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Combined Treatment with Low Concentrations of Aqueous and Gaseous Chlorine Dioxide Inactivates *Escherichia coli* O157:H7 and *Salmonella* Typhimurium Inoculated on Paprika

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Combined treatment with gaseous and aqueous chlorine dioxide (ClO_2) was performed to improve the microbiological safety and quality of paprika. A single treatment of 50 ppmv ClO_2 gas for 30 min decreased the populations of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium by 2.33 and 2.91 log CFU/g, respectively. In addition, a single treatment of aqueous ClO_2 (50 ppm) for 5 min decreased these populations by 1.86 and 1.37, respectively. The most dramatic effects were achieved by combined treatment of 50 ppm aqueous and gaseous ClO_2 for 30 min, which decreased populations of *E. coli* O157:H7 and *S.* Typhimurium by 4.11 and 3.61 log CFU/g, respectively. With regard to the qualities of paprika, no adverse effects were elicited by the combined treatment. Thus, combined treatment with aqueous and gaseous ClO_2 is a suitable approach that can be used to improve the microbial safety and quality of paprika.

Keywords: Chlorine dioxide, combined treatment, microbial safety, paprika, pathogenic bacteria

Introduction

Foodborne illness has increased owing to cross-contamination of foods and global warming [1, 2]. Major foodborne diseases are caused by contamination with *Escherichia* and *Salmonella* [3]. These pathogenic bacteria are a major threat to vendors of fresh produce [4].

Salmonellosis is responsible for 19,000 hospital admissions in the USA every year [5]. Most cases of salmonellosis have been attributed to consuming poultry products [6], but the frequency of foodborne disease outbreaks associated with *Salmonella* in fresh produce has increased [7]. *E. coli* O157:H7 is also a threat to humans, as it causes hemorrhagic colitis and hemolytic uremic syndrome [8]. Feces of livestock can contaminate soil or irrigation water, and fruits and vegetables are not thermally processed after harvest. Therefore, they can be easily exposed to pathogenic bacteria through postharvest processing, irrigation water, and workers [9, 10]. In particular, paprika is more susceptible to contamination with pathogenic bacteria [11], and has previously been recalled owing to *Salmonella* contamination in the United States [12]. Therefore, appropriate postharvest treatment to inactivate *S.* Typhimurium and *E. coli* O157:H7 on paprika is needed.

As a chlorine-based treatment, chlorine dioxide (ClO_2) is a substitute for sodium hypochlorite (NaOCl). ClO_2 has more oxidation capacity than NaOCl and it does not generate harmful chemicals such as trihalomethane [13]. Additionally, it is highly soluble in water and does not leave any toxic residue [14]. Various concentrations of gaseous ClO_2 have been used to inactivate pathogenic bacteria on green peppers [15], strawberries [16], Roma tomatoes [17], and blueberries [18]. In addition, many studies on aqueous ClO_2 in fresh produce have been performed [19, 20], but few have been conducted using low concentrations of gaseous ClO_2.

Combined treatment to secure microbial safety is called hurdle technology. To improve microbial inactivation, hurdle technology has been applied to fresh produce [18, 21, 22]. Combined treatment can reduce the concentration of gaseous ClO_2 required to inactivate microbes on fresh produce. However, the effect of combined gaseous and

aqueous ClO_2 treatment has never been evaluated. To address this, we tested whether this combined treatment could inactivate *S.* Typhimurium and *E. coli* O157:H7 that were inoculated on paprika.

Materials and Methods

Preparation of Samples

Paprika (*Capsicum annuum* L.) fruits were obtained from a local farm in Hwasun, Korea, and fully ripened red paprika fruits were chosen and used for the experiments. Harvested paprika fruits were transported to the laboratory within 3 h under refrigerated temperature condition.

Strains and Culture Preparation

Strains of *S.* Typhimurium (KCTC 2514 and ATCC 14028) and *E. coli* O157:H7 (NCTC 12079 and ATCC 43889) were selected for experiments. Each strain of *S.* Typhimurium and *E. coli* O157:H7 was streaked onto tryptic soy agar (TSA; Difco Co., USA) and incubated at 37°C for 24 h. Following incubation, each single colony of *S.* Typhimurium and *E. coli* O157:H7 was added to 25 ml of tryptic soy broth (TSB; Difco Co.) and incubated at 37°C for 24 h with shaking at 150 rpm. The incubated culture was centrifuged at 1,500 ×*g* for 20 min and the cell pellets were separated and then washed twice with 0.1% sterile peptone water.

