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Inactivation of foodborne pathogens on fresh produce by combined treatment with UV-C radiation and chlorine dioxide gas, and mechanisms of synergistic inactivation

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ABSTRACT

This study was conducted to evaluate the antimicrobial effect of the combined treatment of UV-C radiation (UVC) and chlorine dioxide (ClO_2) gas against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on spinach leaves and tomato surfaces and to clarify the mechanisms of the synergistic effect of this combined treatment. In the case of spinach leaves, as treatment time increased the combined treatments of UVC and ClO_2 gas showed additive effects: the total microbial inactivation of the combined treatment was not significantly ($p > 0.05$) different from the sum of individual treatments. On tomatoes, synergistic effects in inactivating *E. coli* O157:H7 and *S.* Typhimurium were observed after combination treatment of UVC and ClO_2 gas (10 ppmv) for 15 min or more. For both pathogens, inactivation achieved with the combination treatment was significantly ($p < 0.05$) higher than the sum of UVC and ClO_2 gas (10 ppmv) inactivation. In the case of *L. monocytogenes*, the synergistic effect was observed after the combination treatment of UVC and ClO_2 gas (10 ppmv) for 20 min. Measuring leakage of UV-absorbing substances and analyzing transmission electron microscopy images provide evidence that damage to the cell membrane and changes to membrane permeability are involved in the synergistic lethal effect of the combination treatment of UVC and ClO_2 gas. Combined treatment of UVC and ClO_2 gas (10 ppmv) did not significantly ($p > 0.05$) affect the color and texture of samples during 7 days of storage.

1. Introduction

The consumption of fresh produce has significantly increased due to heightened public awareness of the importance of healthy eating (Dikici, Koluman, & Calicioglu, 2015). However, with increasing produce consumption, the number of produce-related foodborne outbreaks has also increased (Lynch, Tauxe, & Hedberg, 2009). Particularly, leafy vegetable greens such as lettuce and spinach were associated with 22% of all foodborne illnesses in the United States between 1998 and 2008 (Painter et al., 2013). As leafy vegetables were minimally processed, contamination during harvest or transport can result in products containing foodborne pathogens (Mishra, Guo, Buchanan, Schaffner, & Pradhan, 2016). Tomatoes are another frequent vehicle associated with foodborne outbreaks and have been implicated in 15 multistate outbreaks resulting in 1959 illnesses, 384 hospitalizations, and 3 deaths between 1990 and 2010 (Bennett, Littrell, Hill, Mahovic, & Behravesh, 2015). *Escherichia coli* O157:H7 and *Salmonella* spp. have often been associated with the largest number of leafy vegetable outbreaks, and in the case of tomatoes, all multistate outbreaks have been caused by *Salmonella enterica* (Bennett et al., 2015; Herman, Hall, & Gould, 2015). Also, *L. monocytogenes* is of concern since it has been isolated from tomatoes and spinach (Moreno et al., 2012; Pingulkar, Kamat, & Bongirwar, 2001).

Washing with sanitizers has been used to reduce microbial loads on produce. Several sanitizers have been evaluated to inactivate foodborne pathogens on produce including chlorine (Bari, Inatsu, Kawasaki, Nazuka, & Isshiki, 2002; Bermúdez-Aguirre & Barbosa-Cánovas, 2013), ozonated water (Chaidez, Lopez, Vidales, & Campo, 2007), electrolyzed water (Ding, Rahman, & Oh, 2011; Issa-Zacharia, Kamitani, Miwa, Muhimbula, & Iwasaki, 2011),

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organic acids (Inatsu et al., 2010; Park et al., 2011), aqueous chlorine dioxide (ClO_2) (Pao, Kelsey, & Long, 2009), and hydrogen peroxide (Venkitanarayanan, Lin, Bailey, & Doyle, 2002). However, decontamination of produce by conventional washing and sanitizing is only marginally effective; thus, alternative methods are needed to overcome this limitation (Jin, Yu, & Gurtler, 2017).

Combinations of different technologies, known as hurdle technology, could be an alternative to the limited effectiveness of sanitizer washing. The antimicrobial efficacy of combination treatment of several sanitizers with ultrasound, ultraviolet (UV), pulsed UV light, and mild heat have been reported (Chun & Song, 2013; Huang & Chen, 2011; Rahman, Jin, & Oh, 2011; Sagong et al., 2011; Xu & Wu, 2014). These combination treatments could enhance the antimicrobial efficacy of sanitizer washing, but there still exists a problem in using sanitizer washing. Although sanitizer washing may be a useful tool for reducing potential contamination, it can also introduce or spread contaminants, especially if the water is reused (US FDA, 2008). Also, these combination treatments cannot be applied for microbial control during transportation and storage of produce. Preventing contamination at all stages of production, harvesting, processing, storage, and preparation of fresh produce is important (Beuchat, 2006).

Combination treatment of gaseous sanitizer with UV could be an alternative to conventional sanitizer washing and its combination treatment which has its own limitations as already described. Chlorine dioxide (ClO_2), a strong oxidizing agent with a broad antimicrobial spectrum, has emerged as a promising non-thermal sanitizing technology for fresh produce in recent years (Beuchat, 1998; Bhagat, Mahmoud, & Linton, 2010; Trinetta, Vaid, Xu, Linton, & Morgan, 2012). ClO_2 gas has been evaluated for inactivating foodborne pathogens on several types of fresh produce (Bhagat et al., 2010; Du, Han, & Linton, 2002; Mahmoud & Linton, 2008; Mahmoud, Vaidya, Corvalan, & Linton, 2008; Neal et al., 2012; Park & Kang, 2015; Sy, Murray, Harrison, & Beuchat, 2005; Trinetta, Linton, & Morgan, 2013; Wu & Rioux, 2010). Ultraviolet (UV) radiation, another non-thermal technology, has been approved for use as a sanitizer for surface treatment of foods (US FDA, 2002). Since it can cause cumulative damage to microbial DNA, UV radiation was recommended for use in combination with other techniques (Rame, Chaloupecky, Sojkova, & Bencko, 1997). Antimicrobial effects of the combination of UV radiation with aqueous sanitizers such as hydrogen peroxide (Hadjok, Mittal, & Warriner, 2008), ozone (Selma, Allende, López-Gálvez, Conesa, & Gil, 2008), and sodium hypochlorite (Ha & Ha, 2011) have been reported. However, none of the studies examined the antimicrobial effect of UV-C radiation (UVC) in combination with ClO_2 gas.

