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Antimicrobial effect of chlorine dioxide gas against foodborne pathogens under differing conditions of relative humidity

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ABSTRACT

The objective of this study was to determine the influence of relative humidity (RH) on the antimicrobial effect of chlorine dioxide (ClO_2) gas against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on spinach leaves. Spinach leaves were inoculated with three foodborne pathogens and treated with ClO_2 gas at different concentrations (1, 5, 10, 30, and 50 ppmv) for 0, 5, 10, 15, and 20 min under differing conditions of RH (50, 70, and 90%). Inoculated spinach leaves subjected to ClO_2 gas treatments experienced significant reductions, with pathogen populations reduced more under conditions of 90% than under 50 and 70% RH. Generally, there were no significant differences ($p > 0.05$) in reduction levels of the three foodborne pathogens between 50 and 70% RH. Exposure to 50 ppmv of ClO_2 gas for 20 min resulted in 1.25–1.78 (50% RH) and 2.02 to 2.54 (70% RH) log reductions of the three foodborne pathogens. The levels of the three foodborne pathogens was reduced to below the detection limit (1 log CFU/g) within 15 min when treated with 50 ppmv of ClO_2 gas under 90% RH. The results may help the fresh produce industry to establish effective conditions for ClO_2 gas treatment.

1. Introduction

Foodborne outbreaks related to the consumption of fresh and fresh-cut produce have increased in recent years (FDA, 2011). From 1996 to 2008, about 82 foodborne illness outbreaks were related to the consumption of fresh produce, and among these outbreaks 28 cases were associated with leafy greens, accounting for 949 illnesses and 5 deaths (FDA, 2009). In the EU, the share of all foodborne outbreaks for fresh produces increased from 4.4% in 2009 to 10% in 2010 (Uyttendaele, Jacxsens, & Boxstael, 2014). In 2006, fresh spinach and spinach-containing products were associated with an outbreak of *Escherichia coli* O157:H7 (CDC, 2006; Maki, 2006). Most of the 204 cases and 3 deaths occurred in the United States, and one case occurred in Canada (Jay et al., 2007; Wendel et al., 2009). Also, a total of 33 persons infected with *E. coli* O157:H7 traced to organic spinach and spring mix blend was reported from 5 US states in 2012 (CDC, 2012).

ClO_2 is a strong oxidizing agent with a broad antimicrobial spectrum (Trinetta, Vaid, Xu, Linton, & Morgan, 2012). Its efficacy is largely not affected by pH and organic matter and it does no react with nitrogen compounds to form chloramines (Aieta, Roberts, & Hernandez, 1984; Beuchat, 1998). The mechanism of disinfection by ClO_2 has been postulated by several studies. The most widely accepted antimicrobial mechanism of ClO_2 is damage to protein synthesis and increased permeability of the outer cell membrane (Aieta & Berg, 1986; Roller, Olivieri, & Kawata, 1980).

ClO_2 gas may be more effective for inactivation of foodborne pathogens than aqueous ClO_2 due to its penetration ability (Han, Linton, Nielsen, & Nelson, 2001). Also, ClO_2 gas could be applied for microbial control during transportation and storage of fresh produce. Preventing contamination at all stages of production, harvesting, processing, storage, and preparation of fresh produce is important because postharvest washing of produce with aqueous sanitizers at concentrations low enough to not cause sensory degradation is inadequate for reducing foodborne pathogens (Beuchat, 2006). ClO_2 gas has been evaluated for inactivating foodborne pathogens on fresh produce such as apples (Du, Han, & Linton, 2002), green peppers (Han, Linton, Nielsen, & Nelson, 2000), lettuce (Mahmoud & Linton, 2008; Sy, Murray, Harrison, & Beuchat, 2005), cabbage (Sy et al., 2005), carrots (Gómez-López, Devlieghere, Ragaert, & Debevere, 2007), tomatoes (Bhagat, Mahmoud, & Linton, 2010; Trinetta, Linton, & Morgan, 2013), blueberries (Popa, Hanson, Todd, Schilder, & Ryser, 2007; Sy et al.,