Inoculation of Strains

Prior to inoculation, washed cell pellets from each culture were added to 25 ml of 0.1% aseptic peptone water and then mixed to form an inoculum. Paprika samples were then spot inoculated with 0.5 ml of this inoculum. The inoculated samples were placed in a laminar flow hood for 2 h to allow attachment of the pathogenic bacteria to the surface of paprika samples.

Chemical Treatment

ClO_2 gas treatment (0.03–0.14 mg/l, 10–50 ppmv) was conducted using a ClO_2 gas generating system (CA-300; Purgofarm, Korea) [23]. ClO_2 gas treatment was carried out in a treatment chamber (150 × 100 × 100 cm) for 5, 10, 20, or 30 min. To monitor the ClO_2 gas concentration in the treatment chamber, a ClO_2 gas sensor (Model F12; ATi Inc., USA) was used. A humidifier (CH-5762; Cuckoo Inc., Korea) was used to adjust relative humidity to 90% in a treatment chamber. Aqueous ClO_2 was produced and set at 50 mg/l based on a previous study [13], and its concentration was determined by using the iodometric titration method [24]. Aqueous ClO_2 treatment was conducted for 5 min by submerging the samples at a ratio of 1:5 (w/v). For the combined treatment of gaseous and aqueous ClO_2, the paprika samples were first treated with gaseous ClO_2 and then aqueous ClO_2, based on a preliminary experiment. All treatments were conducted in triplicates.

Quality Measurement

The color of paprika surfaces was measured with a colorimeter (CR-400 Chroma Meter; Konica Minolta Sensing Inc., Japan). Hunter color measurement was carried out for each sample. The standard *L*, *a*, and *b* values were $L = 96.87$, $a = -0.13$, and $b = 2.13$. The chroma (C) and hue angle (h^o) were calculated according to the following equations [25].

$$C = (a^2 + b^2)^{1/2}, \quad h^o = \arctan(b/a) \times (180/\pi)$$

The total soluble solid content of each sample was determined with a refractometer (PR-101α; Atago, Japan). The hardness value was determined by a texture analyzer (TA-XT2; Stable Micro Systems Ltd., UK) with a cylinder probe (10 cm diameter; TA-40; Stable Micro Systems Ltd., UK). the pretest, test, and posttest speeds were 2, 5, and 5 mm/s, respectively. The distance between the probe and sample was 20 mm, and the trigger force was 0.2 N. Vitamin C measurement was carried out using a high-performance liquid chromatography system (Waters Inc., USA) with a UV detector and a C18 column (250 × 4.6 mm, 5 μm; Phenomenex Inc., USA). The mobile phases were buffers A (0.05 M potassium phosphate monobasic) and B (acetonitrile) at a ratio of 6:4. The flow rate of the mobile phase was 1 ml/min, and the detection of vitamin C was carried out at 254 nm. Standard ascorbic acid was used to calculate the vitamin C content of the samples. All treatments were conducted in triplicates.

Microorganism Inactivation Model

To examine the inactivation kinetics of microorganisms by ClO_2 gas treatment, the Weibull model was applied. The Weibull model for non-log linear survival curves can be expressed by the following equation [26].

$$\text{Log}(N/N_0) = -(t/\delta)^{\beta}$$

where N_0 is the initial number of bacteria, N is the number of surviving bacteria after time t, and δ and β are the first reduction time that causes 1 log reduction of surviving population and shape parameter, respectively.

Microbiological Analysis

Following ClO_2 treatment, the samples (20 ± 0.3 g) were placed in a stomacher bag including 180 ml of aseptic peptone water (0.1%) and then smashed for 3 min by using a Stomacher (MIX 2, AES Laboratoire, France). Homogenized samples were then diluted serially with peptone water (0.1%). To analyze the population of each pathogen, 0.1 ml of diluted samples was diffused onto each selective agar and then incubated at 37°C for 48 h. The media for *S.* Typhimurium and *E. coli* O157:H7 were xylose lysine deoxycholate agar (Difco Co.) and sorbitol MacConkey agar (Difco Co.), respectively. All data were depicted as log colony-forming units (CFU)/g.