The objective of this study was to evaluate the antimicrobial effects of ClO_2 gas combined with UVC against *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on spinach leaves and tomatoes. The mechanism of inactivation was investigated by measuring leakage of UV-absorbing substances and analyzing transmission electron microscopy. Also, any changes in color and texture of samples were assessed.

2. Materials and methods

2.1. Bacterial strains and cell suspension

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19586, ATCC 43174, DT 104), and *L. monocytogenes* (ATCC 7644, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection of the Food Hygiene Laboratory at Seoul National University (SNCC; Seoul, Korea), for this study. 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, followed by centrifugation at 4000 × *g* at 4 °C for 20 min, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in BPW, corresponding to approximately 10^7–10^8 CFU/ml. Suspended pellets of the three pathogens were combined to comprise a mixed culture cocktail for use in experiments.

2.2. Sample preparation and inoculation

Spinach and whole tomatoes were purchased from a local market (Seoul, South Korea). These products were washed in running water and dried in a laminar flow biosafety hood (22 ± 2 °C) for 1 h before experiments. Spinach leaves were trimmed to approximately 5 × 2 cm in size, and the outer surface of tomatoes was cut into 5 × 2 cm pieces. Prepared spinach leaves and tomato surface samples were placed on aluminum foil in a laminar flow biosafety hood, and 0.1 ml of culture cocktail was inoculated onto one side of each prepared sample by depositing droplets with a micropipettor at 15–20 locations. After inoculation, samples were dried in the hood for 1 h at 22 ± 2 °C with the fan running.

2.3. Combined treatment of UVC and ClO_2 gas

The combined treatment of UVC and ClO_2 gas was conducted in a treatment system described previously with slight modification (Park & Kang, 2015). ClO_2 gas was prepared using a ClO_2 gas generating system (Daehan E&B, Goyang-si, South Korea). Generated ClO_2 gas was introduced into the polyvinyl chloride treatment chamber (length × width × height, 0.7 m × 0.5 m × 0.6 m), and the concentration of ClO_2 gas in the treatment chamber was continuously monitored and controlled using a ClO_2 gas transmitter (ATi F12, Analytical Technology, U.K.). The ClO_2 gas in the treatment chamber was continuously circulated using a ring blower (HRB–101, Hwanghae electronic, Incheon, South Korea). A commercial ultrasonic nebulizer (H–C976, Osungsa, Changwon–si, South Korea) was used to control relative humidity (RH) in the treatment chamber. A thermohygrometer (YTH-600, Uins, Seoul, South Korea) was used to measure temperature and RH in the treatment chamber. A germicidal UV lamp (G6T5, Sankyo, Japan) with a nominal output power of 6 W was used as a UVC emitting source. The UV lamp was located in the ceiling of the treatment chamber and was allowed to stabilize by turning it on for at least 5 min before experiments.

2.4. Procedures for treating samples

Inoculated spinach leaves and tomatoes were placed in the treatment chamber with the inoculated surfaces facing upwards and covered with a plastic lid. For UVC treatment alone, samples were treated with UVC for 5, 10, 15, or 20 min (radiation intensity, 70.68 μW/cm^2 at the sample location). The UVC (at 253.7 nm wavelength) intensity was measured with a spectrometer (AvaSpec-ULS2048-USB2-UA-50, Avantes, Netherlands). For ClO_2 gas treatments alone, samples were treated with 5 or 10 ppmv ClO_2 gas for 5, 10, 15, or 20 min. For combined treatments, samples were subjected with simultaneous treatment of UVC and ClO_2 gas (5 or 10 ppmv) for 5, 10, 15, or 20 min. All experiments were performed at 22 ± 1 °C, and RH of the treatment chamber was adjusted with distilled water to 90 ± 2% during treatment. When the desired ClO_2 gas concentration and RH were achieved, the plastic lid was removed. Samples were withdrawn from the treatment chamber after 5, 10, 15, or 20 min exposure to each treatment, and treated samples were used to determine surviving bacterial populations. These experiments were repeated three times.

2.5. Bacterial enumeration

Treated spinach leaves (5 ± 0.2 g) or one piece of tomato were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 45 or 30 ml of neutralizing buffer (Difco), respectively. Stomacher bags were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for 2 min. After homogenization, 1 ml aliquots of the sample were tenfold serially diluted in 9 ml of BPW, and 0.1 ml of sample or diluent was 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 numbers of surviving cells were anticipated, 250 μl of undiluted sample was plated onto each of four plates to increase the possibility of detecting the pathogens. The plates were incubated at 37 °C for 24–48 h. After incubation, colonies were counted and calculated as log CFU/g for spinach leaves and log CFU/cm^2 for tomatoes, respectively.

2.6. Leakage of UV-absorbing substances

Membrane damage to *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was evaluated after treatment by quantification of the intracellular UV materials released from each pathogen (Aronsson, Rönner, & Borch, 2005; Virto, Mañas, Álvarez, Condon, & Raso, 2005). Each cell suspension (1 ml) of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was inoculated onto glass petri dishes, and dried in the hood for 1 h at 22 ± 2 °C with the fan running. Each pathogen inoculated onto glass petri dishes was treated with UVC, ClO_2 gas (10 ppmv), and the combined treatment of UVC and ClO_2 gas (UVC-ClO_2) for 15 min; preliminary experiments confirmed that inactivation patterns of three pathogens on glass petri dishes were similar to those on tomato surfaces. Treated cells were resuspended using 10 ml of phosphate-buffered saline (PBS; pH 7.0), and centrifuged at 10,000 × *g* at 4 °C for 10 min. The upper 1 ml of the supernatant was removed and the UV absorbance was measured at a wavelength of 260 and 280 nm with a spectrophotometer (SpectraMax M2e, Molecular Devices, CA, USA). The absorbance was presented as the mean of triplicated measurements.

