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Inactivation of foodborne pathogens on alfalfa and radish seeds by sequential treatment with chlorine dioxide gas and dry heat

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ABSTRACT

This study was conducted to evaluate the antimicrobial effect of sequential treatment with chlorine dioxide (ClO_2) gas and dry heat against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium on alfalfa and radish seeds. Inoculated alfalfa and radish seeds were treated with 150 ppmv of ClO_2 gas for 1 h followed by 70 or 80 °C dry heat for 0, 1, 3 or 5 h. Dry heat treatment alone at 80 °C for 5 h resulted in 3.08 and 3.23 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds, respectively. ClO_2 gas treatment alone for 1 h resulted in 1.22–1.58 and 1.45 to 1.61 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Subsequent dry heat treatment (80 °C) for 5 h caused more than 5.32 and 5.29 log reduction of *E. coli* O157:H7 and *S.* Typhimurium, respectively. On radish seeds, dry heat treatment at 80 °C for 5 h resulted in 2.49 and 2.27 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively, and sequential treatment with ClO_2 gas and dry heat (80 °C) for 5 h caused 4.38 and 4.11 log reduction of *E. coli* O157:H7 and *S.* Typhimurium, respectively. The germination rate of seeds did not significantly decrease after sequential treatment except for radish seeds sequentially treated with ClO_2 gas and dry heat at 80 °C for 5 h.

1. Introduction

The consumption of sprouted seed products has increased in recent decades because of their nutritional value (Nei, Latiful, Enomoto, Inatsu, & Kawamoto, 2011; Xiao, Nou, Luo, & Wang, 2014). However, consumption of sprouts has been associated with a number of foodborne disease outbreaks. Most outbreaks have been associated with alfalfa, mung bean, and radish seed sprouts contaminated with *Escherichia coli* O157:H7 and *Salmonella* spp. (Bang, Kim, Kim, Beuchat, & Ryu, 2011b; NACMCF, 1999). In the U. S., there were at least 33 outbreaks linked to sprouted seed products between 1998 and 2010 (Dechet et al., 2014). Sprouts from an organic farm were determined as a source of an *E. coli* O104:H4 outbreak in 2011, Germany, which caused about 4000 infections and 53 deaths (Uphoff et al., 2014). Also, sprouts-associated outbreaks in Japan (Watanabe et al., 1999), Canada (CFIA, 2005), Sweden, Finland, and Denmark (Emberland et al., 2007) have been reported.

Although the contamination levels of seeds are usually low in numbers (Jaquette, Beuchat, & Mahon, 1996; Taormina, Beuchat, & Slutsker, 1999), the optimal temperature and humidity condition during the sprouting process are favorable for the rapid growth of pathogens (Feng, Churey, & Worobo, 2007; Stewart, Reineke, Ulaszek, Fu, & Tortorello, 2001; Taormina & Beuchat, 1999). Therefore, assuring the absence of foodborne pathogens on seeds is regarded as a critical control point (Weiss & Hammes, 2005). The U.S. Food and Drug Administration (FDA) recommends the application of 20,000 ppm of free chlorine from calcium hypochlorite during seed soaking (NACMCF, 1999). However, the effect of chlorine treatment has been reported to be limited (Holliday, Scouten, & Beuchat, 2001; Lang, Ingham, & Ingham, 2000; Montville & Schaffner, 2004). Also, chlorine can combine with organic substances in water leading to the formation of harmful by-products such as trihalomethanes (THMs) (Dunnick & Melnick, 1993).

The application of dry heat treatments has been one of the most studied methods for the decontamination of seeds. Dry heat treatment could be easily applied in a seed sanitizing process.

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However, dry heat treatment alone requires 1–6 days to achieve more than 5 log reductions of *E. coli* O157:H7 and *Salmonella* (Feng et al., 2007; Hu, Churey, & Worobo, 2004; Neetoo & Chen, 2011). Combining two or more types of treatments, either simultaneously or in sequence, has been applied to achieve greater reductions in numbers of pathogens on seeds. Combined treatments of dry heat and other chemical or physical treatments have been studied including ethanol, oxalic acid, electrolyzed water (Bari, Nei, Enomoto, Todoriki, & Kawamoto, 2009), high hydrostatic pressure (Neetoo & Chen, 2011; Neetoo, Pizzolato, & Chen, 2009), and gamma irradiation (Bari et al., 2009), and each showed different levels of inactivation.

