

The risk of contamination by spoilage and pathogenic microorganisms in RTD food is of concern because these products are consumed raw without further preparation or cooking. Orange juice (OJ), one of the most prevalent sources for bioactive compounds, is an RTD beverage that is usually pasteurized for safety. The current standard for OJ processing and preservation requires reducing microorganism concentration by 5-log cycles (USFDA 2001). Thermal treatment of OJ at 90 °C for 1 min is currently used to prevent microbial spoilage and inactivate the pectin methylesterase (PME) more than 90–98% (Eagerman and Rouse 1976). However, thermal pasteurization can adversely affect texture, destroy heat-sensitive nutritional components (such as vitamins), degrade bioactive compounds, and cause unfavorable flavor (Arreola et al. 1991; Vikram et al. 2005; Lee et al. 2015). Vitamin C concentration has reduced by 50% after pasteurization at 90 °C for

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2 min (Vikram et al. 2005). Therefore, nonthermal techniques are attractive to industry because they can potentially maintain food safety with minimal quality loss (Fridman et al. 2005; Tiwari et al. 2009a; Misra et al. 2011).

Increasing consumer demand for safe, minimally processed foods has motivated the food industry to investigate new nonthermal processing techniques. Current alternative commercial techniques, including high pressure processing (HPP) and pulsed electric fields (PEF), are expensive due to capital investment (HPP), up to sevenfold higher than conventional thermal processing, increased labor (HPP), and product type limitation (HPP and PEF) (Sampedro et al. 2014). High voltage atmospheric cold plasma (HVACP) treatment offers distinct advantages for food decontamination, including reduced capital and operational costs (Keener 2016). Specifically, 59% (6.3¢/L) of HPP pasteurization costs are due to capital costs, which are lower for HVACP systems since they have minimal equipment costs due to their simple design and low power requirements. HVACP may also be applied to both solid and liquid foods, providing additional flexibility compared to other techniques. Plasma consists of highly energetic species, including photons, electrons, positive and negative ions, free radicals, and excited or nonexcited molecules and atoms (Deng et al. 2006). Atmospheric cold plasma (ACP) is generated far from thermal equilibrium, so the electron temperature is much higher than the ion and neutral temperatures (Lang et al. 2016). Therefore, ACP treatment is particularly attractive for food treatment since it does not require extreme process conditions compared to classical preservation methods, such as heat treatments, which adversely impact food texture and bioactive nutrients. While ACP has been commonly used in industrial processes for surface modification, such as electronic cleaning, bonding plastics, or binding dye to textile fibers, its potential remains untapped in the food industry (Napartovich 2001; Fernández et al. 2013; Mai-Prochnow et al. 2014). HVACP treatment can significantly reduce foodborne pathogens and spoilage microorganisms (Coventry et al. 2009; Ehlbeck et al. 2011; Ziuzina et al. 2014; Misra et al. 2014; Pankaj et al. 2014).

The novel, in-package HVACP technology used in this study applies high voltage (up to 90 kV) to either dry air or modified atmospheric gas (65% oxygen and 30% carbon dioxide, 5% nitrogen) with the package material as a dielectric barrier (Shi et al. 2017). The cold plasma was generated by applying a high voltage with low average current (0.2–1.0 mA) through the gas inside the package. This high voltage creates a strong electric field that generates free electrons that interact with nearby gas molecules (O_2, N_2, CO_2, etc.) to form a quasi-stable charged gas species or plasma. The resulting reactive gas species (RGS), such as NO, NO_2, O, ozone (O_3), and hydroxyl radicals (OH), have bactericidal, fungicidal, and sporicidal characteristics (Lu et al. 2008; Ikawa et al. 2010; Xu et al. 2016). The HVACP process generates much higher concentrations of RGS than other plasma devices because of its higher voltages while the containment of RGS in the package enhances its effectiveness (Patil et al. 2014). Many groups have studied the mechanism of bacterial inactivation by ACP (Gallagher et al. 2007; Cooper et al. 2010; Fröhling et al. 2012; Kvam et al. 2012; van Gils et al. 2013; Zhang et al. 2015). The bactericidal effect arises due to breaking down or causing surface lesions on the bacterial cell wall, loss of membrane integrity or membrane permeabilization, or damaging membrane or intracellular proteins and nucleic acids (Ziuzina et al. 2013; Alkawareek et al. 2014; Han et al. 2015). In the HVACP process, the RGS convert back to the original package gas (e.g., air) within a few hours, leaving no chemical residuals while significantly reducing microorganisms when present (Keener 2016). So far, few studies report ACP-induced microbial decontamination in OJ for larger volumes (up to 50 mL compared to past studies at 50 μL (Shi et al. 2011a) using high voltage (up to 90 kV, rather than <50 kV (Shi et al. 2011) using dielectric barrier discharges (DBDs) to generate ACP, nor for exploring HVACP induced physical and chemical effects in OJ. Therefore, this study provides one of the first feasibility analyses of using HVACP for microorganism inactivation in a relatively large scale (up to 50 mL) of OJ.

The present study assesses the use of HVACP to inactivate *S. enterica* in OJ and the resulting change in OJ's physical and chemical properties. HVACP was generated in a sealed bag, packed with air or modified air (MA65). The effect of plasma exposure model (direct or indirect), treatment time, and post-storage of HVACP were explored on *S. enterica* decontamination. We evaluated OJ quality by assessing pH, Brix, color, the activity of PME, and vitamin C content.

Materials and Methods

Sample Preparation and Bacterial Inoculation

S. enterica serovar Typhimurium ATCC 14028 was obtained from the Department of Food Science, Purdue University. *S. enterica* strains were grown overnight in tryptic soy broth (TSB, Difco™, MD, USA) at 37 °C for 24 h, in a shaking bath. Samples of OJ were inoculated at 0.1% with the overnight culture to achieve an initial population of approximately 5 log colony-forming units (cfu)/mL. The initial cell concentration effect on *S. enterica* inactivation was investigated and optimized to maximize *S. enterica* reduction and exclude cell concentration effects. The optimization procedure included inoculating OJ with an initial concentration of 5–8 log cfu/mL of *S. enterica* and comparing the inactivation rate after HVACP treatment at 90 kV for 2 mins. Correlation between the *D* value and initial cell concentration (log) is linear (Fernández et al. 2012).

The xylose lysine deoxycholate (XLD, Difco™, MD, USA) selective media was used to enumerate organisms in pre- and post-processed samples. Preparing a tenfold dilution series of samples for analysis required using peptone water (Bacto, MD, USA) and plating out 0.1-mL aliquots of relevant dilutions on solid media. Plates were incubated at 37 °C for 24 h and survivors (cfu/mL) were enumerated. Analyzing duplicate plates for each dilution yielded the number of cfu with the counts reported as the average of three independent HVACP treatments. Treated and untreated samples were enumerated immediately and following storage at 4 °C for 24 and 48 h to assess the impact of post-treatment storage.