2.7. Transmission electron microscopy analysis

Transmission electron microscopy (TEM) analysis was conducted after UVC, ClO_2 gas (10 ppmv), and UVC-ClO_2 gas (10 ppmv) treatment for 15 min to investigate structural damage to pathogen cells. Treated *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells inoculated onto glass petri dishes as described above were resuspended using 10 ml of PBS and collected by centrifugation at 4000 × *g* at 4 °C for 10 min. The cells were fixed at 4 °C for 4 h in modified Karnovsky's fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). After primary fixation, each sample was centrifuged and washed three times with 0.05 M sodium cacodylate buffer (pH 7.2) at 4 °C for 10 min. Cells were postfixed with 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) at 4 °C for 2 h and briefly washed twice with distilled water at room temperature. The washed cells were stained overnight with 0.5% uranyl acetate at 4 °C. The cells were then dehydrated at room temperature using a graded ethanol series (10 min each in 30, 50, 60, 70, 95, and 100%), finishing with three consecutive 100% ethanol washes. The transition was performed with 100% propylene oxide at room temperature for 15 min. The cells were then infiltrated for 2 h with a 1:1 solution of propylene oxide and Spurr's resin, and then placed in Spurr's resin overnight. In order to get specimen blocks, the polymerization of the resin was conducted in an oven at 70 °C for 24 h. Specimens were sectioned (70-nm thick) by means of an ultramicrotome (MT-X; RMC, Tucson, AZ, USA) and then stained with 2% uranyl acetate for 7 min, followed by Reynolds' lead citrate for 7 min. The sections were then observed with a transmission electron microscope (Libra 120; Carl Zeiss, Heidenheim, Germany).

2.8. Measurement of color and texture of samples

After combined treatment with UVC and ClO_2 gas (10 ppmv), uninoculated spinach leaves and tomatoes were stored at 7 °C for 7 days to identify quality changes during storage following treatments. Hunter's L, a, b values of the sample were measured with a Minolta colorimeter (model CR300, Minolta Co., Osaka, Japan) at 3 locations on each sample. The texture of spinach leaves and tomatoes was measured with a texture analyzer (TA-CT3, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a blade set and cylinder probe with a 4 mm diameter, respectively. Twenty grams of spinach leaves were placed onto the press holder with the stems positioned perpendicular to the path of the blade, and a blade was moved down at 2 mm/s (path length 10 mm). For tomatoes, the loading rate and path length were also set at 2 mm/s and 10 mm. Maximum force (N) was recorded using Texturepro CT software (Brookfield Engineering Laboratories, Inc.). Three measurements were performed for each treatment with independently-prepared samples.

2.9. Statistical analysis

All experiments were done in triplicate. 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

3.1. Effects of UVC, ClO_2 gas, and UVC-ClO_2 gas treatments on populations of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes*

The reduction in numbers of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves during UVC, ClO_2 gas, and simultaneous application of both technologies is presented in Tables 1 and 2. Generally, antimicrobial effects of UVC-ClO_2 gas (5 ppmv) treatment were not superior to those of individual treatments during 15 min. After 20 min treatment, UVC-ClO_2 gas (5 ppmv) treatment showed the additive effect: the total microbial inactivation of the combined treatment was not significantly ($p > 0.05$) different from the sum of individual treatments (Gallo, Pilosof, & Jagus, 2007). Treatment with UVC for 20 min caused 1.85, 2.02, and 1.87 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Exposure to 5 ppmv of ClO_2 gas for 20 min resulted in 2.19, 2.17, and 1.58 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. UVC-ClO_2 gas (5 ppmv) treatment resulted in 4.38, 3.73, and 3.14 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

Similarly, UVC-ClO_2 gas (10 ppmv) treatment showed additive effects in the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* after 20 min treatment. Exposure to 10 ppmv of ClO_2 gas for 20 min resulted in 3.56, 3.61, and 3.23 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. UVC-ClO_2 gas (10 ppmv) treatment caused 5.17, 5.41, and 4.32 log

Table 1
Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves treated with UV-C radiation (UVC), 5 ppmv ClO_2 gas, and both technologies simultaneously (UVC-ClO_2 gas).

Treatment time	Log reduction (log CFU/g)								
	E. coli O157:H7			*S.* Typhimurium			*L. monocytogenes*		
	UVC	ClO_2 gas (5 ppmv)	UVC-ClO_2 gas	UVC	ClO_2 gas (5 ppmv)	UVC-ClO_2 gas	UVC	ClO_2 gas (5 ppmv)	UVC-ClO_2 gas
5 min	1.16 ± 0.01Aa[b]	1.34 ± 0.24Aa	1.94 ± 0.19Ab	1.35 ± 0.13Aa	1.09 ± 0.22Aa	1.67 ± 0.02Ab	1.13 ± 0.43Aa	0.74 ± 0.35Aa	1.44 ± 0.38Aa
10 min	1.53 ± 0.17ABa	1.86 ± 0.40ABa	2.29 ± 0.55Aa	1.56 ± 0.07ABa	1.57 ± 0.14Ba	1.78 ± 0.08Ab	1.54 ± 0.46Aa	0.82 ± 0.35Aa	1.63 ± 0.54Aa
15 min	1.69 ± 0.31ABa	1.95 ± 0.23ABa	2.46 ± 0.20Ab	2.01 ± 0.26Ba	1.76 ± 0.30BCa	2.25 ± 0.14Ba	1.71 ± 0.49Aab	1.26 ± 0.33ABa	2.13 ± 0.40Ab
20 min	1.85 ± 0.49Ba	2.19 ± 0.39Ba	4.38 ± 0.19Bb	2.02 ± 0.42Ba	2.17 ± 0.26Ca	3.73 ± 0.04Cb	1.87 ± 0.50Aa	1.58 ± 0.49Ba	3.14 ± 0.25Bb

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after treatment.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table 2
Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves treated with UV-C radiation (UVC), 10 ppmv ClO_2 gas, and both technologies simultaneously (UVC-ClO_2 gas).