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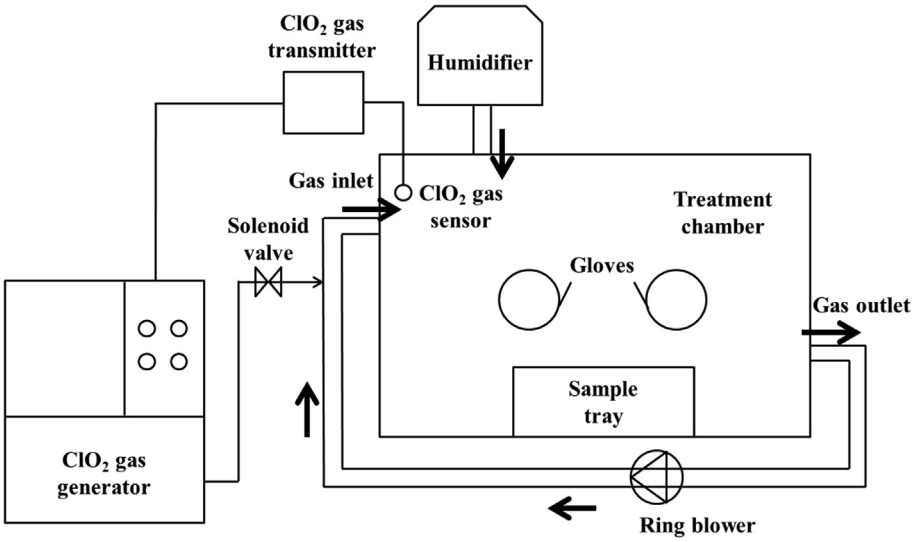

Fig. 1. Schematic diagram of the ClO_2 gas treatment system used in this experiment.

2005), and strawberries (Han, Shelby, Schultze, Nelson, & Linton, 2004).

Gas concentration, relative humidity (RH), treatment time, and temperature can affect the antimicrobial effect of ClO_2 gas, and especially, the combination of gas concentration and RH shows a synergistic effect (Han, Floros, et al. 2001). Several studies have been conducted under conditions of high RH (>80%) to evaluate the antimicrobial effect of ClO_2 gas, but there have been no studies to evaluate the inactivation tendency of ClO_2 gas relative to conditions of RH and treatment time (Bhagat et al., 2010; Bhagat, Mahmoud, & Linton, 2011; Gómez-López, Ragaert, Jeyachchandran, Debevere, & Devlieghere, 2008; Popa et al., 2007; Vandekinderen et al., 2009).

This study evaluated the effect of varying levels of RH on the antimicrobial effect of chlorine dioxide (ClO_2) gas against *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on spinach leaves. Also, any changes in color and texture of spinach leaves were assessed.

2. Materials and methods

2.1. Bacterial strains

Three isolates 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.

2.2. Culture preparation and sample inoculation

All strains of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were cultured individually in 5 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 °C for 24 h, harvested by centrifugation at 4000 × *g* for 20 min at 4 °C, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in sterile BPW, corresponding to ca. 10^7–10^8 CFU/ml. Mixed pathogen culture cocktails were prepared by blending together equal volumes of all test strains. These cell suspensions were used in subsequent experiments.

Spinach was purchased from a local market (Seoul, South Korea) on the day of experiments.

Spinach leaves were trimmed to approximately 5 × 3 cm in size. Spinach leaves were then placed on aluminum foil in a laminar flow hood, and 0.1 ml of culture cocktail was inoculated onto each leaf by depositing droplets with a micropipettor at 15–20 locations. After inoculation, spinach leaves were dried in a laminar flow biosafety hood for 1 h at 22 ± 2 °C.

2.3. ClO_2 gas treatment system

Fig. 1 shows ClO_2 gas treatment system used in this experiment. 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). 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 humidifier (H–C976, Osungsa, Changwon-si, South Korea) was used to control RH in the treatment chamber. A thermohygrometer (YTH-600, Uins, Seoul, South Korea) was used to measure RH and temperature in the treatment chamber.