Sample Packages

Pasteurized orange juice (Tropicana, FL) free of any preservatives and freshly squeezed orange juice (Sunkist, CA) were purchased from a local grocery store. We only use freshly squeezed orange juice to study the enzyme inactivation; all other experiments were performed with pasteurized orange juice. We placed 25- and 50-mL OJ samples (control and inoculated with *S. enterica*) onto a Petri dish (diameter: 85 mm) in a storage box, flushed with filling gas for 3 min, and sealed it with dry air (<5% relative humidity) or MA65 (65%O_2 + 30%N_2 + 5%CO_2, <5% relative humidity) using a Cryovac® B2630T (Sealed Air, NC, USA) high barrier film to retain the plasma and RGS. The gas composition in the box has been tested using detector tubes (Dräger-Tubes®, Houston, TX) to confirm its purity of filling gas (air or MA65).

High Voltage Atmospheric Cold Plasma Treatment

The HVACP device (Fig. 1) is an atmospheric low temperature plasma generator employed for in-package plasma treatment (Yepez and Keener 2016). Electric field voltages up to 90 kV were applied (Patil et al. 2014). The HVACP system included a transformer (Phenix Technologies, MD, USA) with an electrical energy input voltage of 120 V (AC) at 60 Hz. A combination of dielectric barriers was assembled to achieve maximum RGS generation for each gas-package combination. We placed the package between two 15.24-cm-diameter aluminum electrodes with a gap of 4.44 cm and the two Cuisinart® (Cuisinart, NJ, USA) polypropylene layers (355 × 272 × 2.20 mm) above and below the package as additional dielectric barriers. We directly and indirectly exposed 25 and 50 mL of OJ to 90 kV for 30, 60, and 120 s. The treated samples were stored in a refrigerator at 4 °C for 24 h to study the dependence of microorganism

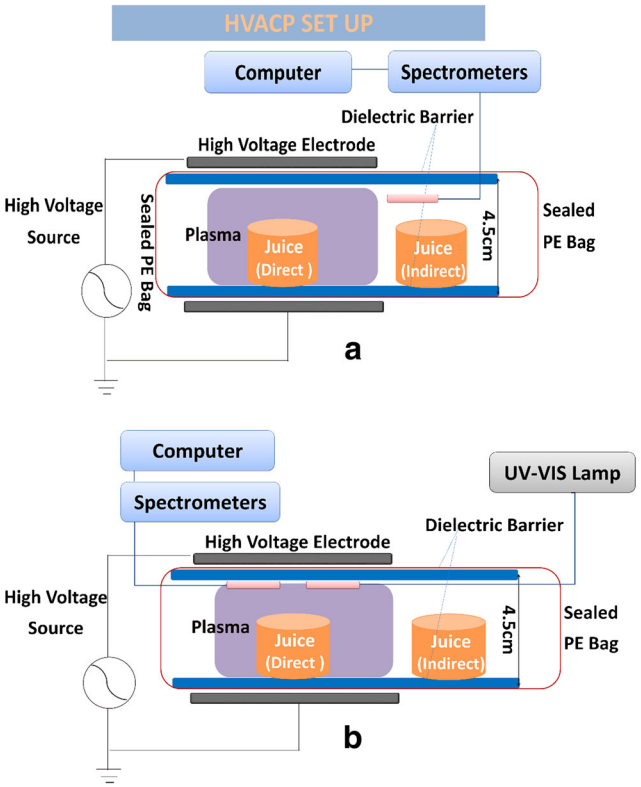

Fig. 1 Schematic of the experimental setup employed for high voltage atmospheric cold plasma (HVACP) treatment of orange juice (OJ) (direct and indirect). **a** Optical emission spectroscopy (OES). **b** Optical absorption spectroscopy (OAS)

population on post-treatment storage. XLD agar (Difco) was used to enumerate *S. enterica*.

Optical Emission Spectroscopy and Optical Absorption Spectroscopy

Optical emission spectroscopy (OES) was performed to characterize the reactive gas species generated in the plasma during the treatment and optical absorption spectroscopy (OAS) to investigate the gas composition post-discharge (Moiseev et al. 2014; Brayfield et al. 2016). OES and OAS were measured using a HR2000+ Spectrometer and 400-mm optical fibers produced by Ocean Optics (FL, USA). The fibers had a numerical aperture of 0.22 and were optimized for use in the ultraviolet and visible portion of the spectrum with a wavelength range between 190 nm and 1100 nm. The distance between the optical fiber and the plasma chamber was 140 mm. The OES spectra were corrected for background noise and recorded every 30 s during the HVACP treatment at 90 kV. The data was recorded and analyzed using OceanView Optics Software (Dunedlin, FL). We identified the peaks by comparing them with the NIST Atomic Spectra Database (Kramida et al. 2016).

The post-discharge gas composition was measured using OAS. Using a UV-visible (UV-Vis) deuterium-hydrogen lamp

as light source, we measured the transmitted light with an Ocean Optics (HR2000+) spectrometer. The optical probes (insulated UV-Vis collimators) were aligned inside the sealed package for all experiments, with an optical path length of 2.4 cm between the probes. Based on Moiseev's method, the Beer–Lambert law was used to calculate the concentrations of ozone (O_3) and nitrogen oxides (NO_2, NO_3, N_2O_4) by averaging concentration along a wavelength interval where each species has an absorption cross-section maximum or values much higher than other absorbent species (Moiseev et al. 2014).

Color, pH, and Brix Measurement

Juice color was measured using a HunterLab colorimeter (ColorFlex model A60-1010-615, Hunter Associates Inc., Reston, VA) at 20 ± 1 °C. The instrument (65°/0° geometry, D25 optical sensor, 10° observer) was calibrated using white (L = 92.8; a = −0.8, b = 0.1) and black reference tiles. Expressing color values as L^* (whiteness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness) allows us to calculate the color difference (ΔE) in OJ after treatment by

$$\Delta E = \sqrt{\left(L^*-L_0\right)^2 + \left(a^*-a_0\right)^2 + \left(b^*-b_0\right)^2}, \quad (1)$$

where L_0, a_0, and b_0 are the color values of control juice samples. Depending on the magnitude of ΔE, one can characterize the development in color difference during storage time as not noticeable (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5–3.0), well visible (3.0–6.0), and great (6.0–12.0) (Cserhalmi et al. 2006). We performed the color measurement in triplicate.

The pH of treated and untreated orange juice samples was measured using a digital pH meter (Orion model 420A, Allometrics Inc., Seabrook, TX). Continually stirred samples (10 mL) were measured at 20 °C.

We measured Brix using an ATAGO™ refractometer (Digital Hand-Held Pocket Refractometer PAL-1) at 20 °C. The refractometer of the prism was cleaned with distilled water after each analysis.