Treatment time	Log reduction (log CFU/g)								
	E. coli O157:H7			*S.* Typhimurium			*L. monocytogenes*		
	UVC	ClO_2 gas (10 ppmv)	UVC-ClO_2 gas	UVC	ClO_2 gas (10 ppmv)	UVC-ClO_2 gas	UVC	ClO_2 gas (10 ppmv)	UVC-ClO_2 gas
5 min	1.32 ± 0.15Aa[b]	1.15 ± 0.19Aa	2.29 ± 0.06Ab	1.44 ± 0.02Aa	1.36 ± 0.19Aa	1.72 ± 0.06Ab	0.96 ± 0.28Aa	0.56 ± 0.28Aa	1.66 ± 0.41Ab
10 min	1.69 ± 0.01ABa	2.44 ± 0.26Bb	3.16 ± 0.25Bc	1.65 ± 0.08ABa	2.37 ± 0.12Bb	2.22 ± 0.16Bb	1.37 ± 0.31ABa	1.61 ± 0.43Ba	2.36 ± 0.13Bb
15 min	1.85 ± 0.47ABa	3.01 ± 0.34Cb	3.73 ± 0.48Cb	2.10 ± 0.13Ba	2.99 ± 0.28Cb	3.36 ± 0.05Cc	1.55 ± 0.36ABa	2.38 ± 0.38Cb	2.96 ± 0.31Bb
20 min	2.01 ± 0.33Ba	3.56 ± 0.34Db	5.17 ± 0.23Dc	2.10 ± 0.45Ba	3.61 ± 0.46Db	5.47 ± 0.32Dc	1.70 ± 0.38Ba	3.23 ± 0.35Db	4.32 ± 0.52Cc

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after treatment.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

UVC-ClO_2 gas (5 ppmv) treatment showed clear additive effects on tomatoes earlier than on spinach leaves (Table 3). Most UVC-ClO_2 gas (5 ppm) treatments showed a more significant reduction than that of each treatment applied individually following 5 min treatment. After 15 min treatment, UVC-ClO_2 gas (5 ppmv) treatment produced an additive effect in inactivating *E. coli* O157:H7 and *S.* Typhimurium. Treatment with UVC for 20 min caused 2.02, 1.96 and 1.58 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells were reduced by 20 min treatment of ClO_2 gas (5 ppmv), showing 2.34, 2.24, and 1.57 log reductions, respectively. UVC-ClO_2 gas (5 ppmv) treatment resulted in 4.80, 4.28, and 2.70 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

After 15 min treatment, UVC-ClO_2 gas (10 ppmv) treatment showed the synergistic effect in inactivating *E. coli* O157:H7 and *S.* Typhimurium; the total microbial inactivation of the combined treatment was significantly ($p < 0.05$) higher than the sum of individual treatments (Gallo et al., 2007) (Table 4). UVC-ClO_2 gas (10 ppmv) treatment for 15 min achieved 5.62 and 5.46 log reductions in *E. coli* O157:H7 and *S.* Typhimurium, respectively. For both pathogens, UVC-ClO_2 gas (10 ppmv) treatment produced a more significant ($p < 0.05$) reduction than the sum of UVC and ClO_2 gas (10 ppmv) inactivation after exposure times of 15 min or more. In case of *L. monocytogenes*, the synergistic effect was observed after UVC-ClO_2 gas (10 ppmv) treatment for 20 min.

3.2. Leakage of UV-absorbing substances

Leakage of UV-absorbing substances from *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells measured at 260 nm is shown in Fig. 1A, C, and E. The levels of UV-absorbing substances in ClO_2 gas (10 ppmv) and UVC-ClO_2 gas (10 ppmv) treated cells were much greater than those of UVC treated cells. Increasing the treatment time resulted in increased levels of UV-absorbing substances when they were treated with ClO_2 gas (10 ppmv) and UVC-ClO_2 gas (10 ppmv). Among them, leakage of UV-absorbing substances began to be remarkable after 15 min treatment of UVC-ClO_2

Table 3
Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes treated with UV-C radiation (UVC), 5 ppmv ClO_2 gas, and both technologies simultaneously (UVC-ClO_2 gas).

Treatment time	Log reduction (log CFU/cm^2)								
	E. coli O157:H7			*S.* Typhimurium			*L. monocytogenes*		
	UVC	ClO_2 gas (5 ppmv)	UVC-ClO_2 gas	UVC	ClO_2 gas (5 ppmv)	UVC-ClO_2 gas	UVC	ClO_2 gas (5 ppmv)	UVC-ClO_2 gas
5 min	1.44 ± 0.15Aa[b]	1.27 ± 0.33Aa	2.21 ± 0.36Ab	1.48 ± 0.27Ab	0.96 ± 0.15Aa	2.27 ± 0.13Ac	0.78 ± 0.38Aa	0.86 ± 0.13Aa	1.38 ± 0.51Aa
10 min	1.73 ± 0.46Aa	1.61 ± 0.18ABa	2.57 ± 0.15Ab	1.62 ± 0.32ABa	1.20 ± 0.22ABa	2.63 ± 0.23Bb	1.18 ± 0.30ABa	1.04 ± 0.35ABa	1.91 ± 0.14Bb
15 min	1.80 ± 0.30Aa	1.92 ± 0.17BCa	4.31 ± 0.10Bb	1.93 ± 0.23ABa	1.62 ± 0.50Ba	3.38 ± 0.23Cb	1.48 ± 0.08Ba	1.36 ± 0.11BCa	2.36 ± 0.06BCb
20 min	2.02 ± 0.13Aa	2.34 ± 0.18Cb	4.80 ± 0.14Cc	1.96 ± 0.07Ba	2.24 ± 0.27Ca	4.28 ± 0.13Db	1.58 ± 0.10Ba	1.57 ± 0.15Ca	2.70 ± 0.05Cb

[a] Log reduction = population (log CFU/cm^2) before treatment − population (log CFU/cm^2) after treatment.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table 4
Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes treated with UV-C radiation (UVC), 10 ppmv ClO_2 gas, and both technologies simultaneously (UVC-ClO_2 gas).