2.4. ClO_2 gas treatment

Inoculated spinach leaves were placed on sterile plastic sample tray inside the treatment chamber as presented Fig. 1 and covered with a plastic lid. Samples were treated with 1, 5, 10, 30, and 50 ppmv ClO_2 gas for 20 min at 22 ± 2 °C. The RH of the treatment chamber was adjusted to 50, 70, and 90% with an accuracy of ±2%. When the desired ClO_2 gas concentration and RH were achieved, the plastic lid was removed and the inoculated side of spinach leaves was exposed to ClO_2 gas. Spinach leaves were withdrawn from the treatment chamber after 1, 5, 10, 15, and 20 min exposure to ClO_2 gas, and treated samples were used to determine surviving bacterial populations. These experiments were repeated three times.

2.5. Bacterial enumeration

Treated spinach leaves (10 ± 0.2 g) were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada)

containing 90 ml of neutralizing buffer (Difco). 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 diluents was spread-plated onto 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. When low bacterial numbers were anticipated, 250 μl of undiluted cell suspension was plated onto four plates of each respective medium. The plates were incubated at 37 °C for 24–48 h. After incubation, colonies were counted and calculated as log CFU/g.

2.6. Color and texture measurement

Treated spinach leaves were stored in Ziploc® bag at 4 °C for 7 days to identify changes in quality during storage following treatments. Colors of spinach leaves were measured with a Minolta colorimeter (model CR300, Minolta Co., Osaka, Japan) at 3 locations on each leaf. Hunter's *L*, *a*, *b* values indicated lightness, redness, and yellowness of the sample, respectively. The texture of spinach leaves was evaluated with a texture analyzer (TA-CT3, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a blade set probe. Twenty grams of sample was 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. Maximum force was recorded using Texturepro CT software (Brookfield Engineering Laboratories, Inc.). Three measurements were performed for each treatment with independently-prepared samples.

2.7. Statistical analysis

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

3. Results

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

Tables 1–5 show the effects of ClO_2 gas treatment against the three pathogens on spinach leaves. Population before treatment was ca. 6–7 log CFU/g for *E. coli* O157:H7 and *S.* Typhimurium, and ca. 5–6 log CFU/g for *L. monocytogenes*. When spinach leaves were treated with 1 ppmv ClO_2 gas, reduction levels of the three pathogens were not significantly ($p > 0.05$) different between varying levels of RH (Table 1). After treatments under 50, 70, and 90% RH for 20 min, 0.83, 1.01, and 1.12 log reductions of *E. coli* O157:H7 were achieved, respectively. *S.* Typhimurium was reduced by 0.82, 0.90, and 1.22 log after 20 min treatment under 50, 70, and 90% RH, respectively. One ppmv ClO_2 gas treatment under 50, 70, and 90% RH for 20 min resulted in 0.57, 0.68, and 1.02 log reductions of *L. monocytogenes*, respectively.

As ClO_2 gas concentration increased, treatment under 90% RH resulted in more significant differences ($p < 0.05$) in reduction levels of the three foodborne pathogens than ClO_2 gas treatment under 50 and 70% RH. Generally, there were no significant differences ($p > 0.05$) in reduction levels of three foodborne pathogens between treatments of 50 and 70% RH. Treatment with 5 ppmv of ClO_2 gas for 20 min caused 0.84 to 1.34 (50% RH), 1.32 to 1.41 (70% RH), and 1.94 to 2.36 (90% RH) log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*. Exposure to 10 ppmv of ClO_2 gas for 20 min resulted in 1.20–1.32 (50% RH), 1.53 to 2.09 (70% RH), and 3.56 to 3.63 (90% RH) log reductions of the three foodborne pathogens. Treatment with 30 ppmv of ClO_2 gas for 20 min caused 1.25 to 1.54 (50% RH) and 1.55 to 1.76 (70% RH) log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*. The numbers of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were reduced to below the detection limit (1 log CFU/g) within 15 min when treated with 30 ppmv of ClO_2 gas under 90% RH. Exposure to 50 ppmv of ClO_2 gas for 20 min resulted in 1.25–1.78 (50% RH) and 2.02 to 2.54 (70% RH) log reductions of the three foodborne pathogens. The levels of the three foodborne pathogens were reduced to below the detection limit (1 log CFU/g) within 15 min when treated with 50 ppmv of ClO_2 gas under 90% RH.