Vitamin C Determination

We used a high-performance liquid chromatography (HPLC) system to measure the total vitamin C content, including ascorbic acid (AA) and dehydroascorbic acid (DHAA), based on Ayhan et al. (2001). A Hewlett-Packard liquid chromatograph (Wilmington, DE) equipped with an auto-sampler and a detector at 254 nm was used. We calculated the HPLC chromatograph peak area using a Hewlett-Packard integrator (HP3396 Series II). A reversed-phase C-18 column (5-μm particle size, 4.6-mm diameter, 250-mm length, Hewlett-Packard) and a Hewlett-Packard C-18 guard column separated the AA using methanol and acidified water (10:90, *v*/*v*) as a mobile phase. The water was acidified (0.01%, *v*/*v*) with phosphoric acid (90%). The mobile phase was filtered using a 0.45-μm membrane filter (Micron Separations Inc., Westboro, MA) and degassed using ultrasound before passing through the column at a flow rate of 0.5 ± 0.01 mL/min. We observed a standard calibration curve by using L-ascorbic acid (Sigma Chemical Co., St. Louis, MO) in concentrations ranging from 5 to 80 mg/100 mL. The OJ was derivatized with dithiothreitol before analysis to reduce the DHAA to the AA. We centrifuged the OJ at 8000×*g* for 10 min in a Beckman Microfuge E (Beckman Instruments Inc., Palo Alto, CA) to remove pulp and coarse cloud particles. We injected 10 μL of the supernatant into the column using the HPLC auto-sampler. We determined the reproducibility of six time analyses per each orange juice sample based on a relative standard deviation ±5%.

Pectin Methyl Esterase

PME activity was determined by titrating the liberated carboxyl group at pH 7.5 (30 °C) based on the methods reported by Rouse and Atkins (1955). After mixing the OJ sample (from fresh squeezed juice) well, we transferred 5 mL into 50 mL of a 1% pectin substrate solution in 0.2 M sodium chloride. The sample was titrated to pH 7.5 with 0.2 N NaOH, which was maintained for 30 min by titrating 0.05 N NaOH to the sample. The volume of 0.05 N NaOH consumed during this time was recorded. We determined the PME activity, P_a, in PME units (PMEu) per gram by

$$P_a = \frac{V_N N_N}{m \times (30\ min)}, \quad (2)$$

where V_N is the volume of NaOH in milliliter, N_N is the normality of NaOH, and m is the mass of the sample in grams.

Statistical Methods

We used SAS Version 10.1 (Statistical Analysis Software, Cary, NC) to analyze all resultant gas concentrations and microbial populations. Statistical analysis of results utilized a general linear model (GLM) for unbalanced data sets and Analysis of Variance (ANOVA) procedure for balanced data. We determined mean differences by using Duncan's LSD Test for separations of means showing significant differences ($P < 0.05$). The mean and standard deviation were calculated for the log reduction of microorganisms for each set of experiments. A 95% confidence interval was used for all procedures.

Results

Effect of HVACP on the Microbial Population in OJ

Effect of Gas Composition

Figure 2 shows the effect of the gas composition on the *S. enterica* inactivation. A 2-min HVACP treatment with air or MA65 as the fill gas followed by 24 h of post-treatment storage resulted in *S. enterica* inactivation. In this study, we determine the inactivation of *S. enterica* by using XLD to enumerate the population difference before and after treatment. The sublethally injured cells, which can reproduce and form colonies on Tryptic Soy Agar (TSA) but not in XLD, were not counted as surviving/viable bacteria after HVACP treatment because they lost metabolic activity (Ulmer et al. 2000).

The population of *S. enterica* in OJ decreases with treatment time. Immediately after subjecting OJ to 2 min of HVACP direct treatment, *S. enterica* population decreased by 0.65 and 1.75 log in air and MA65, respectively. The population of *S. enterica* continued to decrease after 24 h of post-treatment storage (incubation OJ without opening the package at 5 °C). Following 24 h of post-treatment storage, the population of *S. enterica* in 50 mL of OJ subjected to 2 min HVACP treatment decreased by 2.81 log (air) and approximately 5 log (MA65) without recovery. This indicates that HVACP-generated RGS remain in the package and can inactivate *S. enterica* after removing the electricity. MA65 is more effective in inactivating *S. enterica* in OJ compared with air, which may correlate to the different RGS generated during HVACP treatment.

Direct vs. Indirect

Figure 2 shows that both direct and indirect HVACP treatment can achieve microbial decontamination. Other studies (Ziuzina et al. 2013; Han et al. 2015) demonstrated direct plasma treatment more efficiently inactivated *S. enterica* than indirect treatment. There is no significant difference ($P < 0.05$) between direct and indirect treatment for short (<1 min) HVACP treatment time. A 2-min direct HVACP treatment of 50 mL OJ resulted in a 1.75-log (MA65) reduction immediately (0 h) after treatment while indirect treatment induced a 0.85-log (MA65) reduction (Fig. 2a). Applying a 24-h post-treatment storage after 2 min HVACP with MA65 reduced *S. enterica* population by 3.78 log with indirect exposure and by greater than 5 log (under the population threshold for detection) with direct exposure (Fig. 2a). In Fig. 2b, the high microbial inactivation in direct (MA65-D-24h) compared with indirect (MA65-IN-24h) exposure at 120 s may arise due to undetectable RGS, UV light, electroporation (Corrales et al. 2008), or other short-lived species (many of which are not available for OJ during indirect treatment) that may also have microbiocidal effect. This high inactivation effect in direct treatment has been reported previously (Stoffels et al. 2008; Misra et al. 2012; Han et al. 2015). Stoffels et al. (2008) reported that during direct treatment, the most reactive short-lived species (such as charged species and certain radicals) have the highest probability of reaching the surface. Also, bombardment of charged RGS in direct treatment (Dobrynin et al. 2009) may facilitate RGS diffusion into liquid to generate microbiocidal compounds (Johnson 2004; Oehmigen et al. 2010; Kim et al. 2014). An electric field will enhance the mass transfer by electroporation (Corrales et al. 2008), which facilitates RGS diffusion. Dobrynin et al. (2009) reported that the