Treatment time	Log reduction (log CFU/cm^2)								
	E. coli O157:H7			*S.* Typhimurium			*L. monocytogenes*		
	UVC	ClO_2 gas (10 ppmv)	UVC-ClO_2 gas	UVC	ClO_2 gas (10 ppmv)	UVC-ClO_2 gas	UVC	ClO_2 gas (10 ppmv)	UVC-ClO_2 gas
5 min	1.69 ± 0.30Aa[b]	1.63 ± 0.28Aa	1.85 ± 0.09Aa	1.71 ± 0.06Ab	1.20 ± 0.06Aa	2.00 ± 0.26Ab	0.86 ± 0.36Aa	0.82 ± 0.50Aa	1.22 ± 0.19Aa
10 min	1.85 ± 0.34ABa	2.42 ± 0.17Ba	3.86 ± 0.54Bb	1.85 ± 0.20Aa	1.76 ± 0.23Ba	3.28 ± 0.53Bb	1.26 ± 0.42ABa	1.38 ± 0.47Aa	1.91 ± 0.08Ba
15 min	2.05 ± 0.06ABa	2.72 ± 0.04Bb	5.62 ± 0.27Cc	2.16 ± 0.38Aa	2.58 ± 0.37Ca	5.46 ± 0.51Cb	1.55 ± 0.25Ba	2.13 ± 0.22Bb	2.73 ± 0.03Cc
20 min	2.27 ± 0.15Ba	3.81 ± 0.05Cb	ND	2.20 ± 0.24Aa	3.85 ± 0.37Db	ND	1.66 ± 0.14Ba	3.13 ± 0.16Cb	ND

[a] Log reduction = population (log CFU/cm^2) before treatment − population (log CFU/cm^2) after treatment.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

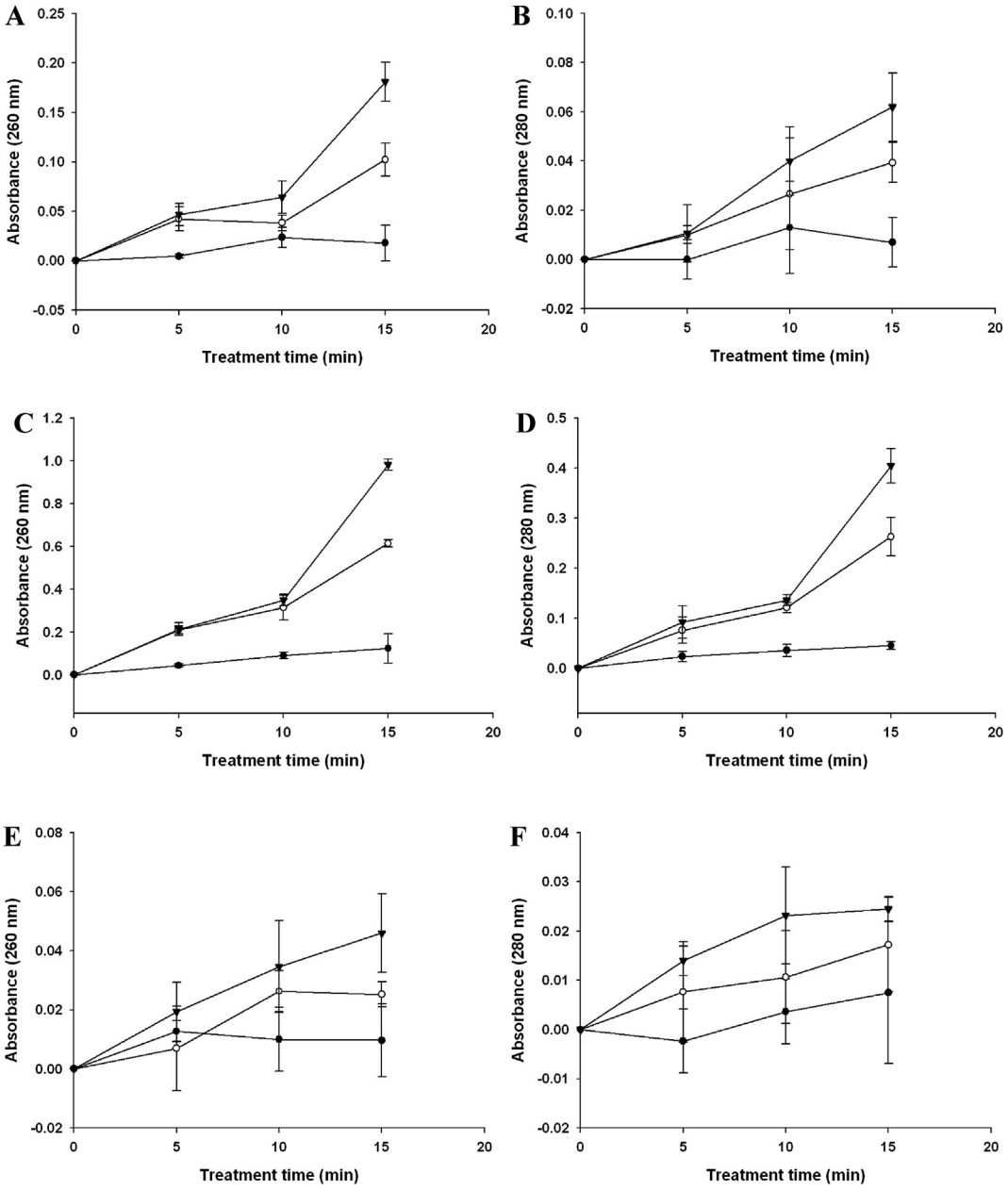

Fig. 1. Leakage of UV-absorbing substances from *E. coli* O157:H7 (A, B), *S.* Typhimurium (C, D), and *L. monocytogenes* (E, F) cells treated with UVC, ClO2 gas (10 ppmv), and UVC-ClO2 gas as a function of treatment time. Symbols: ●, treated with UVC; ○, treated with ClO2 gas (10 ppmv); ▼, treated with UVC-ClO2 gas.

gas (10 ppmv). Leakage of UV-absorbing substances of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells treated with UVC-ClO_2 gas (10 ppmv) was significantly ($p < 0.05$) higher than the sum of levels of UV-absorbing substances treated with UVC and UVC-ClO_2 gas (10 ppmv) after 15 min treatments. Similar patterns were observed in levels of UV-absorbing substances measured at 280 nm (Fig. 1B, 1D, and F). In the case of *S.* Typhimurium, leakage of UV-absorbing substances treated with UVC-ClO_2 gas (10 ppmv) for 15 min was significantly ($p < 0.05$) higher than the sum of levels of UV-absorbing substances treated with UVC and UVC-ClO_2 gas (10 ppmv).

