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Effect of temperature on chlorine dioxide inactivation of *Escherichia coli* O157:H7, *Salmonella* typhimurium, and *Listeria monocytogenes* on spinach, tomatoes, stainless steel, and glass surfaces

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ABSTRACT

The objective of this study was to evaluate how treatment temperature influences the solubility of ClO_2 gas and the antimicrobial effect of ClO_2 gas against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on produce and food contact surfaces. Produce and food contact surfaces inoculated with a combined culture cocktail of three strains each of the three foodborne pathogens were processed in a treatment chamber with 20 ppmv ClO_2 gas at 15 or 25 °C under the same conditions of absolute humidity (11.2–12.3 g/m^3) for up to 30 min. As treatment time increased, ClO_2 gas treatment at 15 °C caused significantly more ($p < 0.05$) inactivation of the three pathogens than treatment at 25 °C. ClO_2 gas treatment at 25 °C for 30 min resulted in 1.15 to 1.54, 1.53 to 1.88, and 1.00 to 1.78 log reductions of the three pathogens on spinach leaves, tomatoes, and stainless steel No.4, respectively. ClO_2 gas treatment at 15 °C for 30 min caused 2.53 to 2.88, 2.82 to 3.23, and 2.37 to 3.03 log reductions of the three pathogens on spinach leaves, tomatoes, and stainless steel No.4, respectively. Treatment with ClO_2 gas at 25 °C for 20 min resulted in 1.88 to 2.31 log reductions of the three pathogens on glass while > 5.91 to 6.82 log reductions of these pathogens occurred after 20 min when treated at 15 °C. Residual ClO_2 levels after gas treatment at 15 °C were significantly ($p < 0.05$) higher than those at 25 °C. The results of this study can help the food processing industry establish optimum ClO_2 gas treatment conditions for maximizing the antimicrobial efficacy of ClO_2 gas.

1. Introduction

Chlorine dioxide (ClO_2) has emerged as a promising non-thermal sanitizing technology in recent years (Bhagat et al., 2010). ClO_2 is a strong oxidizing agent, and functions as a selective oxidant by a one-electron transfer mechanism where it attacks electron-rich centers in organic molecules and is reduced to the ClO_2^- ion (Hoehn et al., 1996). The mechanism of inactivation by ClO_2 is oxidative attack on cell membrane proteins and enzymes and increased membrane permeability (Aieta and Berg, 1986). Also, it penetrates the cell membrane and damages proteins and enzymes within the cell (USDA, 2002). Studies on application of ClO_2 gas to fresh produce, such as blueberries (Sun et al., 2014), spinach (Neal et al., 2012; Park and Kang, 2015), potatoes (Wu and Rioux, 2010), oranges (Bhagat et al., 2011), tomatoes (Bhagat et al., 2010; Trinetta et al., 2013), lettuce (Mahmoud and Linton, 2008), mung bean sprouts (Prodduk et al., 2014), carrots (Sy et al., 2005), and cantaloupe (Mahmoud et al., 2008) have been reported. Also, the antimicrobial effect of ClO_2 gas against pathogens on food contact surfaces such as stainless steel (Vaid et al., 2010; Trinetta et al., 2012), wood, plastic (Han et al., 2003), polyvinyl chloride, and glass (Li et al., 2012, Morino et al., 2011) have been evaluated. However, most of these studies only evaluated the antimicrobial effect of ClO_2 gas against several foodborne pathogens on various sample surfaces according to gas concentration and treatment time.

The antimicrobial efficacy of ClO_2 gas is affected by intrinsic factors such as sample surface characteristics (roughness and hydrophobicity) and extrinsic factors such as gas concentration, treatment time, and relative humidity (RH) (Han et al., 2001; Park and Kang, 2017). Especially, Park and Kang (2016) reported that residual ClO_2 on produce surfaces increased with increasing RH and the amount of residual ClO_2 on produce is correlated with the level of inactivation of pathogens. Also, Park and Kang (2017) reported that surface hydrophobicity is a more important factor relative to bacterial inactivation by ClO_2 gas than is surface roughness. Water contact angles of selected produce and food contact surfaces were highly negatively correlated with log reductions of foodborne pathogens: that is, the more hydrophobic the