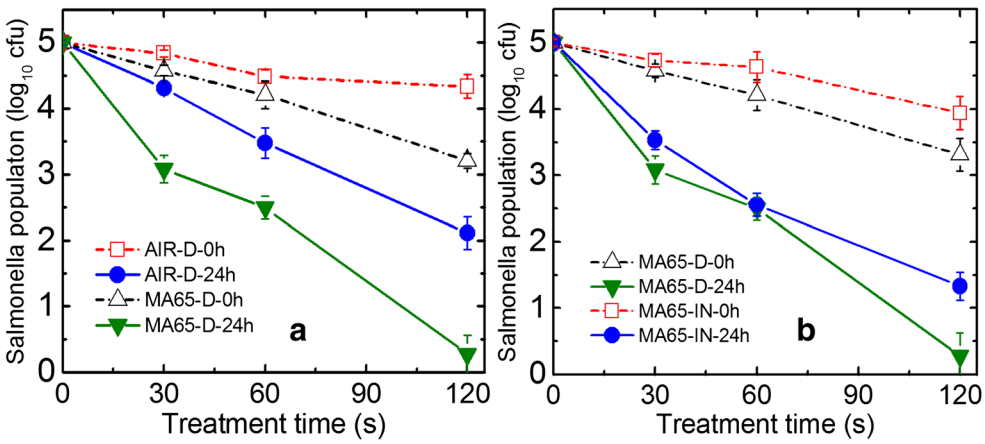

Fig. 2 The survival of *Salmonella enterica* serovar Typhimurium (*S. enterica*) population ($\log_{10}$ CFU) is as a function of treatment time. **a** Fifty milliliters of OJ packed with air or MA65 is treated with HVACP at 90 kV. OJ subject to direct HVACP treatment: air packed (*white square*); (*white up-pointing triangle*) MA65 packed. HVACP-treated OJ after 24 h post-storage: (*black circle*) air packed; (*black down-pointing triangle*) MA65 packed. **b** Fifty milliliters of OJ packed with MA65 is treated with HVACP at 90 kV directly or indirectly. OJ subject to HVACP treatment: (*white square*) direct treatment; (*white square*) indirect treatment. HVACP-treated OJ after 24 h post-storage: (*black down-pointing triangle*) direct treatment; (*black circle*) indirect treatment. Values represent the mean and standard deviations of three replicates

microbiocidal effect of plasma treatment in liquid may be enhanced due to energetic ion bombardment. In the 50-mL OJ sample, there is no significant difference between direct and indirect treatment for short treatment times. Differences detected following 24 h of post-storage incubation (Fig. 2b) indicate that the long-living RGS, including hydrogen peroxide and ozone, contribute to the microbiocidal effect of HVACP. Therefore, both direct and indirect treatment can achieve a 3.5–5-log *S. enterica* reduction in 50 mL OJ with 2 min HVACP treatment and 24-h post-storage.

Effect of Sample Height and Exposure Surface Area

We next investigated the effect of sample height and exposure surface area on the *S. enterica* inactivation efficiency of HVACP treatment. With the same exposure surface area, the inactivation of *S. enterica* in 25-mL OJ (sample a, Fig. 3a) is higher than 50-mL OJ (sample b, Fig. 3b). Within 30 s, HVACP direct treatment achieved greater than 5 log reduction (detection limit) of *S. enterica* in sample (a); however, it required 120 s to achieve 5 log reduction in sample (b) with double height (10 mm). With the same sample volume (50 mL), the inactivation of *S. enterica* with the double exposure surface (Fig. 3c) in OJ is higher than the single exposure

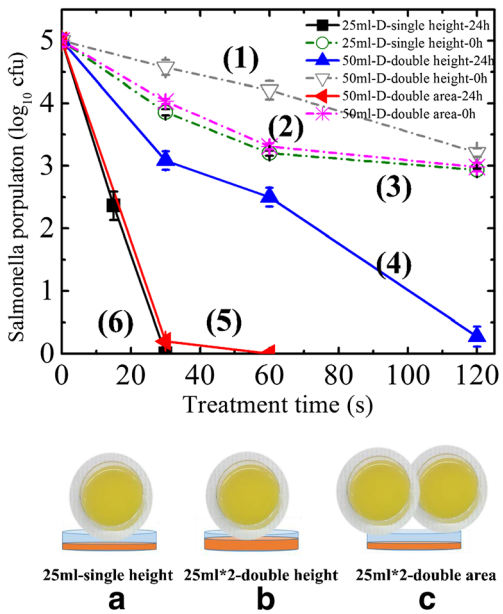

Fig. 3 The survival of *S. enterica* population ($\log_{10}$ CFU) is influenced by exposure surface and sample height. Twenty-five milliliters or 50 mL of OJ packed with MA65 treated directly with HVACP at 90 kV. The survival of *S. enterica* population ($\log_{10}$ CFU) is as a function of treatment time. OJ subject to HVACP treatment: (*1*) 50-mL OJ with double height (9 mm); (*2*) 50-mL OJ with double area (56 $cm^2 \times 2$); (*3*) 25-mL OJ with single height (4.5 mm). HVACP-treated OJ after 24-h post-storage: (*4*) 50-mL OJ with double height; (*5*) 50-mL OJ with double area; (*6*) 25-mL OJ with single area (56 cm^2). Values represent the mean and standard deviations of three replicates test

surface of OJ (Fig. 3b). Within 60 s, HVACP achieved greater than 5 log reduction of *S. enterica* in sample (c). This indicates that for a certain volume of OJ, increased exposure surface and minimized sample thickness will achieve a high *S. enterica* inactivation efficiency, which may accelerate RGS diffusion into OJ. Modeling reaction mechanisms consisting of 79 gas phase species, 83 liquid phase species, 1680 gas phase reactions, and 448 liquid reactions, Lietz and Kushner (2016) showed that RGS diffused through the liquid and induced additional chemical reactions at the surface or inside the liquid to generate additional species. Bruggeman et al. (2016) reported that the RGS can react at or penetrate through the plasma-gas/liquid interface and dissolve into the bulk liquid, initiating secondary chemical processes. This agrees with other reports (Patil and Bourke 2016; Surowsky et al. 2016). Microbial inactivation efficiency of plasma is influenced by the sample depth, sample volume, and food composition (Surowsky et al. 2016). Therefore, increasing the exposure surface and minimizing OJ sample thickness are crucial for maximizing *S. enterica* decontamination using HVACP.

Optical Emission Spectroscopy

We used OES to characterize the different main emissions of the RGS generated by air and MA65 during direct and indirect treatment. Figure 4 and Table 1 show that the recorded spectrum consists of various molecular and atomic nitrogen and/or oxygen species. During treatment, both direct and indirect treatment can generate RGS, which have microbiocidal effects. The major peaks in the spectra correspond to the emissions of excited species of atomic nitrogen and atomic oxygen, including the nitrogen second positive system N2(C-B) (Ricard et al. 2013), the first negative system N2+ (B-X) (at 336, 357, 380, 390, 405, 426 nm), and optical transitions of the O atom, including 616 nm and 777 nm (Choi et al. 2005; Machala et al. 2007). The OH peak around 309 nm was also identified. Figure 4 indicates that using MA65 as the fill gas generates primarily reactive oxygen species (ROS) while air generates reactive nitrogen species (RNS), which may contribute to the different microbial decontamination efficiency. At the same time, the different active ions and free radicals reach the liquid phase and produce various biologically active reactive species (RS) in the liquid phase (Dobrynin et al. 2009; Zhang et al. 2015), including long-lived RS, such as hydrogen peroxide (H_2O_2), ozone (O_3), and nitrate ion (NO_3^-), and short-lived RS, such as hydroxyl radical (OH^-), superoxide (O_2^-), and singlet oxygen (Arjunan et al. 2011). The signal will disappear after removing the field from the sample due to the short life of the atomic species and will either generate long-living species through complicated reactions or react with the liquid phase. There are more than 75 species generated among 500 reactions as a function of treatment time and