Statistical Analysis

Statistical significance was conducted with SAS software (ver. 8.4; SAS Institute, Inc., USA). Analysis of variance and Duncan's multiple range test were conducted to analyze significant

differences at $p < 0.05$. For these tests, data from at least three replicates are presented as the mean ± standard deviation.

Results and Discussion

Effect of ClO_2 Gas Treatment

First, we examined the ability of ClO_2 gas treatment (10 to 50 ppmv) to reduce the populations of pathogenic bacteria inoculated on paprika (Figs. 1–3). The initial microbial populations of *S.* Typhimurium and *E. coli* O157:H7 were 5.84 and 6.34 log CFU/g, respectively. Among all 5 min treatments, only 50 ppmv was able to reduce the population of *E. coli* O157:H7. This result indicates that ClO_2 gas treatment at concentrations lower than 50 ppmv for 5 min is insufficient to inactivate pathogenic bacteria that have been inoculated on paprika surfaces. On the contrary, a significant inhibitory effect was observed when the treatment was lengthened to between 10 and 30 min. In the case of *E. coli* O157:H7, the inhibitory effect increased gradually up to 20 ppmv ClO_2 and more rapidly at 30 ppmv. At 30 ppmv, ClO_2 gas treatment for 30 min reduced the population of *E. coli* O157:H7 by 2.06 log CFU/g, compared with that in the control. The effect was most robust with a 50 ppmv ClO_2 treatment for 30 min. Specifically, this treatment reduced the microbial population by 2.33 log CFU/g, compared with that of the control.

A similar pattern was also seen with *S.* Typhimurium, with a gradual decrease in microbial populations at gas concentrations between 10 to 30 ppmv, and a more significant

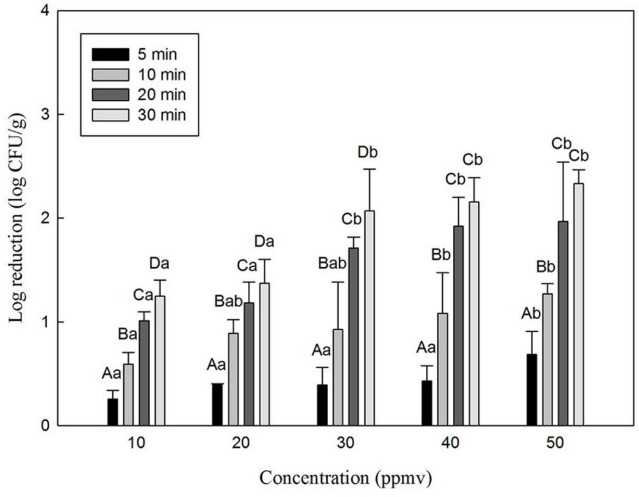

Fig. 1. Effect of ClO_2 gas treatment on the inactivation of *E. coli* O157:H7 inoculated on paprika.

Mean values with different letters in the same concentration (A–D) and time (a–b) are significantly ($p < 0.05$) different. Data are the mean ± standard deviation ($n = 3$).

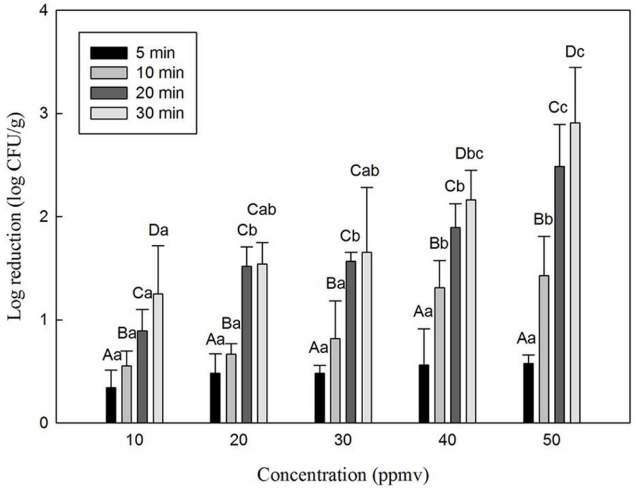

Fig. 2. Effect of ClO_2 gas treatment on the inactivation of *S.* Typhimurium inoculated on paprika.