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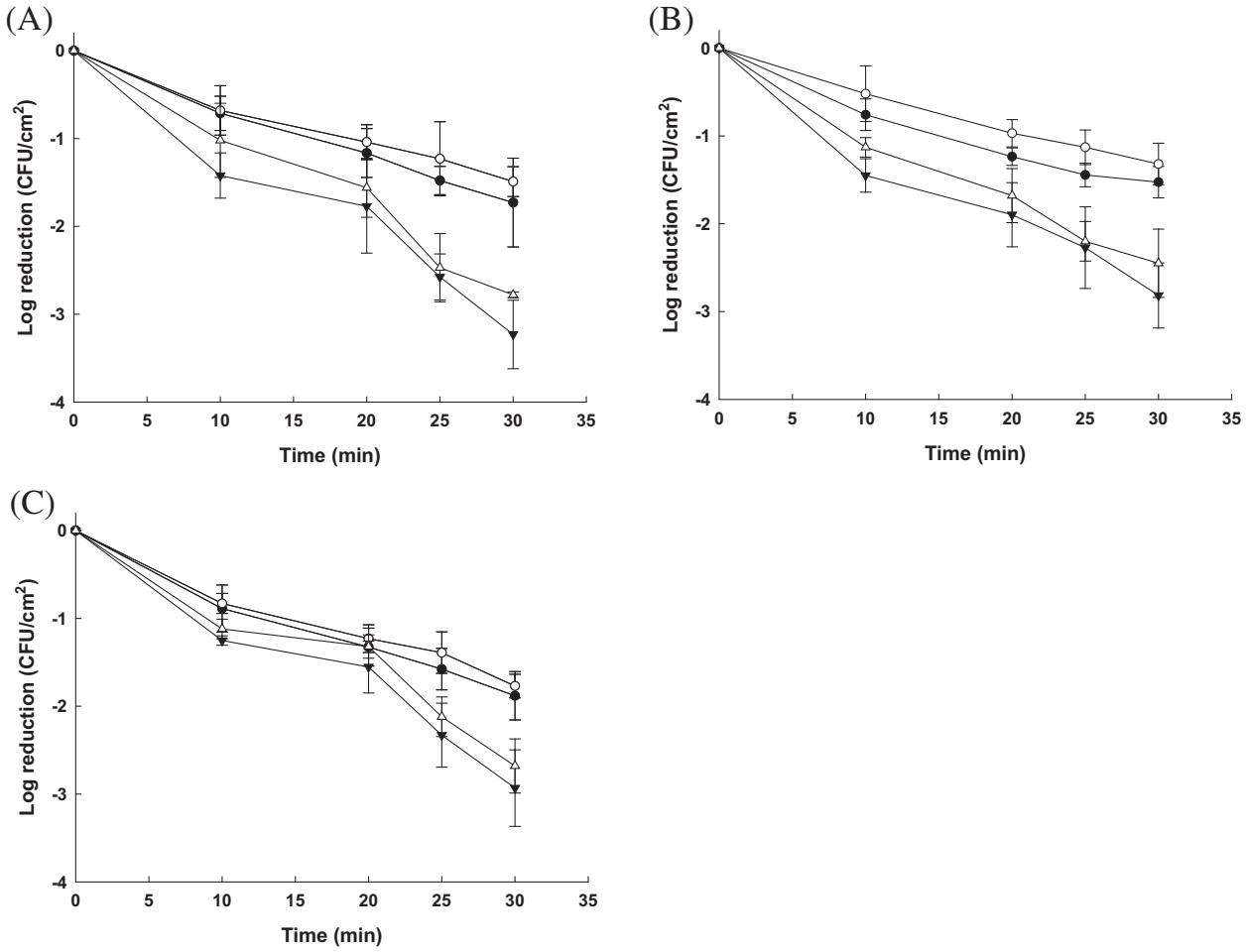

Fig. 1. Log reduction of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and L. *monocytogenes* (C) on tomatoes treated with 20 ppmv ClO2 gas at 15 °C (○, total cells; ●, healthy cells) and 25 °C (△, total cells; ▼, healthy cells). Each point represents the mean for triplicates, and error bars show standard errors.

surface, the lower the reduction of the three pathogens. Differences in reduction levels according to hydrophobicity may be due to different levels of hydration of each sample surface. As is well known, ClO_2 gas has high solubility in water, so it acts similar to aqueous ClO_2 for inactivating microorganisms (Linton et al., 2006).

Treatment temperature may be an important factor affecting antimicrobial efficacy of ClO_2 gas because it could affect ClO_2 gas solubility and reactivity simultaneously. In the case of aqueous ClO_2, inactivation efficacy generally increases with increasing treatment temperature (Taylor et al., 1999; Vicuña-Reyes et al., 2008). However, there have been no studies considering the influence of treatment temperature on the inactivation efficacy of ClO_2 gas. Although Han et al. (2001) evaluated the correlations between temperature (5 to 25 °C) and RH (55 to 95%), absolute humidity (AH) should be used to compare the effect of different treatment temperatures on the microbial inactivation of ClO_2 gas. The objective of this study was to determine how ClO_2 gas treatment temperature influences the antimicrobial effect of ClO_2 gas against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on produce and food contact surfaces.