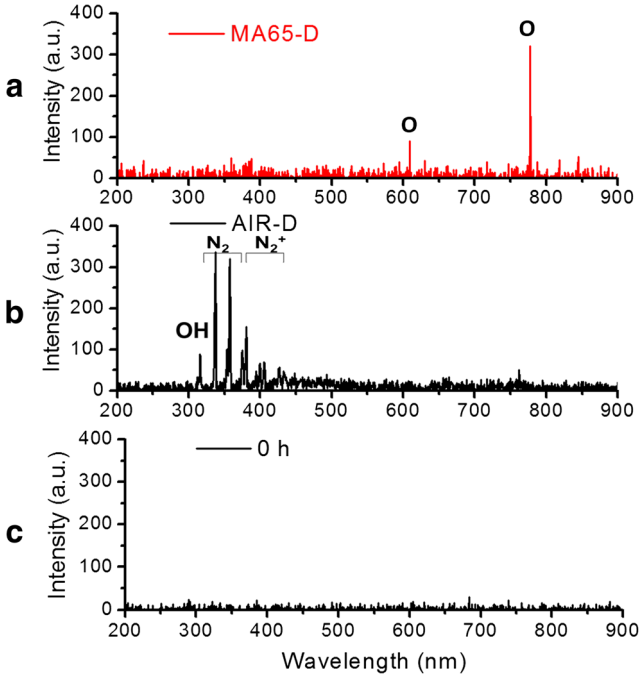

Fig. 4 Optical emission spectroscopy results of MA65 (**a**) and air (**b**) packed OJ during HVACP treatment (direct) at 90 kV for 2 min. **c** Optical emission spectroscopy of control

energy scale (Gordillo-Vázquez 2008), which have microbiocidal effects and are undetected by OES.

Optical Absorption Spectroscopy

Figure 5a shows the typical absorbance during HVACP treatment with the intensity increased with increasing treatment time. The concentration of O_3 and nitrogen oxides (NO_2, NO_3, N_2O_4) was calculated based on their optical absorbance (Moiseev et al. 2014; Ishikawa 2016), as shown in Fig. 5b. Within 2 min of HVACP direct treatment, O_3 concentrations were 1660 ppmv in MA65 and 990 ppmv in dry air (5% humidity) packed samples. These high concentrations of O_3, as well as NO_2, NO_3, and N_2O_4 generated by MA65, may contribute to its higher microbial inactivation rate than air. Therefore, using MA65 which has a higher concentration of O_2, as the plasma generation gas enhances its microbial inactivation effectiveness, as observed in other studies (Sureshkumar et al. 2010; Xu et al. 2016; Wan et al. 2017). Upon turning off the HVACP, the OAS signal slowly decreased (fitting second-order polynomial model—MA65: $y = 0.3315x^2 - 42.692x + 1891.9$, $R^2 = 0.9977$; air: $y = 0.2821x^2 - 28.278x + 902.32$, $R^2 = 0.9627$) indicating the correlated RGS do not disappear immediately and can continue inactivating bacteria during post-treatment. This is consistent with the extra reduction during the post-treatment (Figs. 2 and 3). However, HVACP generate numerous additional species that could contribute to the observed mechanisms and should be considered, such as HONO, HO_2NO_2, and HNO_3 (Brayfield et al. 2016), which currently are not measurable with OAS.

Table 1 The major peaks in the spectra correspond to the emissions of excited species (Ricard et al. 2013)

Wavelength (nm)	Species
309	OH
336	N_2
357	N_2
380	N_2
390	N_2^+
405	N_2^+
426	N_2^+
616	O_2^+
673	O_2^+
686	O_2^+
777	O

Structure Analysis Using Scanning Electron Microscope

Figure 6 shows a scanning electron microscope (SEM) image of *S. enterica* cells in the control and in OJ subjected to HVACP treatment for 120 s. *S. enterica* in the control have relatively smooth bacterial cell walls (Fig. 6a), while the cell surface wrinkles and the cell lyses for HVACP-treated *S. enterica* in both air and MA65 packed OJ samples (Fig. 6b, c). This is consistent with other reports (Kuo et al. 2006; Ziuzina et al. 2013; Han et al. 2015). Cell morphology has changed compared to the control. In Fig. 6c and its replications, SEM showed cell alterations in HVACP-treated bacteria surface morphology and loss of integrity. Severe physical damage, including etching and irregular surfaces on the *S. enterica* cells, also occurred. Han et al. (2015) reported that HVACP induced microorganism inactivation occurred due to RGS either reacting with the cell membrane or damaging intracellular components (e.g., nucleic acids, proteins/enzymes). The mechanism of various RGS on the bacteria could be investigated by checking the effect on cell membrane integrity: absorbance of intracellular components is at 260 nm for nucleic acid, at 280 nm for protein.

Effect of HVACP on the Physical and Chemical Properties of OJ

Pectin Methylesterase

Figure 7a shows the residual activity of the PME as a function of treatment time. The activity of PME decreased with increasing HVACP treatment time using either air or MA65 as the fill gas. Twenty-four-hour post-treatment storage further inactivates PME by 73–76% compared to its activity in fresh