Mean values with different letters in the same concentration (A–D) and time (a–c) are significantly ($p < 0.05$) different. Data are the mean ± standard deviation ($n = 3$).

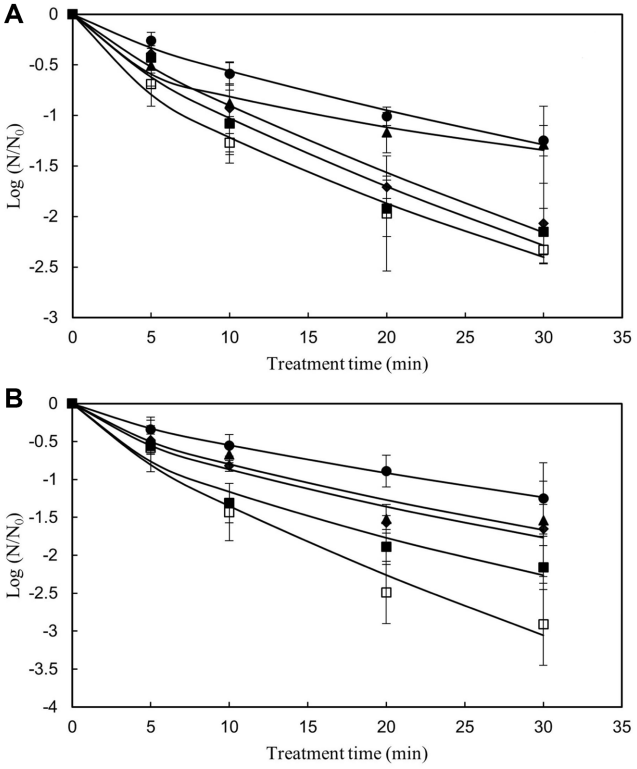

Fig. 3. Survival plots of pathogenic bacteria by ClO_2 gas treatment.

(**A**) *E. coli* O157:H7; (**B**) *S.* Typhimurium. ●, 10 ppmv; ▲, 20 ppmv; ◆, 30 ppmv; ■, 40 ppmv; □, 50 ppmv.

increase at 50 ppmv; the latter concentration decreased the population of *S.* Typhimurium by 2.91 log CFU/g. Han *et al.* [15] reported an approximately 3 log CFU/g reduction when 227 ppmv ClO_2 gas was used for 30 min against *E. coli* O157:H7 inoculated on green peppers. Wu and Kim [27] also reported that 15 ppmv ClO_2 gas treatment for 30 min reduced the population of *S.* Typhimurium inoculated on blueberries by about 3 log CFU/g.

To further examine the germicidal effect of ClO_2 gas treatment, survival curves of *E. coli* O157:H7 and *S.* Typhimurium were fitted with a Weibull model (Fig. 3) and the parameters were calculated (Table 1). Non-log linear survival curves were shown for all treatments and both pathogens (Fig. 3), and the survival plots exhibited typical upward concavity, resulting in β below 1.0. The β values for ClO_2 gas treatments were in the range between 0.46 and 0.79, indicating that the inhibitory effect of ClO_2 gas treatment increases slowly with treatment time. If β is above 1.0, the survival plots should have downward concavity and the inhibitory effect would have increased rapidly with treatment time. In addition, the δ parameters decreased with the increase of ClO_2 gas concentration. In particular, the δ parameter for the 10 ppmv ClO_2 gas treatment against *E. coli* O157:H7 and *S.* Typhimurium was 21.45 and 22.54 min, respectively, which means the time required to decrease pathogenic bacteria 10-fold, whereas δ for the 50 ppmv ClO_2 gas treatment was 7.27 and 6.67 min, respectively.