2. Materials and methods

2.1. Bacterial strains and culture preparation

Three isolates each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, and DT 104), and L. *monocytogenes* (ATCC 19111, ATCC 19115, and ATCC 15313) obtained from the bacterial culture collection of the Food Safety Engineering Laboratory at Seoul National University (SNCC; Seoul, South Korea) were used in this study. All three strains of *E. coli* O157:H7 were human feces isolates. *S.* Typhimurium ATCC 43971 was derived from an existing strain. *L. monocytogenes* strains ATCC 19111, 19,115, and 15,313 were isolated from poultry, human, and rabbit subjects, respectively. All strains of *E. coli* O157:H7, *S.* Typhimurium, and L. *monocytogenes* were cultured individually in 10 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 °C for 24 h and harvested by centrifugation at 4000 ×*g* for 20 min at 4 °C. The final pellets were resuspended in sterile buffered peptone water (BPW; Difco), corresponding to approximately 7.0–8.0 log CFU/ml. Then, suspended pellets of the three pathogens were combined to comprise a mixed culture cocktail containing approximately equal numbers of cells of each strain of *E. coli* O157:H7, *S.* Typhimurium, and L. *monocytogenes*.

2.2. Sample preparation and inoculation

Spinach and tomatoes were purchased from a local market (Seoul, South Korea), washed in running water, then dried in a laminar flow biosafety hood (22 ± 2 °C) for 1 h before experiments to remove surface moisture. Spinach and tomatoes used in this study were previously screened to ensure no presumptive *E. coli* O157:H7, *Salmonella*, or L. *monocytogenes*-like colonies were recovered from un-inoculated samples. Spinach leaves and tomato surfaces were cut into 5 × 2 cm pieces, and the upper side of spinach leaves and outer surface of tomatoes were wiped with clean tissue paper (Kimtech Science Wipers, Yuhan-