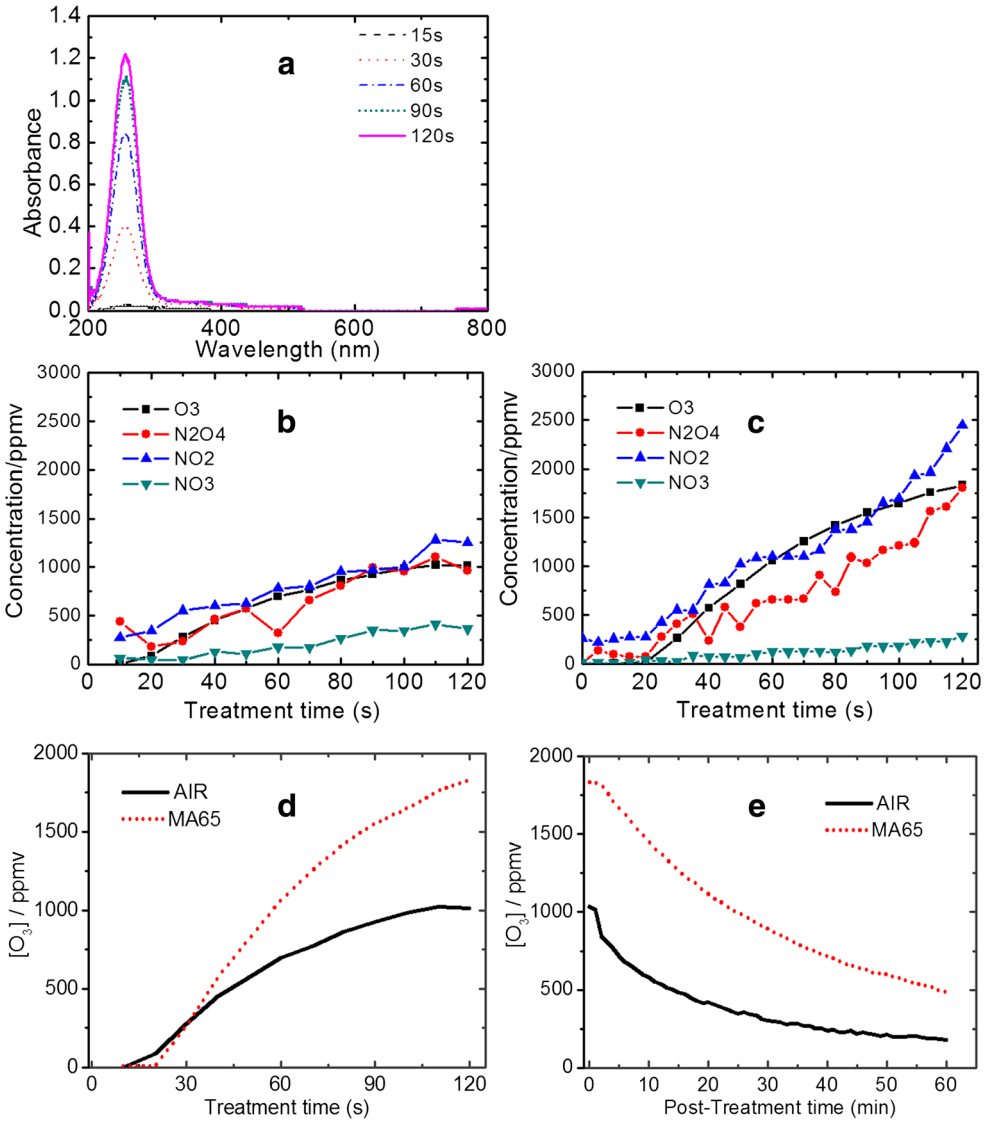

Fig. 5 **a** Optical absorption spectroscopy (OAS) signals of MA65 packed OJ during HVACP treatment (direct) at 90 kV for 2 min. **b** (air), **c** (MA65) Concentration of reactive gas species (O_3—*black*, NO_2—*blue*, NO_3—*green*, N_2O_4—*red*) of MA65 packed OJ during HVACP treatment (direct) were calculated from OAS signals of Fig. 5a, based on the Beer–Lambert law by averaging concentration along a wavelength interval. **d** O_3 concentrations in air (*straight line*) and MA65 (*black circle*) packed OJ (50 mL) during HVACP treatment (direct) at 90 kV for 2 min. **d** O_3 concentrations during post-treatment storage

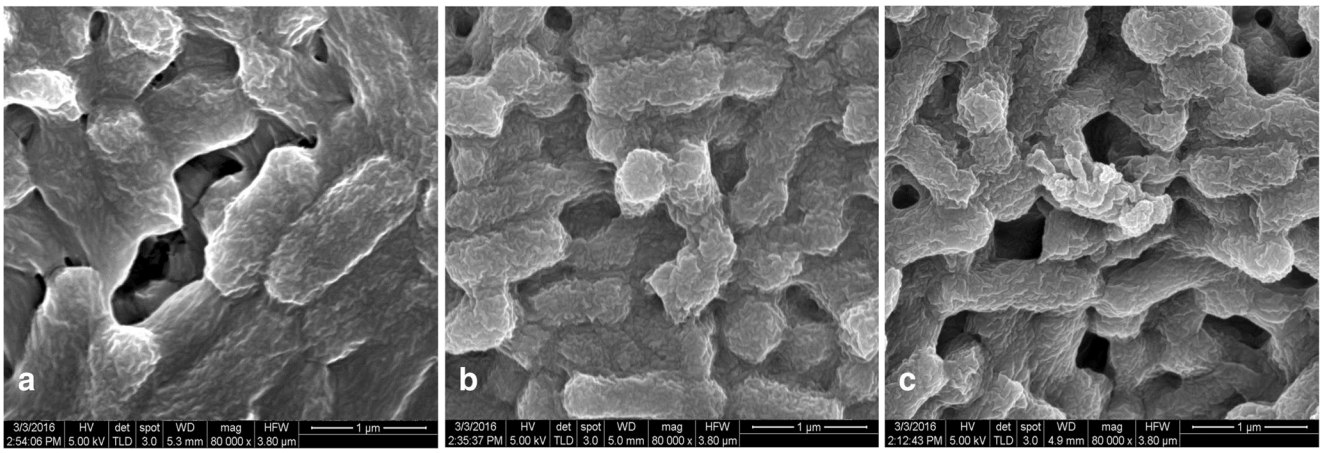

Fig. 6 Scanning electron microscope image of *S. enterica* cells in the control and OJ subjected to HVACP treatment (direct) at 90 kV for 120 s. *S. enterica* were isolated from OJ by centrifugation and washed with phosphate buffer saline. **a** Control. **b** Air packed. **c** MA65 packed OJ