3.2. Effects of ClO_2 gas treatment on the quality of spinach leaves

No significant ($p > 0.05$) differences in Hunter's color values (L^*, a^*, b^*) were observed between untreated spinach leaves (control) and those treated with 1–30 ppmv of ClO_2 gas during storage at 4 °C for 7 days (data not shown). However, differences in color values between control and spinach leaves exposed to 50 ppmv of ClO_2 gas were observed during storage (Table 6). The L^* value of spinach leaves treated with 50 ppmv of ClO_2 gas significantly ($p < 0.05$) increased after 7 days. The a^* value of spinach leaves treated with 50 ppmv of ClO_2 gas under RH 70 and 90% conditions significantly ($p < 0.05$) decreased after 3 days. After 7 days of storage, the a^* value of all spinach leaves treated with 50 ppmv of ClO_2 gas significantly ($p < 0.05$) decreased. The b^* value of spinach leaves treated with 50 ppmv of ClO_2 gas under 70 and 90% RH significantly ($p < 0.05$) increased after 3 days, and the b^* value of all spinach leaves treated with 50 ppmv of ClO_2 gas significantly ($p < 0.05$) increased after 7 days. There were no significant

Table 1
Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves after 1 ppmv ClO_2 gas treatments.

Treatment time (min)	Log reduction (log CFU/g)								
	E. coli O157:H7			*S.* Typhimurium			*L. monocytogenes*		
	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%
1	0.15(0.28)Aa[b]	0.37(0.03)Aa	0.30(0.22)Aa	0.20(0.29)Aa	0.25(0.18)Aa	0.26(0.09)Aa	0.20(0.23)Aa	0.10(0.15)Aa	0.22(0.22)Aa
5	0.53(0.17)ABa	0.63(0.15)ABa	0.52(0.35)ABa	0.22(0.30)Aa	0.41(0.24)Aa	0.44(0.36)ABa	0.38(0.04)ABa	0.32(0.25)Aa	0.53(0.12)ABa
10	0.58(0.16)ABa	0.75(0.08)BCa	0.69(0.51)ABa	0.35(0.39)Aa	0.49(0.05)Aa	0.69(0.49)ABCa	0.46(0.05)ABa	0.61(0.50)Aa	0.68(0.39)ABCa
15	0.75(0.33)Ba	0.88(0.30)BCa	0.89(0.22)ABa	0.47(0.64)Aa	0.59(0.17)ABa	0.94(0.26)BCa	0.56(0.14)Ba	0.64(0.56)Aa	0.80(0.30)BCa
20	0.83(0.08)Ba	1.01(0.02)Ca	1.12(0.39)Ba	0.82(0.04)Aa	0.90(0.27)Ba	1.22(0.29)Ca	0.57(0.23)Ba	0.68(0.50)Aa	1.02(0.05)Ca

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after ClO_2 gas treatment. Values are means ($n = 3$) and values in parentheses represent the standard deviations (SD) of the means.
[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table 2
Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves after 5 ppmv ClO_2 gas treatments.

Treatment time (min)	Log reduction (log CFU/g)								
	E. coli O157:H7			*S.* Typhimurium			*L. monocytogenes*		
	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%
1	0.46(0.70)Aa[b]	0.60(0.32)Aa	0.67(0.24)Aa	0.46(0.10)Aa	0.69(0.39)Aa	0.55(0.16)Aa	0.38(0.31)Aa	0.57(0.06)Aa	0.38(0.24)Aa
5	0.55(0.70)Aa	0.89(0.41)ABa	1.11(0.40)ABa	0.51(0.04)Aa	0.86(0.55)Aa	1.01(0.18)Aa	0.51(0.11)ABa	0.70(0.11)ABa	0.56(0.13)Aa
10	0.96(0.36)Aa	1.12(0.50)ABa	1.68(0.42)BCa	0.57(0.05)Aa	1.03(0.27)Aa	1.72(0.35)Bb	0.56(0.07)ABa	0.97(0.13)BCb	1.12(0.21)Bb
15	1.19(0.41)Aa	1.33(0.18)ABa	2.01(0.03)CDb	0.78(0.06)Ba	1.29(0.37)Aab	1.83(0.52)Bb	0.56(0.15)ABa	1.21(0.31)CDb	1.41(0.15)Bb
20	1.34(0.47)Aa	1.40(0.48)Ba	2.36(0.46)Db	1.06(0.13)Ca	1.41(0.24)Ab	2.33(0.31)Bb	0.84(0.16)Ba	1.32(0.12)Db	1.94(0.15)Cc