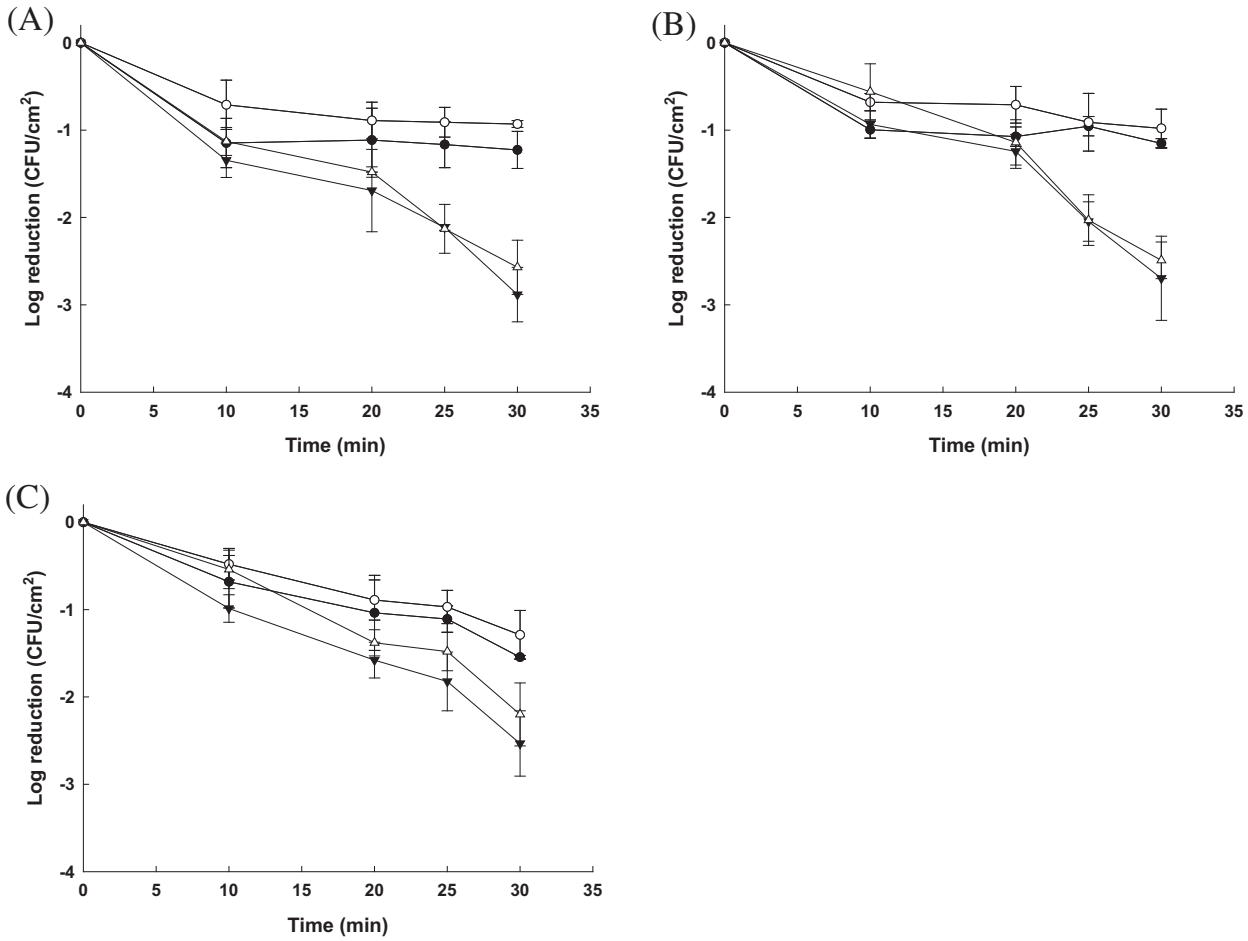

Fig. 2. Log reduction of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) on spinach treated with 20 ppmv ClO2 gas at 15 °C (○, total cells; ●, healthy cells) and 25 °C (△, total cells; ▼, healthy cells). Each point represents the mean for triplicates, and error bars show standard errors.

Kimberly Inc., Seoul, South Korea) to remove juice exuding from cut produce. Type 304 stainless steel (SS) with No.4 finish (Ian industry, Ansan-si, South Korea) and glass (Corning Inc., NY, USA) were cut into coupons (5 × 2 cm), immersed in 70% ethanol for 20 min, and rinsed with distilled water. After washing, coupons were dried in a laminar flow biosafety hood (22 ± 2 °C) for 1 h to remove surface moisture. Each coupon was used only once in order to avoid any surface changes. Prepared samples were placed on aluminum foil in a laminar flow hood, and 0.1 ml of culture cocktail was inoculated onto one side of the sample (upper side of spinach leaves and outer surface of tomatoes) by depositing droplets with a micropipettor at 15–20 locations. After inoculation, samples were dried in the laminar flow biosafety hood for 1 h at 22 ± 2 °C.

2.3. ClO_2 gas treatment

ClO_2 gas treatment was conducted in a treatment system described previously (Park and Kang, 2015). ClO_2 gas was produced in a ClO_2 gas generating system (Daehan E&B, Goyang-si, South Korea) and introduced into a polyvinyl chloride treatment chamber (length × width × height, 0.7 m × 0.5 m × 0.6 m). The ClO_2 gas concentration in the treatment chamber was continuously monitored and controlled using a ClO_2 gas transmitter (ATi F12, Analytical Technology, U.K.). ClO_2 gas in the treatment chamber was circulated using a ring blower (HRB-101, Hwanghae electronic, Incheon, South Korea). Inoculated samples were placed in the treatment chamber with the inoculated surfaces facing up and covered with a plastic lid. Samples were treated with 20 ppmv ClO_2 gas for up to 30 min. The RH of the treatment chamber was adjusted to 92 ± 1% at 15 ± 0.5 °C (AH 11.4–12.3 g/m^3) and 51 ± 1% at 25 ± 0.5 °C (AH 11.2–12.3 g/m^3) using a commercial ultrasonic nebulizer (H−C976, Osungsa, Changwon-si, South Korea). When the desired ClO_2 gas concentration and RH were achieved, the plastic lid was removed and samples were exposed to the treatment. A thermohygrometer (Center 342, CENTER Electronic Technology Co., LTD, Taiwan) was used to measure RH and temperature in the treatment chamber.

Absolute humidity was calculated for the RH and temperature data observed (Seinfeld and Pandis, 2006; Huang and Gustin, 2015):

$$\mathrm{AH} = \frac{P^0_{H_2O} \times \frac{RH}{100} \times 18\frac{g}{mol}}{R \times T}$$

where

$$\mathrm{P}^0_{H_2O} = 1013.25\, exp(13.3185A - 1.92A^2 - 0.6445A^3 - 0.1299A^4)$$

$$A = 1 - \frac{373.15}{T}$$

where AH is absolute humidity (g/m^3), RH is relative humidity, R is the ideal gas law constant, and T is absolute temperature in degrees Kelvin.