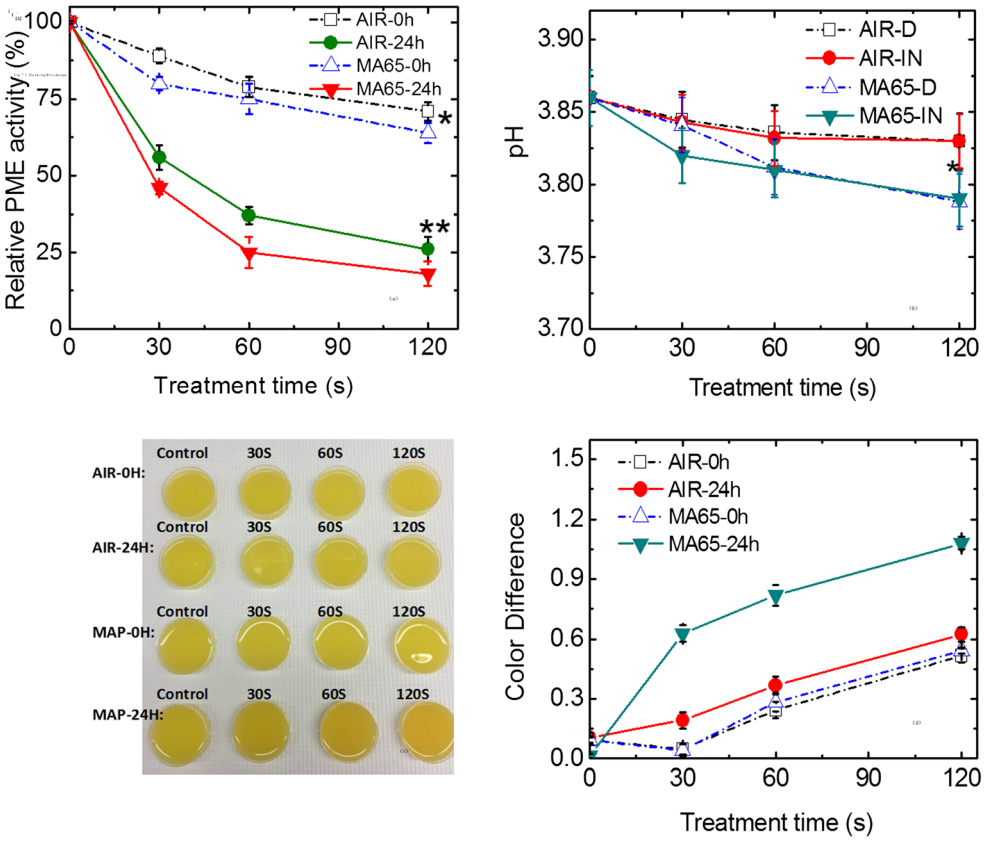

Fig. 7 Pectin methyl esterase (*PME*) activity. pH of OJ packed with air and MA65 subjected to HVACP treatment (direct) at 90 kV for 120 s. Color observation. The color difference (ΔE) of OJ packed with air and MA65 subjected to HVACP treatment (direct) at 90 kV up to 120 s. Color of OJ subjected to HVACP treatment at 0 h and after 24-h post-storage was compared. *Asterisk*: ANOVA test results indicate significant difference between 0- and 24-h post-storage ($P < 0.05$). Values represent the mean and standard deviations of three-replicate test

OJ. A 16% residual PME activity was observed after OJ pasteurization at 66 °C for 60 s, and 99% PME was inactivated at 90 °C for 60 s (Sadler et al. 1992). Therefore, a higher PME inactivation may be achieved by increasing HVACP treatment intensity. The inactivation of PME may relate to its structural modification, which was caused by RGS, and the RS generated in the liquid phase. The mechanism of PME inactivation by HVACP treatment has not been reported before. It is estimated that ROS initiated structure modification of PME by oxidative attack leads to loss of enzyme functionality, which is consistent with other reports (Yin et al. 2005; Henselová et al. 2012; Tappi et al. 2014). Takai et al. (2012) confirmed that hydroxyl radicals (OH), superoxide anion radicals (O_2^-), hydroperoxy radicals (HOO), and nitric oxide (NO) generated from plasma sources may modify reactive side chains of the amino acids, such as cysteine, and aromatic rings of phenylalanine, tyrosine, and tryptophan, leading to loss of enzyme activity. Figure 7a also indicates that using MA65 as the fill gas more efficiently inactivates PME, which may relate to its higher ROS species concentration. For instance, the concentration of O_3 in MA65 (1810 ppmv) is higher than in air (930 ppmv) at 120 s, indicating a higher oxidative capacity. However, the generation of hydroxyl radicals and nitrous/nitric acid in the liquid phase (Oehmigen et al. 2010; Van Gils et al. 2013; Jablonowski 2015) may also accelerate the PME inactivation. The RS transported from the gas phase into the liquid transform via successive reactions into products that inactivate enzymes (Misra et al. 2016). Therefore, RGS and RS generated in the liquid inactivate PME in OJ during HVACP treatment.

pH, Brix, and Color

HVACP treatment reduced pH from 3.86 to 3.80 (Fig. 7b); Brix did not change significantly ($P < 0.05$) in OJ after HVACP treatment (data not shown). The pH decrease might be due to the formation of nitric acid (HNO_3) and nitrous acid (HNO_2) in the liquid phase (Oehmigen et al. 2010). Helmke et al. (2011) reported that pH following plasma treatment depended strongly on treatment time, which is consistent with the results in Fig. 7b. Further analysis of the acid profile may better explain the mechanism of the pH shift since OJ has its own buffering capacity, consisting of citric acid, malic acid, and ascorbic acid.

Figure 7c shows pictures of OJ with air/MA65 before and after post-storage, demonstrating the color of OJ subject to HVACP treatment. The United States Department of Agriculture (USDA) assigned 40 points out of a scale of 100 points for color for the commercial classification of OJ. Grade A OJ must have a color number between 36 and 40 points, while grade B OJ has color numbers ranging from 32 to 35 points (Lee and Coates 2003; Meléndez-Martínez et al. 2005).

However, according to Cserhalmi et al. (2006), practically, it is more valuable to compare the color difference (ΔE) values of OJ. Figure 7d shows ΔE, which is the sum of the square of the changes in L^*, a^*, and b^*. We consider ΔE because the consumer will more likely perceive the total change in color than the individual L^*, a^*, or b^* values. Treating OJ with HVACP for 120 s yielded ΔE of 0.51 (air) and 0.54 (MA65), which were classified as slightly noticeable (0.5–1.5). The largest ΔE for HVACP-treated OJ was 1.08, which is still within the range of slightly noticeable (0.5–1.5), following 24 h of post-treated storage of OJ packed with MA65 (Cserhalmi et al. 2006). Although some color parameters presented statistical difference ($P > 0.05$), the characteristic juice color remained in the expected ranges for OJ with a slight increase in ΔE. Different plasma-gas resources (e.g., helium) or reduced treatment time may mitigate the slight color change.

Vitamin C Content

Vitamin C content is an important parameter to evaluate the nutritional quality of OJ (Tiwari et al. 2009b). Vitamin C is heat-labile and easily destabilized during thermal processing or post-storage (Polydera et al. 2003; Sánchez-Moreno et al. 2005). In this study, vitamin C content is the total concentration of AA and DHAA, which is consistent with industry requirements and other studies (Polydera et al. 2003; Vervoort et al. 2011). Figure 8 shows the vitamin C content after HVACP treatment compared with untreated OJ. The vitamin C concentration decreases as a function of increasing treatment time: from 53 mg/100 mg (control) to 41 mg/100 mg (in air) and 24 mg/100 mg (in MA65), after 2 min HVACP treatment subject 24 h post-storage. A minimum of 30 mg of vitamin C per 100 mL of juice is required to meet the commercial standard (US Food and Drug Administration 2016). This loss of vitamin C may arise due to the high concentration of ROS and RNS in the RGS generated by HVACP.