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after ClO_2 gas treatment. Values are means ($n = 3$) and values in parentheses represent the standard deviations (SD) of the means.
[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table 3
Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves after 10 ppmv ClO_2 gas treatments.

Treatment time (min)	Log reduction (log CFU/g)								
	E. coli O157:H7			*S.* Typhimurium			*L. monocytogenes*		
	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%
1	0.39(0.09)Aa[b]	0.75(0.33)Aa	0.85(0.25)Aa	0.40(0.12)Aa	0.76(0.04)Aa	0.72(0.54)Aa	0.41(0.05)Aa	0.45(0.20)Aa	0.41(0.02)Aa
5	0.50(0.21)Aa	0.93(0.07)Ab	1.22(0.15)Ab	0.57(0.52)Aa	0.97(0.14)ABa	1.08(0.54)Aa	0.39(0.42)Aa	0.76(0.33)ABa	0.71(0.27)Ba
10	0.91(0.13)Ba	1.56(0.16)Bb	2.51(0.32)Bc	0.67(0.18)ABa	1.21(0.13)Bb	2.32(0.20)Bc	0.49(0.05)Aa	0.90(0.54)ABCa	1.76(0.16)Bb
15	1.14(0.31)BCa	1.77(0.14)BCb	3.08(0.41)Cc	0.76(0.19)ABa	1.23(0.14)Bb	2.94(0.23)BCc	0.91(0.42)ABa	1.21(0.46)BCa	2.54(0.30)Cb
20	1.32(0.22)Ca	2.09(0.40)Cb	3.63(0.37)Cc	1.20(0.21)Ba	1.68(0.24)Ca	3.56(0.51)Cb	1.21(0.39)Ba	1.53(0.28)Ca	3.38(0.29)Db

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after ClO_2 gas treatment. Values are means ($n = 3$) and values in parentheses represent the standard deviations (SD) of the means.
[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table 4
Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves after 30 ppmv ClO_2 gas treatments.

Treatment time (min)	Log reduction (log CFU/g)								
	E. coli O157:H7			*S.* Typhimurium			*L. monocytogenes*		
	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%
1	0.65(0.36)Aa[b]	0.73(0.15)Aa	1.06(0.43)Aa	0.50(0.08)Aa	0.64(0.19)Aa	0.98(0.10)Ab	0.71(0.25)Aa	0.86(0.19)Aa	0.62(0.17)Aa
5	0.82(0.16)ABa	0.78(0.22)ABa	1.99(0.37)Bb	0.64(0.11)ABa	0.88(0.21)Aa	1.69(0.08)Bb	1.07(0.40)ABa	1.05(0.13)ABa	1.33(0.06)Ba
10	1.12(0.34)ABCa	1.13(0.32)Ba	4.02(0.50)Cb	0.88(0.10)BCa	1.16(0.03)Ba	3.76(0.32)Cb	1.12(0.13)ABa	1.16(0.01)BCa	2.76(0.07)Cb
15	1.30(0.37)BCa	1.53(0.09)Ca	>5.78Db	1.07(0.29)CDa	1.42(0.03)Ca	>5.68Db	1.17(0.19)ABa	1.30(0.04)Ca	>4.86Db
20	1.54(0.13)Ca	1.70(0.19)Ca	>5.78Db	1.25(0.10)Da	1.76(0.11)Db	>5.68Dc	1.45(0.18)Ba	1.55(0.07)Da	>4.86Db

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after ClO_2 gas treatment. Values are means ($n = 3$) and values in parentheses represent the standard deviations (SD) of the means.
[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table 5
Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves after 50 ppmv ClO_2 gas treatments.