2.4. Bacterial enumeration

Treated spinach leaves and tomatoes were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 30 ml of neutralizing buffer (Difco). Stomacher bags were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for

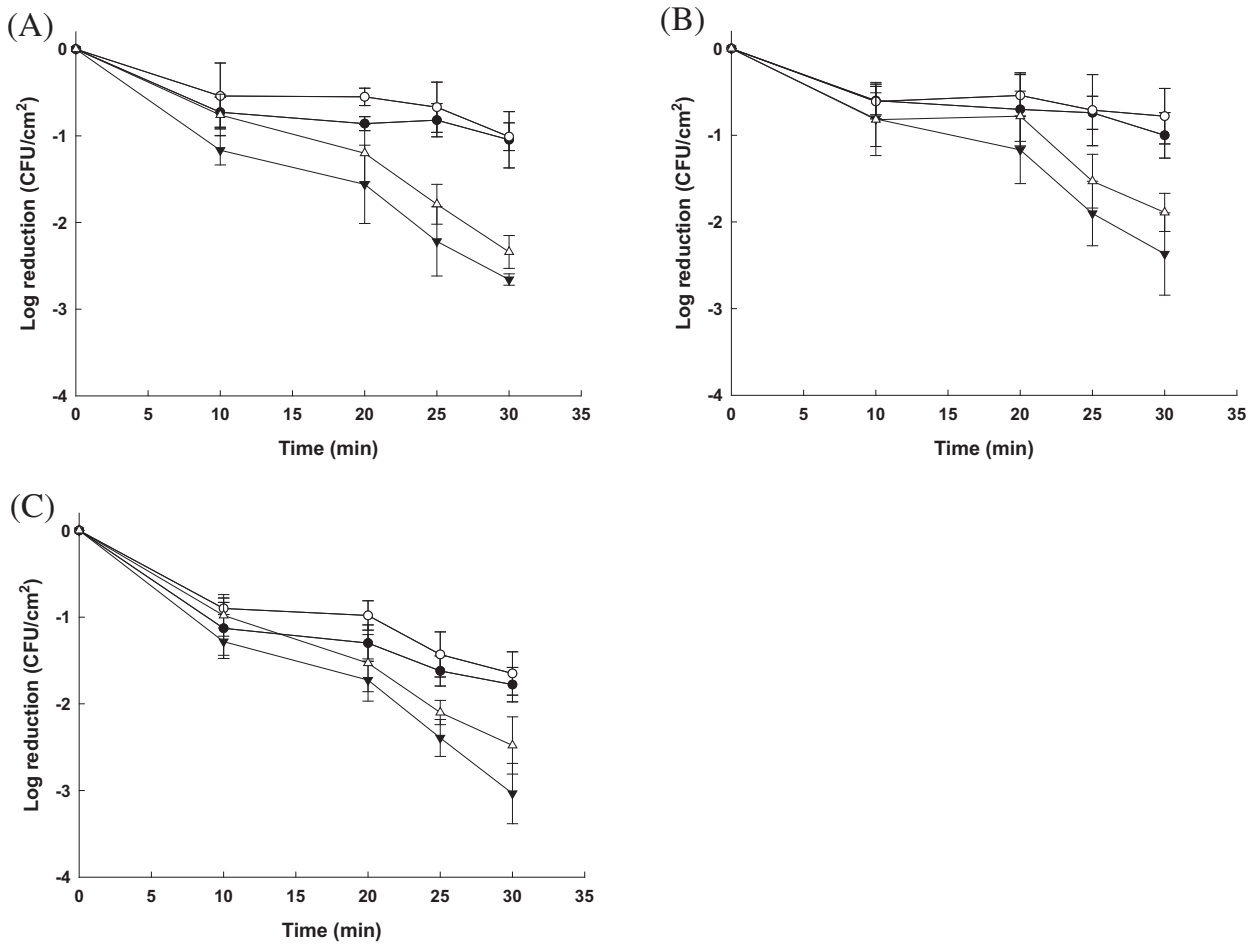

Fig. 3. Log reduction of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) on stainless steel No.4 treated with 20 ppmv ClO2 gas at 15 °C (○, total cells; ●, healthy cells) and 25 °C (△, total cells; ▼, healthy cells). Each point represents the mean for triplicates, and error bars show standard errors.