Bang, Kim, Kim, Beuchat, and Ryu (2011c) reported sequential treatments with aqueous chlorine dioxide (ClO_2) followed by drying, and dry heat eliminated *E. coli* O157:H7 on radish seeds without decreasing the germination rate. ClO_2 is a strong oxidizer with a broad antimicrobial spectrum (Beuchat, 1998; Trinetta, Vaid, Xu, Linton, & Morgan, 2012), and ClO_2 gas is more effectively inactivates pathogens than aqueous ClO_2 due to its penetrability (Han, Linton, Nielsen, & Nelson, 2001). The objective of this study was to investigate the efficacy of sequential treatments of ClO_2 gas and dry heat for eliminating *E. coli* O157:H7 and *Salmonella* Typhimurium on alfalfa and radish seeds, and evaluate the impact of this sequential treatment on seed viability.

2. Materials and methods

2.1. Bacterial strains and cell suspension

Stock cultures of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890) and *S.* Typhimurium (ATCC 19585, ATCC 43971, and DT 104) were obtained from the bacterial culture collection of the School of Food Science, Seoul National University (Seoul, South Korea). Working cultures were prepared by streaking stock cultures onto tryptic soy agar (TSA; Difco, BD, Sparks, MD, USA), incubating at 37 °C for 24 h and storing at 4 °C. A single colony of each strain of *E. coli* O157:H7 and *S.* Typhimurium were inoculated individually into 5 ml of tryptic soy broth (TSB; Difco) and incubated at 37 °C for 24 h. Overnight culture (1 ml) of each strain was spread onto three TSA plates to produce a bacterial lawn, which was followed by incubation at 37 °C for 24 h. Ten ml of buffered peptone water (BPW; Difco) was added to each plate, and cell suspensions were made by rubbing the agar surface with a sterile swab (3M pipette swab, 3M Korea Ltd.) to dislodge cells. Cell suspensions (ca.10^{11}–10^{12} CFU/ml) were combined to construct a mixed culture cocktail, and combined with BPW to yield 200 ml of culture cocktail in total with a final cell population of ca. 10^9–10^{10} CFU/ml.

2.2. Inoculation of seeds

Alfalfa and radish seeds (Danong Co. Ltd., Gyeonggi-do, South Korea) were purchased from a local market (Seoul, South Korea) and stored at room temperature. Seeds used in this study were previously screened to ensure no presumptive *E. coli* O157:H7 or *Salmonella*-like colonies were recovered from un-inoculated samples. Two hundred grams of alfalfa or radish seeds were added to 200 ml of mixed cell suspension and gently agitated for 10 min. The cell suspension was drained, and the seeds were placed onto aluminum foil and dried in a laminar flow hood at 22 ± 1 °C for 18 h before use in experiments. Moisture content (dry basis) of seeds was measured using a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH).

2.3. Sequential treatment with ClO_2 gas and dry heat

ClO_2 gas treatment was conducted in a treatment system described previously (Park & Kang, 2015). ClO_2 gas was generated by a ClO_2 gas system (Daehan E&B, Goyang-si, South Korea) and generated ClO_2 gas was introduced into the treatment chamber. The ClO_2 gas in the treatment chamber was continuously circulated using a ring blower (HRB-101, Hwanghae electronic, Incheon, South Korea) and the concentration of ClO_2 gas was continuously monitored and controlled using a ClO_2 gas transmitter (ATi F12, Analytical Technology, U.K.). Ultrasonic nebulizer (H-C976, Osungsa, Changwon-si, South Korea) was used to control RH in the treatment chamber. RH and temperature in the treatment chamber was continuously monitored with a thermohygrometer (YTH-600, Uins, Seoul, South Korea).