The germicidal effect of ClO_2 gas is based on its high oxidation capacity, which readily damages bacterial cell membranes. In addition, the gas inhibits enzyme activity in pathogenic bacteria [19]. To achieve microbial inactivation, two strategies are commonly applied: either a high concentration short time (HCST) or a low concentration long time (LCLT) treatment [16, 26, 27]. A HCST treatment is favorable for rapid inactivation of pathogenic bacteria on fresh produce. However, high concentrations of gaseous ClO_2 may be explosive and should be handled carefully [29]. Because of this limitation, the LCLT treatment provides an alternative method for inactivation of pathogenic bacteria. In addition, low concentration gaseous ClO_2 (0–0.12 mg/l) was not toxic in long-term animal experiments [30]. Based on these results, we suggest that a 50 ppmv ClO_2 gas treatment is an effective approach for improving the microbial safety of paprika.

Table 1. Weibull model parameters for inactivation of *E. coli* O157:H7 and *S.* Typhimurium, inoculated on paprika, by ClO_2 gas treatment.

Concentration (ppmv)	Microorganism	RMSE[a]	R^2	δ[b]	β[c]
10	*E. coli* O157:H7	0.03	0.99	21.45	0.76
	S. Typhimurium	0.01	0.99	22.54	0.74
20	*E. coli* O157:H7	0.04	0.99	15.78	0.46
	S. Typhimurium	0.10	0.97	14.03	0.67
30	*E. coli* O157:H7	0.05	0.98	11.39	0.79
	S. Typhimurium	0.08	0.98	12.47	0.65
40	*E. coli* O157:H7	0.10	0.98	9.64	0.73
	S. Typhimurium	0.09	0.99	7.82	0.61
50	*E. coli* O157:H7	0.03	0.99	7.27	0.62
	S. Typhimurium	0.11	0.99	6.67	0.74

[a]RMSE, root mean square error.

[b]δ, the first reduction time that causes 1 log reduction of microbial population.

[c]β, shape parameter.

Effects of Aqueous ClO_2 and Combined Treatment

We next tested whether the germicidal effect of gaseous ClO_2 treatment could be enhanced when combined with aqueous ClO_2 (Table 2). This is important, since the persistence of injured but viable cells on fresh produce as a result of inadequate sanitizing processes can affect food safety [31]. Such combined treatment is referred to as a hurdle technology, as the repeated stress to which the bacteria are exposed significantly reduces the likelihood that viable cells remain.

The initial microbial populations of *S.* Typhimurium and *E. coli* O157:H7 inoculated on paprika were 5.06 and 5.68 log CFU/g, respectively. For the combined treatment, paprika was first treated with gaseous ClO_2 and then with aqueous ClO_2. The sequence of this combined treatment

Table 2. Effect of the combined treatment of aqueous and gaseous ClO_2 on the inactivation of *E. coli* O157:H7 and *S.* Typhimurium inoculated on paprika.

(log CFU/g)

Treatment[a]		*E. coli* O157:H7	*S.* Typhimurium
Control		5.68 ± 0.35A	5.06 ± 0.18A
Water washing		5.04 ± 0.07A	4.40 ± 0.40B
Aqueous ClO_2 50 ppm		3.82 ± 0.17B	3.69 ± 0.26C
Combined treatment	5 min	3.30 ± 0.75BC	2.86 ± 0.24D
	10 min	3.02 ± 0.52CD	2.59 ± 0.47DE
	20 min	2.60 ± 0.07D	2.33 ± 0.29E
	30 min	1.57 ± 0.68E	1.45 ± 0.52F

[a]Combined treatment, aqueous ClO_2 50 ppm + gaseous ClO_2 50 ppmv.

Means with different letters (A–F) in the same column are significantly ($p < 0.05$) different.