2 min. Treated stainless steel No.4 and glass coupons were transferred to sterile 50-ml conical centrifuge tubes containing 30 ml of neutralizing buffer and 3 g of glass beads (425–600 μm; Sigma-Aldrich, St. Louis, MO, USA), and then vortexed for 2 min. After homogenization, 1 ml aliquots withdrawn from stomacher bags or 50-ml conical centrifuge tubes were tenfold serially diluted in BPW, and 0.1 ml of appropriate diluents were spread-plated onto each selective medium. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), and Oxford agar base (OAB; Difco) with antimicrobic supplement (Difco) were used as selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and L. *monocytogenes*, respectively. Where low levels of surviving cells were expected, 1 ml aliquots withdrawn from stomacher bags were divided between four plates of each medium to lower the detection limit and spread-plated. The plates were incubated at 37 °C for 24–48 h. Colonies were enumerated after incubation and counts expressed as log CFU/cm^2.

For the resuscitation of injured cells of *E. coli* O157:H7, phenol red agar base (Difco) with 1% sorbitol (SPRAB) was used (Rhee et al., 2003). One hundred microliters of appropriate diluents were spread-plated onto SPRAB and incubated at 37 °C for 24 h. Injured cells of *S.* Typhimurium and L. *monocytogenes* were enumerated using the overlay (OV) method described by Kang and Fung (1999, 2000): 0.1 ml of appropriate diluents were spread-plated onto TSA and incubated at 37 °C for 2 h to allow injured cells to resuscitate before overlaying with 7 mL of XLD (OV-XLD) or MOX (OV-MOX) for *S.* Typhimurium or L. *monocytogenes*, respectively. The plates were incubated at 37 °C for 22 h.

2.5. Residual ClO_2 on surfaces after treatment

Spinach leaves, whole tomatoes (90–110 g), and SS No.4 coupons were exposed to 20 ppmv ClO_2 gas for 30 min under the same treatment conditions described above. Glass was exposed to these conditions for 15 min. After treatment, spinach leaves (10 ± 0.2 g), whole tomatoes, SS No.4 and glass coupons (5 × 2 cm) were immediately rinsed with 100 ml of sterile distilled water in sterile stomacher bags and massaged by hand for 5 min. Ten milliliters of sample were removed from each stomacher bag and tested by the DPD (N, N-diethyl-*p*-phenylenediamine) method using a Hach DR/820 Colorimeter (Hach, Loveland, CO) (Trinetta et al., 2011). Residual ClO_2 in rinse water were reported as mg/L. These experiments were conducted in triplicate.

2.6. Statistical analysis

All experiments were repeated three times. Data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA), and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

3. Results

Figs. 1–4 show log reductions of *E. coli* O157:H7, *S.* Typhimurium, and L. *monocytogenes* on spinach, tomatoes, SS No.4, and glass surfaces after treatment with 20 ppmv ClO_2 gas at 15 and 25 °C. As treatment time increased, significant differences ($p < 0.05$) were observed between inactivation levels under different temperature conditions. ClO_2