Treatment time (min)	Log reduction (log CFU/g)								
	E. coli O157:H7			*S.* Typhimurium			*L. monocytogenes*		
	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%	RH 50%	RH 70%	RH 90%
1	0.74(0.15)Aa[b]	0.50(0.16)Aa	0.77(0.20)Aa	0.66(0.33)Aa	0.60(0.15)Aa	1.09(0.32)Aa	0.64(0.08)Aa	0.78(0.25)Aa	0.76(0.23)Aa
5	1.10(0.21)Ba	0.82(0.28)Aa	2.47(0.10)Bb	0.92(0.09)ABa	0.73(0.07)ABa	2.34(0.59)Bb	1.26(0.41)Ba	1.28(0.08)Ba	1.98(0.24)Bb
10	1.31(0.15)BCa	1.44(0.16)Ba	5.08(0.20)Cb	1.08(0.26)Ca	1.30(0.23)BCa	5.19(0.23)Cb	1.31(0.03)Ba	1.42(0.07)Ba	4.71(0.18)Cb
15	1.50(0.17)Ca	1.80(0.26)Ba	>5.53Db	1.21(0.09)Ca	1.49(0.11)Ca	>5.56Db	1.55(0.27)BCa	1.89(0.14)Ca	>4.85Db
20	1.58(0.02)Ca	2.45(0.35)Cb	>5.53Dc	1.25(0.04)Ca	2.54(0.65)Db	>5.56Dc	1.78(0.09)Ca	2.02(0.35)Ca	>4.85Db

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after ClO_2 gas treatment. Values are means ($n = 3$) and values in parentheses represent the standard deviations (SD) of the means.
[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table 6
Changes in color values[a] of spinach leaves treated with ClO_2 gas during storage at 4 °C for 7 days.

Day	Treatment			
	Control	50 ppmv		
	–	RH 50%	RH 70%	RH 90%
L^*				
0	33.66 ± 0.91A[b]	33.02 ± 0.75A	33.45 ± 1.37A	33.05 ± 1.00A
3	33.54 ± 0.87A	33.66 ± 1.06A	34.32 ± 0.36A	33.60 ± 0.79A
7	33.11 ± 0.43A	36.58 ± 0.85B	36.33 ± 0.51B	36.31 ± 0.69B
a^*				
0	−7.32 ± 0.97A	−7.31 ± 0.51A	−7.27 ± 0.33A	−7.20 ± 0.69A
3	−7.36 ± 0.61A	−7.32 ± 0.35A	−8.65 ± 0.33B	−8.75 ± 0.58B
7	−7.17 ± 0.31A	−8.97 ± 0.71C	−8.78 ± 0.64BC	−8.39 ± 0.42BC
b^*				
0	11.70 ± 0.62A	11.03 ± 0.92A	11.28 ± 0.80A	11.36 ± 0.74A
3	11.22 ± 0.67A	11.42 ± 0.62AB	13.30 ± 0.40BC	13.00 ± 0.65C
7	10.49 ± 1.38A	14.34 ± 0.90B	14.36 ± 0.55B	14.29 ± 0.50B

[a] Color parameters are lightness (L^*), redness (a^*), and yellowness (b^*).
[b] Means ± standard deviations from three replications. Within the same storage time, means with different uppercase letters within a row are significantly different ($p < 0.05$).

($p > 0.05$) differences in texture between untreated spinach leaves (control) and those treated with ClO_2 gas during storage at 4 °C for 7 days (data not shown).