3.3. Transmission electron microscopy analysis

TEM images were used to analyze the morphological changes occurring in *S.* Typhimurium cells treated with UVC, ClO_2 gas (10 ppmv), and simultaneous application of both technologies, as seen in Fig. 2. Individual treatments of *S.* Typhimurium cells with UVC and ClO_2 gas (10 ppmv) was shown to cause some changes (Fig. 2B and C) compared to untreated cells (Fig. 2A), such as the uneven distribution and aggregation of internal cellular substances. Also, slight separations of the cell membrane from cytoplasm were observed. These phenomena were much more pronounced in the case of *S.* Typhimurium cells treated with UVC-ClO_2 gas (10 ppmv) (Fig. 2D). Severe rupturing of cell membranes and leakage of intracellular contents were observed.

3.4. The quality changes of spinach leaves and tomatoes during storage

The a^* value of spinach leaves decreased while the b^* value increased during storage. However, there were no significant ($p > 0.05$) differences in Hunter's color values (L^*, a^*, b^*) between untreated samples (control) and those treated with UVC-ClO_2 gas (10 ppmv) during storage at 7 °C for 7 days (Table 5). In the case of tomatoes, the L^* value decreased and the b^* values increased during storage. However, there were also no significant ($p > 0.05$) differences in Hunter's color values (L^*, a^*, b^*) between the control and those treated with UVC-ClO_2 gas (10 ppmv) during storage. Table 6 shows the effects of UVC-ClO_2 gas (10 ppmv) treatment on the texture of spinach leaves and tomatoes. There were no significant ($p > 0.05$) differences in texture between control and treated samples during storage at 7 °C for 7 days.

4. Discussion

Antimicrobial effects of ClO_2 gas on produce, including spinach and tomatoes, have been reported. *Salmonella* and *E. coli* O157:H7 on spinach leaves treated with 2.1 mg/l ClO_2 gas (generated by a sachet) for 1 h were reduced by 0.6 and 0.7 log CFU/g, respectively

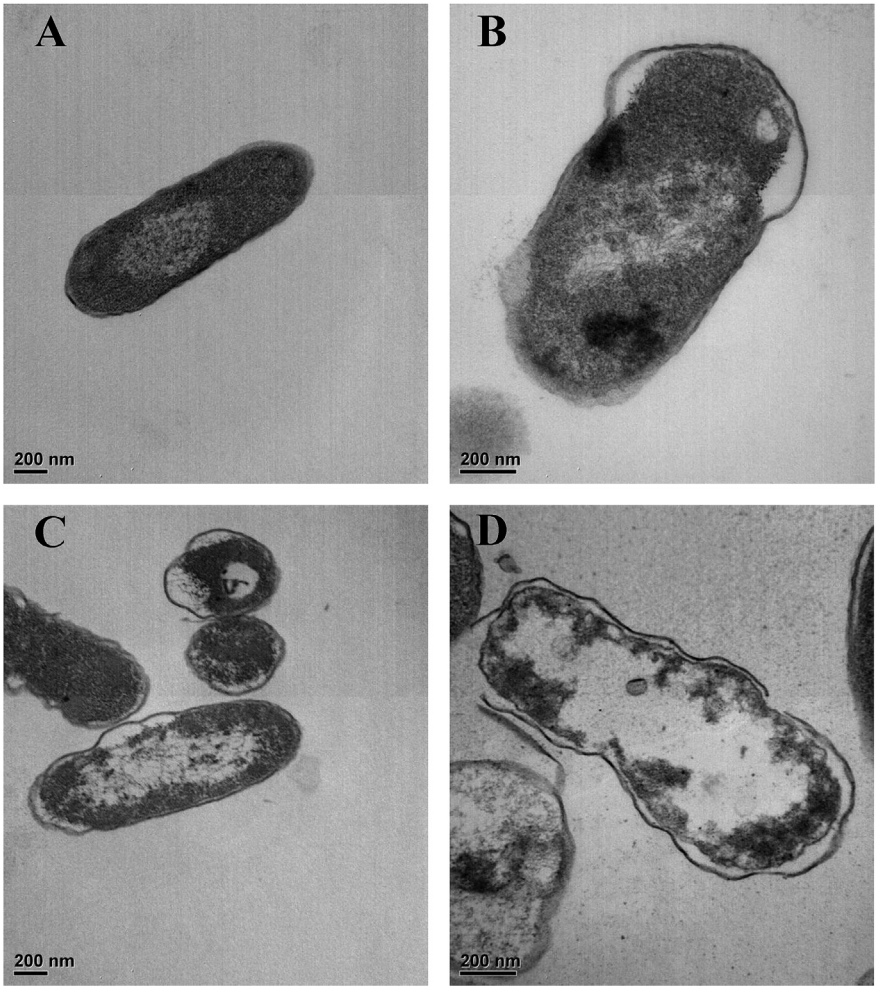

Fig. 2. Transmission electron microscopy images of *S.* Typhimurium treated with UVC, ClO2 gas, and UVC-ClO2 gas for 15 min. (A), nontreated cells; (B) UVC treated cells; (C), ClO2 gas (10 ppmv) treated cells; (D), UVC-ClO2 gas treated cells.

Table 5
Changes in color values[a] of spinach leaves and tomatoes treated in combination with UVC and ClO_2 gas (10 ppmv) during storage at 7 °C for 7 days.