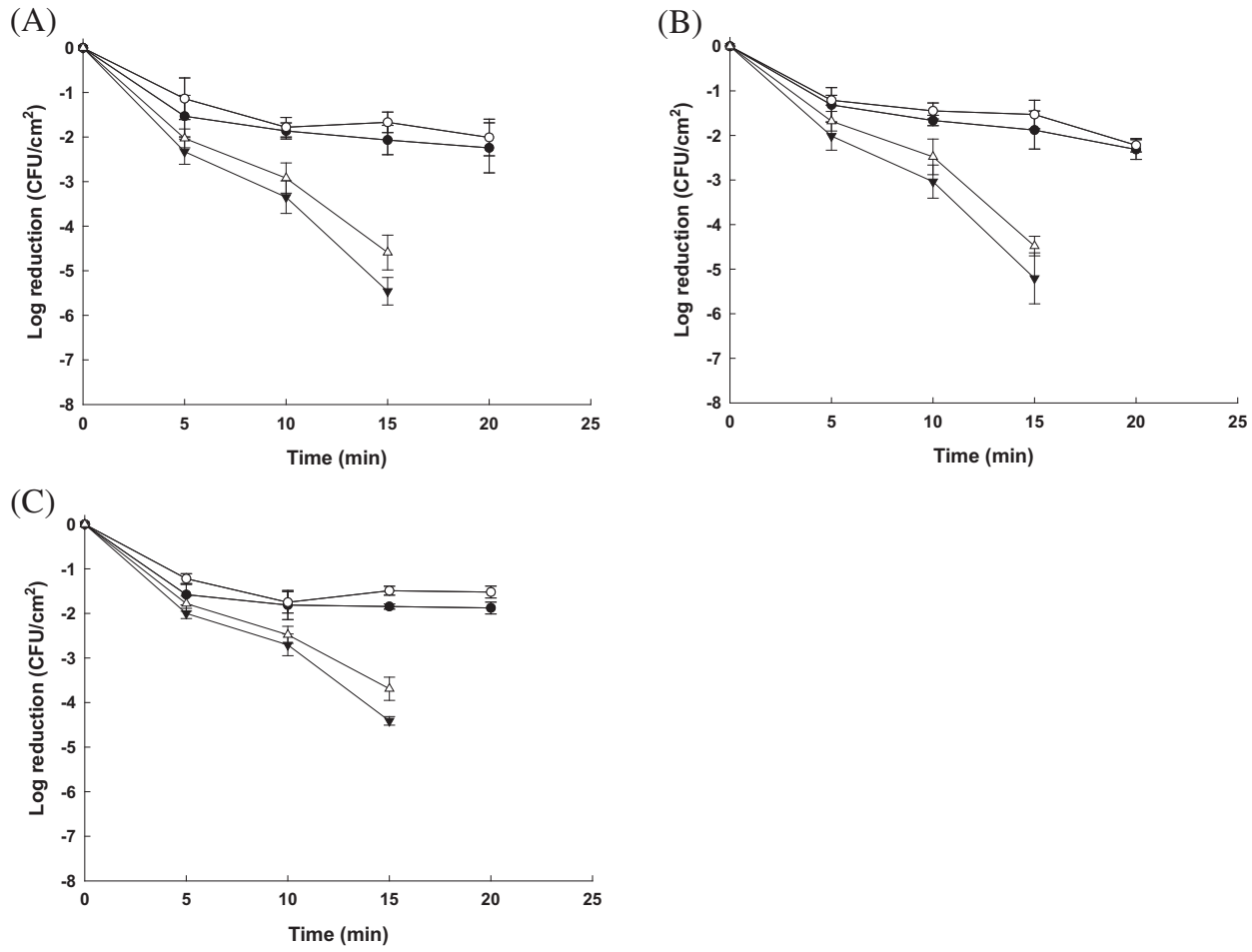

Fig. 4. Log reduction of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) on glass treated with 20 ppmv ClO2 gas at 15 °C (○, total cells; ●, healthy cells) and 25 °C (△, total cells; ▼, healthy cells). Each point represents the mean for triplicates, and error bars show standard errors.

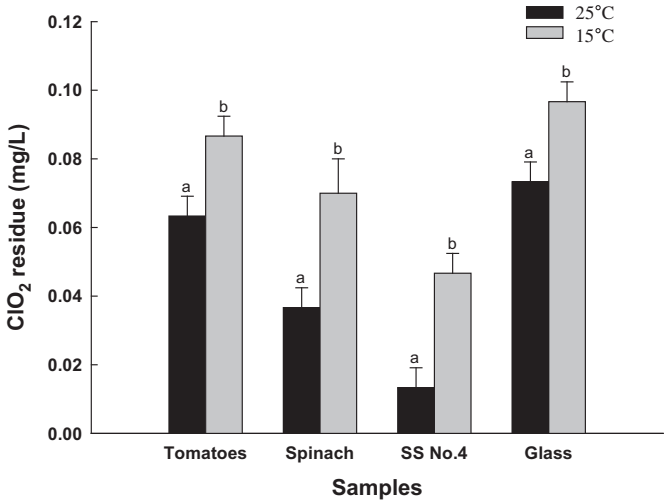

Fig. 5. Residual ClO2 on sample surfaces immediately after treatments with 20 ppmv ClO2 gas under different temperatures. Bars represent means for triplicates, and error bars standard errors. Different letters (a, b) for each sample indicate significantly different ($p < 0.05$) values.

gas treatment at 15 °C caused significantly more ($p < 0.05$) inactivation of the three pathogens than ClO_2 gas treatment at 25 °C. ClO_2 gas treatment at 15 °C for 30 min caused significant ($p < 0.05$) further reductions of 0.99 to 1.65, 1.05 to 1.50, and 1.25 to 1.61 log CFU/cm^2 of the three pathogens on spinach leaves, tomatoes, and stainless steel No.4, respectively, compared to 25 °C treatment. Treatment with ClO_2 gas at 25 °C for 20 min resulted in 1.88 to 2.31 log reductions of the three pathogens on glass while > 5.91 to 6.82 log reductions of these pathogens occurred after 20 min when treated with ClO_2 gas at 15 °C. Generally, ClO_2 gas treatment at neither temperature produced injured cells of any of the pathogens (data not shown).