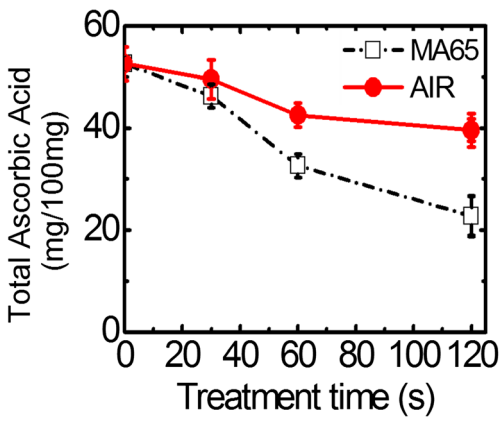

Fig. 8 The vitamin C content in OJ packed with air and MA65 subjected to direct HVACP treatment at 90 kV up to 120 s and stored for 24 h. Values represent mean and standard deviations of three-replicate test

AA is sensitive to O_3 and easily degraded by other free radicals (García-Viguera and Bridle 1999; Tiwari et al. 2008). Traditional thermal processing frequently uses higher temperature to establish more excessive PME inactivation but inevitably induces vitamin C breakdown. Vikram et al. (2005) reported that OJ subject conventional thermal treatment at 90 °C for 2 min, the retention of vitamin C was less than 50%. Since HVACP is a nonthermal processing technology, it may better preserve vitamin C by using a different fill gas to generate RGS and may inactivate PME while preserving vitamin C concentration.

Discussion

The effect of HVACP on *S. enterica* inactivation in OJ depends on the mode of plasma exposure, gas type, exposure surface area, and treatment time. It is hypothesized that HVACPs primarily inactivate microorganisms in liquid food by creating RGS and subsequent RS (generated by RGS in the liquid phase through chemical reactions) which induce DNA/protein alteration, integrity loss, and cell lysis (van Gils et al. 2013; Han et al. 2014; Zhang et al. 2015). Charged species were identified as the major contributors to the microbiocidal effect. ROS, including hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and the superoxide anion (O_2^-), induce DNA breakdown inside the cells (Dobrynin et al. 2009). A combination of plasma-induced membrane pore formation and peroxidation may lead to cell damage/apoptosis and loss of bioactivity and functionality (Dobrynin et al. 2009; Kvam et al. 2012). Various CAP-microorganism inactivation models have been proposed, including direct destruction of microbial genetic material by UV irradiation, cell surface etching induced by RGS, volatilization of compounds, and intrinsic photodesorption of UV photons (Korachi and Aslan 2013) and that is followed by RS formation in the liquid phase (Moisan et al. 2001; Schnabel et al. 2014). Shi et al. (2011) reported that ROS and RNS played dominant roles in microbial inactivation by reacting with various macromolecules on the microorganisms' outer surfaces (such as membrane lipids) or inside the microorganisms (protein and nucleic acids) to induce microbial death or injury in OJ. Therefore, the higher concentrations of ROS and RNS generated by HVACP, characterized by OES and OAS (Fig. 5), may enhance microbial inactivation. In this study, treatment factors, such as using MA65 with high O_2 concentration to generate effective RGS (Fig. 4) or using direct treatment with a large surface exposure combined with sufficient treatment time, may facilitate interactions between RGS and *S. enterica* to optimize microbial inactivation (Figs. 2 and 3).

The quality of OJ, including PME activity, pH, color, and vitamin C content, depends on HVACP treatment time, fill gas, exposure model, and post-storage time. This is primarily

related to the specific RGS formed and their concentrations under various conditions. ROS and RNS initiate multiple chain reactions, resulting in numerous chemical reactions and species, including the following (Surowsky, et al. 2015):

$$OH + NO \rightarrow NO_2^- + H^+ \quad (3)$$

$$O + H_2O \rightarrow 2OH^- \quad (4)$$

$$2OH \rightarrow H_2O_2 \quad (5)$$

$$OH + H_2O_2 \rightarrow OOH + H_2O \quad (6)$$

$$OH + NO_2^- \rightarrow OH^- + NO_2 \quad (7)$$

Surowsky et al. (2015) reported the following chemical reactions occur at the gas–liquid interface: (1) acid–base reactions, (2) oxidation reactions, (3) reduction reactions caused by reductive species (e.g., H and HO radicals), and (4) photochemical reactions initiated by UV radiation from the plasma. The pH decrease in Fig. 7b occurred due to the formation of nitrous and nitric acids, excited nitrogen species, and their products (NO_2^-, NO_3^-), as well as singlet oxygen during plasma treatment, which is more predominant in nonbuffered solutions (Misra et al. 2011). Oxidation reactions initiated by ROS and RNS are the most important plasma-related reactions which may inactivate microorganisms and degrade organic compounds. This may be responsible for the vitamin C loss and color alteration (Tiwari et al. 2008). In this study, using MA65 as process gas combined with a longer treatment time is preferred for inactivating PME and *S. enterica* in OJ. However, color retention was greatly reduced during subsequent storage using MA65 as process gas, whereas the color was less affected by the use of ambient air, which was consistent with previous studies (Klockow and Keener 2009). Therefore, adjusting the gas composition to control the gas RGS formation can minimize both color alteration and vitamin C loss. Therefore, elucidating the inactivation mechanism and reaction rate between HVACP to PME, vitamin C, and other chemical compounds will enable the development of an efficient HVACP system for decontaminating *S. enterica* in OJ without quality alteration.

Conclusion

This study examined the impact of HVACP treatment on *S. enterica* deactivation in OJ and OJ quality. The novelty of this study addressed using a high voltage (up to 90 kV) to treat higher volumes of OJ (up to 50 mL), instead of using 30 kV and 50 μL, as in previous studies (Shi et al. 2011). Efficient *S. enterica* inactivation has been achieved by using either air or MA65 as the fill gas in HVACP treatment of OJ. We achieved more than 5 log reduction of *S. enterica* in 25 mL OJ within 30 s of direct or indirect HVACP treatment using either air or MA65 as fill gas. For 50 mL OJ, 120 s of direct HVACP treatment and 24 h storage induced a 2.9-log reduction of *S. enterica* in air and a 4.7-log reduction in MA65 gas. Therefore, MA65 more effectively inactivates *S. enterica* in OJ compared to air, likely due to the different RGS generated during HVACP treatment. Using MA65 as the fill gas, extending treatment time, and expanding exposure surface can maximize inactivation of *S. enterica* population and decrease the PME activity of HVACP treatment. No significant color, Brix, nor pH change occurred following 120-s HVACP treatment. The concentration of vitamin C decreased by 22% (in air) after 120-s HVACP direct treatment in 25-mL air packed OJ compared with untreated OJ. Therefore, we conclude that HVACP treatment can be an effective nonthermal technology to control, or potentially eliminate, *Salmonella* in OJ with minimal quality alteration.

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