4. Discussion

Several sanitation methods have been evaluated to inactivate foodborne pathogens on spinach. Treatment with sodium hypochlorite (100 ppm) for 5 min reduced levels of *E. coli* O157:H7 by 1.1 log CFU/g (Lee & Baek, 2008). Guentzel, Lam, Callan, Emmons, and Dunham (2008) reported a dipping treatment of spinach at 100 and 120 ppm total residual chlorine for 10 min resulted in a 4.0–5.0 log CFU/ml reduction of bacterial counts for *E. coli*, *S.* Typhimurium, *Staphylococcus aureus*, *L. monocytogenes*, and *Enterococcus faecalis*. Lactic, citric, malic, tartaric, and acetic acid (2%) resulted in 1.5–1.8 log CFU/g reduction of *E. coli* O157:H7 on baby spinach after 5 min of treatment (Huang & Chen, 2011). Neal et al. (2012) reported 1 mg/L ozone treatment for 30 min reduced levels of *Salmonella* and *E. coli* O157:H7 on spinach leaves by 1.0 and 0.6 log CFU/g, respectively. Aqueous chlorine dioxide (100 ppm) reduced levels of *E. coli* O157:H7 on spinach by 2.6 log CFU/g (Lee et al., 2008).

In the present study, ClO_2 gas showed a significant antimicrobial effect against foodborne pathogens on spinach leaves. ClO_2 gas treatment used in this study showed a higher reduction of inoculated foodborne pathogens on spinach leaves compared to other studies. Neal et al. (2012) evaluated the antimicrobial effect of ClO_2 gas in inactivating *Salmonella* and *E. coli* O157:H7 on spinach leaves using a ClO_2 gas generating sachet. *Salmonella* and *E. coli* O157:H7 on spinach leaves exposed to 2.1 mg/L ClO_2 gas for 1 h were reduced by 0.6 and 0.7 log CFU/g, respectively. Differences in reduction levels are likely due to different treatment conditions. The effect of ClO_2 gas is known to be influenced by various factors such as humidity, temperature, airflow, and gas generating method (Morino, Fukuda, Miura, & Shibata, 2011; Vandekinderen et al., 2009).

It has been well known that the antimicrobial effect of ClO_2 gas increases with increasing RH. Han et al. (Han, Floros, Linton, Nielsen, & Nelson, 2001) reported that high RH enhanced the efficacy of ClO_2 gas in inactivating *E. coli* O157:H7 on green peppers. When green peppers were treated with 0.3 mg/L ClO_2 gas at 15 °C, reductions increased from 1.93 to 4.00 log CFU/5 g as RH increased from 55 to 95%. Growth of *Lactobacillus buchneri* on stainless steel strips after treatment with 8 mg/L ClO_2 gas for 10 min decreased as RH increased from 56 to 94% (Han, Guentert, Smith, Linton, & Nelson, 1999). Also, several studies evaluated the antimicrobial effect of ClO_2 gas conducted under conditions of high RH (>80%) (Bhagat et al., 2010, 2011; Guentzel et al., 2008; Popa et al., 2007; Vandekinderen et al., 2009). However, there have been no studies which assessed the inactivation tendency of ClO_2 gas according to levels of RH and treatment time. This may be an important factor for practical application of ClO_2 gas by the food processing industry. The results of this study showed that treatment with ClO_2 gas under 90% RH caused more significant ($p < 0.05$) reductions in levels of three foodborne pathogens than ClO_2 gas treatment under 50 and 70% RH at the same ClO_2 gas concentrations. Also, differences in reduction levels between treatments under 90% RH and those under 50 and 70% RH increased as ClO_2 gas concentration increased. Differences in reduction levels may due to different solubility of ClO_2 gas under different levels of RH. ClO_2 gas acts similar to aqueous ClO_2 for inactivating microorganisms due to its high water solubility (Linton, Han, Selby, & Nelson, 2006).

The color of spinach leaves treated with 50 ppmv of ClO_2 gas gradually changed during storage. Also, color changes occurred rapidly in spinach leaves treated under conditions of high RH. Changing color of spinach leaves during storage may be due to the high oxidation capacity of ClO_2 gas (Mahmoud & Linton, 2008). Discoloration of fresh produce following ClO_2 gas treatment has been reported in several studies (Guentzel et al., 2008; Mahmoud & Linton, 2008; Sy et al., 2005). Therefore, treatment conditions should be optimized considering the inactivation effect and desired quality of the food product.

In conclusion, this study showed inactivation patterns of ClO_2 gas varied according to conditions of RH. The results of this study are helpful for fresh produce industry to establish ClO_2 gas treatment conditions.

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