Day	Treatment			
	Spinach leaves		Tomatoes	
	Control	UVC + ClO_2 gas	Control	UVC + ClO_2 gas
L^*				
0	40.59 ± 1.33A[b]	40.79 ± 0.62A	43.84 ± 0.73A	43.69 ± 0.91A
2	40.60 ± 1.10A	40.81 ± 2.23A	44.72 ± 1.21A	43.63 ± 0.64A
4	40.56 ± 1.06A	40.06 ± 1.37A	42.59 ± 0.53A	41.45 ± 1.76A
7	39.60 ± 1.56A	39.27 ± 1.48A	40.85 ± 1.33A	40.20 ± 2.03A
a^*				
0	−7.12 ± 0.84A	−7.77 ± 0.28A	18.42 ± 2.13A	18.29 ± 0.64A
2	−7.09 ± 0.89A	−7.50 ± 0.90A	19.79 ± 2.37A	20.21 ± 1.59A
4	−7.97 ± 0.80A	−8.42 ± 0.73A	19.34 ± 1.23A	19.64 ± 1.02A
7	−8.78 ± 0.55A	−9.30 ± 1.09A	20.47 ± 0.87A	19.85 ± 1.27A
b^*				
0	9.45 ± 1.03A	9.83 ± 0.32A	19.74 ± 1.56A	19.81 ± 0.39A
2	9.20 ± 1.23A	9.78 ± 0.82A	24.48 ± 0.55A	24.72 ± 0.79A
4	10.57 ± 1.59A	10.40 ± 1.26A	25.16 ± 1.95A	25.44 ± 1.53A
7	12.79 ± 1.60A	12.93 ± 1.49A	24.41 ± 0.74A	24.68 ± 1.26A

[a] Color parameters are lightness (L^*), redness (a^*), and yellowness (b^*).
[b] Means ± standard deviations from three replications. Within the same storage time and produce type, means with the same uppercase letters within a row are not significantly different ($p > 0.05$).

Table 6
Maximum force (N) required for breakage of spinach leaves and tomatoes treated in combination with UVC and ClO_2 gas (10 ppmv) during storage at 7 °C for 7 days.

Day	Maximum force (N)			
	Spinach leaves		Tomatoes	
	Control	UVC + ClO_2 gas	Control	UVC + ClO_2 gas
0	47.23 ± 0.93A[a]	47.82 ± 0.84A	10.68 ± 0.84A	10.21 ± 0.77A
2	47.25 ± 0.42A	48.00 ± 2.69A	10.92 ± 1.04A	10.99 ± 1.05A
4	47.18 ± 2.78A	46.67 ± 1.52A	10.21 ± 1.18A	10.76 ± 2.37A
7	46.89 ± 3.12A	45.14 ± 1.86A	9.28 ± 0.22A	9.91 ± 1.58A

[a] Means ± standard deviations from three replications. Within the same storage time and produce type, means with the same uppercase letters within a row are not significantly different (p > 0.05).

(Neal et al., 2012). Bhagat et al. (2010) reported that treatment with 0.5 mg/l ClO_2 gas for 12 min showed more than a 5 log reduction in *Salmonella* and L. *monocytogenes* on tomato skin surfaces. Treatment with 8 mg/l ClO_2 gas for 60s and 10 mg/l ClO_2 gas for 180 s reduced levels of *Salmonella* on Roma tomatoes by 2.94 and 4.87 log CFU/cm^2, respectively (Trinetta et al., 2013). However, the concentration of ClO_2 gas used in previous studies was excessive (about 180–3600 ppmv). These concentrations were much higher than a LC50 value (32 ppmv, 90 mg/m^3) determined for rats as a single exposure (Dobson, 2002).

The antimicrobial effects of UVC on produce decontamination have also been evaluated by several studies. Sommers, Sites, and Musgrove (2010) reported a UVC dose of 5 kJ/m^2 inactivated 3.02 and 2.59 log of *Salmonella* spp. and *L. monocytogenes*, respectively, on the surface of Roma tomatoes. A UVC dose of 4.9 kJ/m^2 achieved 1.79, 2.59, and 1.80 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively, on lettuce (Kim et al., 2013). However, the poor penetration of UV light into solids is a significant limitation of UV light for decontamination of foods (Murdoch, Maclean, MacGregor, & Anderson, 2010). Also, Mukhopadhyay, Ukuku, Juneja, and Fan (2014) reported that produce surface characteristics such as surface roughness and the location of foodborne pathogens on produce greatly influence the efficacy of UVC treatment.

In the present study, UVC was applied as hurdle technology to reduce the concentration of ClO_2 gas. As treatment time increased, the combination of UVC and ClO_2 gas (5 ppmv) caused similar microbial reductions of the three foodborne pathogens when compared with 10 ppmv ClO_2 gas treatments alone. In case of *E. coli* O157:H7, combined treatment with UVC and ClO_2 gas (5 ppmv) was more effective than 10 ppmv ClO_2 gas alone after 20 min of exposure. Park and Kang (2015) revealed that treatment with 30 ppmv of ClO_2 gas for 20 min at 90% RH caused 5.78, 5.68, and 4.86 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively, on spinach leaves. In this study, a combination treatment of UVC and ClO_2 gas (10 ppmv) for 20 min resulted in 5.17, 5.47, and 4.32 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively, on spinach leaves. Also, exposure to 20 ppmv of ClO_2 gas for 15 min at 90% RH caused more than 5.92 and 5.67 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively, on tomatoes (Park, 2016). In the present study, the combination treatment of UVC and ClO_2 gas (10 ppmv) for 15 min resulted in 5.62 and 5.46 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. These results suggest that combined treatment with UVC and ClO_2 gas could reduce the concentration of ClO_2 gas needed, while still ensuring microbial safety.

Combination treatments of UVC with chemical sanitizers have been widely used for inactivating microorganisms in food products (Jiang, Jahangir, Jiang, Lu, & Ying, 2010). Hadjok et al. (2008) reported that a combined treatment of UVC (37.8 mJ/cm^2) and H_2O_2 (1.5% at 50 °C) caused a 4.12 log reduction of *Salmonella* on iceberg lettuce, which was significantly higher compared to UVC or H_2O_2 treatment alone. Combined treatment with UVC and O_3 achieved a maximum total microbial reduction of 6.6 log CFU/ml after 60 min treatment, whereas 4.0 and 5.9 log CFU/ml reductions, respectively, were achieved by UVC and O_3 treatment alone (Selma et al., 2008). The synergistic effect was observed in inactivating *Cronobacter sakazakii*, *Staphylococcus aureus*, *S.* Typhimurium, and *E. coli* when sodium hypochlorite and UV radiation were treated in combination (Ha & Ha, 2011).