Data are presented as the mean ± standard deviation ($n = 3$).

was determined based on preliminary experiments. If aqueous ClO_2 treatment is performed first, the subsequent water residue can cause uneven distribution of ClO_2 gas on the paprika surface due to the high solubility of this gas in water [14]. In particular, under conditions of high relative humidity, ClO_2 gas can easily reach the paprika surface, affecting the inhibitory effect against pathogenic bacteria. Aqueous ClO_2 treatment alone decreased the populations of *S.* Typhimurium and *E. coli* O157:H7 by 1.37 and 1.86 log CFU/g, respectively, compared with those in the control (Table 2). This result is similar to that of Kim *et al*. [13], who reported that aqueous 50 ppm ClO_2 treatment for 5 min reduced the population of *E. coli* O157:H7 and *S.* Typhimurium inoculated on broccoli sprouts by 1.66 and 1.54 log CFU/g, respectively. In contrast, the combined treatment of 50 ppmv ClO_2 gas for 5 min and aqueous ClO_2 reduced the populations of *E. coli* O157:H7 and *S.* Typhimurium by a further 0.52 and 0.83 log CFU/g, respectively, compared with the aqueous ClO_2 treatment alone. These results indicate that gaseous ClO_2 treatment for 5 min had an additional antimicrobial effect. The inhibitory effect increased proportionally with the time of treatment. For example, after 10 min, the population of *E. coli* O157:H7 was reduced by 2.66 log CFU/g, compared with that in the control, following the combined treatment in which 50 ppm ClO_2 gas was used. The decrease was even greater (3.08 log CFU/g) after a 20 min treatment. The maximum germicidal effect was observed after a 30 min combination treatment in which the ClO_2 gas concentration was 50 ppmv, where the reduction of *E. coli* O157:H7 cells was 4.11 log CFU/g. The trend was similar for *S.* Typhimurium. The combined treatment of 50 ppmv ClO_2 gas for 30 min decreased the population of *S.* Typhimurium by 3.61 log CFU/g, compared with that in the control.

The inhibitory effect of the combined treatment in this study increased rapidly with treatment time. These results were also observed in the study of Park and Kang [32], where the inhibitory effect by the combined treatment of gaseous ClO_2 (10 ppmv) and peracetic acid (80 ppm) increased rapidly with treatment time. The combined treatment for 5, 10, 15, and 20 min reduced the population of *E. coli* O157:H7 inoculated on tomatoes by 1.00, 2.60, 3.70, and 5.10 log CFU/g, respectively. In this study, the inhibitory effect of ClO_2 gas single treatment increased slowly with treatment time, whereas the inhibitory effect of the combined treatment increased rapidly. However, it should be noted that an excessive treatment time might affect the food quality negatively. Therefore, the combination treatment of 50 ppm aqueous and gaseous ClO_2 for 30 min is suggested to improve the microbial safety and quality of paprika in this study.

Hurdle technology depends on the type of treatments [33]. Generally, employing two agents with distinct mechanisms of action is preferable, as this lowers the possibility that bacterial resistance will develop. Indeed, using two agents that act in the same manner has been demonstrated to be less effective [32]. Despite this, we find that treatment with gaseous and aqueous ClO_2 treatment is still more effective than either agent alone, and may thus be a useful technique to improve the microbial safety of paprika.

Quality Measurements of Paprika Samples

Quality measurements of paprika samples after the combined treatment of gaseous and aqueous ClO_2 were also taken (Table 3). To apply the combined treatment to food commodities, it is vital that the quality of fresh produce is maintained; this is often achieved by exposing bacteria to a high dose of exogenous stress [34]. However, excessive oxidative stress damages membranes, enzymes, and DNA of plant cells, causing discoloration, loss of nutrition value, and reduced shelf-life in fresh produce [35, 36]. Reyes *et al*. [37] reported that the response sensitivity to oxidative stress depends on the type of fresh produce. In the current study, no adverse effect on paprika quality was observed, and the color and chroma values of paprika samples increased gradually in the expected manner during storage due to postharvest ripening. The hue angle value also reflects the color of samples and ranges from 0^{o} (pure red) to 270^{o} (pure blue) [25]. These values of paprika were maintained at 26^{o} during storage. Although ClO_2 treatment is highly oxidative, it did not affect the color values of paprika samples. Mahmoud and Linton [38] reported that 183 ppmv gaseous ClO_2 treatment for 10 min affected the color values of iceberg lettuces after 7- day storage at 4°C, as its redness value increased. This result indicates that low concentrations of gaseous ClO_2 are preferable for decontamination of food commodities. Our current results meet this criterion. In addition, the treatment did not affect the hardness or total soluble solid content (TSS) of paprika. Vitamin C values did gradually decrease during storage of untreated paprika, but this was not accelerated by the combined treatment. This is similar to the work of Du *et al*. [39], who reported that a 50 ppmv gaseous ClO_2 sachet did not affect the vitamin C and TSS contents of green bell peppers after 40-day storage at 4°C. Furthermore, Aday *et al*. [40] observed no change in strawberry quality after treatment with 9 ppm aqueous ClO_2, even after a 25-day storage at 4°C. Together, these observations show that combined treatment consisting of