Fig. 5 shows levels of residual ClO_2 on each type of sample treated with ClO_2 gas at 15 and 25 °C. Levels of residual ClO_2 after gas treatment at 15 °C were significantly ($p < 0.05$) higher than those treated at 25 °C. After ClO_2 gas treatment at 25 °C for 30 min, residual ClO_2 on spinach, tomatoes, and SS No.4 were 0.04, 0.06, and 0.01 mg/L, respectively. Residual ClO_2 on these same samples after exposure to ClO_2 gas at 15 °C for 30 min were 0.07, 0.09, and 0.05 mg/L, respectively. After 15 min treatment with ClO_2 gas at 25 and 15 °C, residual ClO_2 on glass was 0.07 and 0.10 mg/L, respectively.

4. Discussion

RH is the ratio between the actual water vapor pressure of air and the water vapor pressure of saturated air at a certain temperature, and AH is the actual water content of the air (Shaman and Kohn, 2009; Zhao et al., 2011). RH varies as a function of the temperature or the actual water vapor content of air because the water vapor pressure of saturated air increases exponentially as temperature increases, while AH is irrespective of temperature (Shaman and Kohn, 2009). Therefore, in the present study, experiments were performed under the same AH

conditions to evaluate the effect of temperature on microbial inactivation by ClO_2 gas.

It is known that ClO_2 gas acts similar to aqueous ClO_2 for inactivating microorganisms due to its high solubility in water (Linton et al., 2006). As is the case with other gases, the solubility of ClO_2 increases as temperature decreases. The mole fraction solubility of ClO_2 (at 101.325 kPa partial pressure of gas) is 2.67×10^{-2}, 1.82×10^{-2}, and 1.26×10^{-2} at 15, 25, and 35 °C, respectively (Haller and Northgraves, 1955; Kepinski and Trzeszczynski, 1964). Temperature may also affect the reactivity of sanitizers. Generally, inactivation efficacy of aqueous sanitizers increases with temperature across a moderate temperature range. Aqueous ClO_2 was more effective at reducing *E. coli* O157:H7 and *Pseudomonas aeruginosa* at 20 °C than at 10 °C (Taylor et al., 1999). Increasing temperature within the range of 5–30 °C led to increased inactivation of *Mycobacterium avium* by aqueous ClO_2 (Vicuña-Reyes et al., 2008). In the case of ozone, Larson and Mariñas (2003) reported increased rates of ozone inactivation of *Bacillus subtilis* spores with increasing temperature within a range of 1–30 °C. Increasing temperature within a range 7–22 °C strongly influenced the inactivation rate of *B. subtilis* spores in oxidant demand-free phosphate buffer and also led to a decrease in the CT 2 log (Dow et al., 2006). Steenstrup and Floros (2004) reported that high temperatures resulted in lower D-values and shorter lag times when apple cider was treated with ozone.

Based on these studies, there is no consensus regarding the effect of temperature on the antimicrobial efficacy of ClO_2 gas, because reactivity of ClO_2 gas increases but ClO_2 gas becomes less soluble in water as temperature increases. The simultaneous effect of these two factors (reactivity and solubility) on the antimicrobial efficacy of ClO_2 gas could vary with experimental conditions. In the present study, higher microbial inactivation was observed at 15 rather than 25 °C. Also, residual ClO_2 levels after ClO_2 gas treatment at 15 °C were significantly ($p < 0.05$) higher than those at 25 °C. These results indicate that ClO_2 solubility rather than reactivity has greater influence on the antimicrobial effect of ClO_2 gas. Although the reactivity of ClO_2 gas decreases with reduced temperature, increasing solubilized ClO_2 on sample surfaces may cause greater reductions of foodborne pathogens. Cárdenas et al. (2011) reported that gaseous ozone treatment was more effective at 0 °C than 4 °C for reducing *E. coli* and total aerobic mesophilic heterotrophic microorganisms on beef. They concluded that higher microbial reduction at lower temperature was due to higher solubility of ozone gas in the aqueous phase of the meat tissue.

There were some differences in the levels of microbial inactivation among samples. Especially, greater microbial inactivation was observed on glass compared to other samples. In our previous study (Park and Kang, 2017), the surface of glass displayed a hydrophilic character, while spinach, tomatoes, and stainless steel No.4 had hydrophobic surfaces, and greater microbial inactivation occurred on hydrophilic sample surfaces. The results of the present study were in agreement with our previous study.

In conclusion, this study showed that treatment temperature has a great impact on the inactivation of foodborne pathogens by ClO_2 gas treatment. The results of this study suggest that temperature control could be an alternative to using high concentrations of ClO_2 gas which could cause quality changes of food, accelerated corrosion of processing surfaces or present a safety issue. Our findings can help the food processing industry establish optimum ClO_2 gas treatment conditions for maximizing the antimicrobial effect of ClO_2 gas.

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