In the present study, most combinations of UVC and ClO_2 gas showed only additive effects in the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves as treatment time increased. However, synergistic effects in inactivating *E. coli* O157:H7 and *S.* Typhimurium on tomatoes were observed after combined treatment of UVC and ClO_2 gas (10 ppmv) for 15 min or more. In the case of *L. monocytogenes*, the existence of a synergistic effect was deduced after 20 min treatment. This difference may due to different surface characteristics of spinach leaves and tomatoes. Foodborne pathogens on tomatoes might be more easily exposed to UVC and ClO_2 gas than spinach leaves as tomatoes have smoother surfaces than spinach leaves.

The underlying inactivation mechanisms of the combined treatment of UVC and ClO_2 gas are not well understood. UVC radiation inactivates microorganisms by damaging their nucleic acid, thereby blocking cell replication (Koutchma, 2009). It has also been suggested that photons could interact with cell envelope components and favor the oxidation of unsaturated fatty acid residues of lipids and phospholipids (Koutchma, Keller, Chirtel, & Parisi, 2004; Montgomery, 1985). The mechanism of inactivation by ClO_2 has been postulated by several studies. Antimicrobial effects of ClO_2 are primarily due to oxidative attack on cell surface membrane proteins, including proteins involved in transport (Jeng & Woodworth, 1990). It is related to the denaturation of constituent proteins for cellular integrity and function, and the loss of permeability control of the outer membrane (Berg, Roberts, & Matin, 1986). To clarify the mechanism of the synergistic lethal effect of the combination treatment of UVC and ClO_2 gas, membrane damage to bacterial cells

caused by UVC, ClO_2 gas, and UVC-ClO_2 gas simultaneous treatment was evaluated. Measurement of the leakage of UV-absorbing materials from cytoplasm has been used to verify cell membrane damage since cytoplasmic substances may diffuse out to the suspension via the permeabilized cell wall and membrane (Liu et al., 2016; Ukuku & Geveke, 2010; Virto et al., 2005). In the present study, increasing treatment time of ClO_2 gas (10 ppmv) led to greater leakage of intracellular compounds for all three pathogens, whereas UVC treatment did not increase leakage of cytoplasmic materials. The synergism of UVC-ClO_2 gas simultaneous treatment was observed through spectrophotometric measurements of leakage of UV-absorbing materials (260 nm) of the three pathogens. Significant ($p < 0.05$) differences between the sum of levels of UV-absorbing substances of cells suspensions treated individually with UVC and ClO_2 gas (10 ppmv) and those achieved with combination treatment were observed after 15 min treatments. Quantitative results of cell membrane damage measured by UV-absorbing substances were consistent with TEM analysis (Fig. 2). Also, the results show that there is a correlation between increased leakage of cytoplasmic materials and an increased level of inactivation determined by counts of CFU. These results provide evidence that damage to the cell membrane and changes to membrane permeability are involved in the synergistic lethal effect of the combination treatment of UVC and ClO_2 gas. Also, cell membrane damage or permeability could affect the antimicrobial effect of ClO_2 gas, because for bactericides to be more effective, they must penetrate the cell envelope and attain a concentration high enough to exert their antimicrobial action (Virto et al., 2005). Further research is necessary to elucidate the detailed antibacterial mechanism of the combination treatment of UVC and ClO_2 gas.

In the present study, *L. monocytogenes* was more resistant to UVC, ClO_2 gas, and UVC-ClO_2 gas simultaneous treatment than were *E. coli* O157:H7 and *S.* Typhimurium. It is well known that gram-positive bacteria are generally more resistant to UVC than gram-negative bacteria due to the structure of cell wall (thick layer of peptidoglycan) (Beauchamp & Lacroix, 2012; Kim, Kim, & Kang, 2017). In case of ClO_2 gas, Bhagat et al. (2010) reported that D-values for *Salmonella enterica* and *L. monocytogenes* after ClO_2 gas treatment did not differ significantly ($p > 0.05$). However, some researchers have reported that *L. monocytogenes* was slightly more susceptible to ClO_2 gas treatment than *E. coli* O157:H7 and *S. enterica* (Han, Selby, Schultze, Nelson, & Linton, 2004; Lee, Costello, & Kang, 2004; Mahmoud, Bhagat, & Linton, 2007). The different results in the sensitivity to ClO_2 gas treatment may be due to strain of pathogens used in each study. It is suggested that combination treatment for enough time is necessary to ensure sufficient inactivation of *L. monocytogenes*.

Trinetta, Morgan, and Linton (2010) reported treatment of ClO_2 gas (10 mg/l) for 180 s did not significantly ($p > 0.05$) affect color of Roma tomatoes. Also, Bhagat et al. (2010) showed ClO_2 gas treatment did not significantly ($p > 0.05$) affect the color of hydroponic tomatoes. However, the color of spinach leaves treated with ClO_2 gas (50 ppmv) gradually changed during storage (Park & Kang, 2015) and changing color may be due to the high oxidation capacity of ClO_2 gas (Mahmoud et al., 2008). Several studies reported discoloration of produce following ClO_2 gas treatment (Guentzel, Lam, Callan, Emmons, & Dunham, 2008; Mahmoud & Linton, 2008; Sy et al., 2005). In the present study, there were no significant ($p > 0.05$) differences in color and texture values of spinach leaves and tomatoes between untreated samples and those treated with UVC-ClO_2 gas (10 ppmv) during storage for 7 days. No changes in produce quality may due to the use of a lower concentration of ClO_2 gas (10 ppmv) than other studies. It seems that treatment conditions are important and should be optimized considering the inactivation effect and desired quality of the food product.

5. Conclusion

In conclusion, as treatment time increased the combination treatment of UVC and ClO_2 gas could show additive or synergistic effects in the inactivation of three foodborne pathogens depending on type of produce and ClO_2 gas concentration. The results of this study indicate that the mechanism of the synergistic effect was related to membrane damage, followed by changes to membrane permeability. Food sample quality was maintained during 7 days of storage after combined treatment with all combinations of UVC and ClO_2 gas (10 ppmv). The combination treatment of UVC and ClO_2 gas could be an alternative to conventional washing and sanitizing methods in inactivating foodborne pathogens on produce. Also, the combination treatment of UVC and ClO_2 gas could be applied for microbial control during transportation and storage of fresh produce. The results of this study could help the fresh produce industry ensure microbial safety of produce.

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