Table 3. Change in the quality parameters of paprika after the combined treatment, during storage at 8°C.

Quality parameters		Treatment[a]	Storage time (days)		
			0	3	7
Color values[b]	*L*	Control	30.31 ±1.31^{Aa}	30.53 ± 0.63^{Aa}	30.22 ± 0.75^{Aa}
		Combined treatment	30.30 ± 1.08^{Aa}	30.52 ± 0.59^{Aa}	30.30 ± 0.46^{Aa}
	a	Control	21.00 ± 0.98^{Ab}	21.01 ± 0.66^{Ab}	22.86 ± 0.82^{Aa}
		Combined treatment	20.89 ± 0.93^{Ab}	21.02 ± 0.19^{Ab}	22.82 ± 1.19^{Aa}
	b	Control	10.37 ± 1.06^{Ab}	10.43 ± 0.79^{Ab}	11.18 ± 0.76^{Aa}
		Combined treatment	10.10 ± 0.91^{Ab}	10.31 ± 0.68^{Ab}	11.16 ± 0.98^{Aa}
	C	Control	23.83 ± 0.96^{Ab}	23.63 ± 0.48^{Ab}	25.45 ± 1.05^{Aa}
		Combined treatment	23.71 ± 0.71^{Ab}	23.45 ± 0.19^{Ab}	25.45 ± 1.46^{Aa}
	h^o	Control	26.32 ± 0.26^{Aa}	26.66 ± 1.16^{Aa}	26.03 ± 0.88^{Aa}
		Combined treatment	26.56 ± 0.26^{Aa}	26.30 ± 0.70^{Aa}	26.21 ± 1.01^{Aa}
Hardness (N)		Control	73.32 ± 6.64^{Aa}	73.07 ± 6.63^{Aa}	72.65 ± 8.28^{Aa}
		Combined treatment	73.71 ± 9.30^{Aa}	72.95 ± 9.53^{Aa}	72.12 ± 13.19^{Aa}
Total soluble solid (%)		Control	6.72 ± 0.38^{Aa}	6.75 ± 0.26^{Aa}	6.74 ± 0.14^{Aa}
		Combined treatment	6.73 ± 0.12^{Aa}	6.75 ± 0.13^{Aa}	6.73 ± 0.20^{Aa}
Vitamin C (mg/100 g)		Control	4,125.89 ± 121.05^{Aa}	3,614.98 ± 140.98^{Bb}	3,226.01 ± 29.91^{Ac}
		Combined treatment	3,964.72 ± 13.98^{Aa}	3,890.21 ± 97.66^{Aa}	3,165.37 ± 110.83^{Ab}

[a]Combined treatment, aqueous ClO_2 50 ppm + gaseous ClO_2 50 ppmv.

[b]C, chroma; h^o, hue angle.

Means with different letters in the same column (A–B) and row (a–b) are significantly ($p < 0.05$) different. Data are presented as the mean ± standard deviation ($n = 3$).

low concentrations of aqueous and gaseous ClO_2 is a useful technique for ensuring the microbiological safety and quality of paprika.

In conclusion, the combined treatment of gaseous and aqueous ClO_2 effectively reduced the populations of *E. coli* O157:H7 and *S.* Typhimurium inoculated on paprika. The overall qualities of paprika samples were not affected during storage following the combined treatment. Considering that the combined treatment of low concentrations of gaseous and aqueous ClO_2 has never been studied previously, these results clearly suggest that the combination treatment of 50 ppm aqueous and gaseous ClO_2 for 30 min can be used as an emerging hurdle technology to ensure the microbial safety of paprika without impairing quality.

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