A tray (polypropylene, diameter × height, 180 × 75 mm) containing inoculated alfalfa or radish seeds (80 g) was fixed onto a vortex mixer (WiseMix VM-10; Daihan Wisd., Gangwon, South Korea) and placed in the treatment chamber. Seeds were treated with 150 ppmv ClO_2 gas at 22 ± 1 °C for 1 h. During treatment, seeds in the tray were continuously rotated by the vortex mixer at 1000 rpm and relative humidity (RH) of the treatment chamber was adjusted with distilled water to 90 ± 2%. Seeds used for dry heat treatment alone were treated the same way but without ClO_2 gas to adjust moisture content before dry heat treatment. Treated seeds were divided into 10 g portions and placed onto polystyrene trays (length × width × height, 89 × 89 × 25 mm). These trays were transferred to an oven (OV-11, JEIO Tech, Seoul, Korea Rep.) with the temperature set at 70 or 80 °C. Alfalfa or radish seeds were heated for 0, 1, 3, or 5 h.

2.4. Enumeration of E. coli O157:H7 and S. Typhimurium

Each treated seed sample (10 g) was immediately transferred to a sterile filter stomacher bag (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 90 ml of neutralizing buffer (Difco), and homogenized for 2 min with a stomacher (EASY MIX, AES Chemunex, Rennes, France). After homogenization, a 1 ml aliquot of sample was tenfold serially diluted in 9 ml of BPW, and 0.1 ml of sample or diluent was spread-plated onto Sorbitol MacConkey agar (SMAC; Difco) and Xylose Lysine Desoxycholate agar (XLD; Difco) for the enumeration of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Where low numbers of surviving cells were anticipated, 250 μl of undiluted sample was plated onto each of four plates. All plates were incubated at 37 °C for 24 h, and colonies were counted.

Phenol red agar base (Difco) with 1% sorbitol (SPRAB) was used for the resuscitation of injured *E. coli* O157:H7 (Rhee, Lee, Hillers, Mccurdy, & Kang, 2003). One hundred microliter of sample or diluent was spread-plated onto SPRAB and incubated at 37 °C for 24 h. Injured cells of *S.* typhimurium were enumerated using the overlay (OV) method proposed by Kang and Fung (Kang & Fung, 1999, 2000). One hundred microliter of sample or diluent was spread-plated onto TSA and incubated at 37 °C for 2 h to allow injured cells to resuscitate before overlaying with 7 ml of XLD (OV-XLD). The plates were incubated at 37 °C for 22 h after the overlay solidified. Where low numbers of surviving cells were anticipated, 250 μl of undiluted cell suspension was plated onto four plates of each respective medium.

2.5. Determination of seed germination rate

Treated or untreated (control) seeds ($n = 200$) were placed on sterile cheesecloth in petri dishes (90 mm diameter), and periodically provided with distilled water to maintain the amount of moisture required for sprouting. The seeds were incubated at room

temperature (22 ± 1 °C) for 5 days. Only seeds with a hypocotyl protruding were counted as a sprout, and ruptured or swollen seeds were not counted. The germination rate was determined as the proportion of sprouted seeds to the total number of seeds. Experiments were performed in triplicate.

2.6. Statistical analysis

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

3. Results

3.1. Effects of sequential treatment with ClO_2 gas and dry heat on populations of E. coli O157:H7 and S. Typhimurium

Dry heat treatment at 70 °C for 1 h caused significant ($p < 0.05$) log reductions of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds, but thereafter, no further significant ($p > 0.05$) reductions occurred during 5 h treatment (Table 1). Dry heat treatment at 70 °C for 5 h resulted in 2.39 and 1.82 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Exposure to 150 ppmv of ClO_2 gas for 1 h resulted in 1.22 and 1.45 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Subsequent dry heat treatment (70 °C) for 5 h caused 4.17 and 3.70 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Dry heat treatment at 80 °C for 5 h resulted in 3.08 and 3.23 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds, respectively (Table 2). ClO_2 gas treatment followed by dry heat treatment (80 °C) for 5 h caused more than 5.32 and 5.29 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively.

Dry heat treatment at 70 °C was less effective at reducing populations of *E. coli* O157:H7 and *S.* Typhimurium on radish seeds compared to alfalfa seeds, showing 1.72 and 1.43 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively, after 5 h treatment (Table 3). Sequential treatment with ClO_2 gas and dry heat treatment caused 3.33 and 2.69 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Dry heat treatment at 80 °C for 5 h resulted in 2.49 and 2.27 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. ClO_2 gas treatment followed by dry heat treatment (80 °C) for 5 h caused 4.38 and 4.11 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively (see Table 4).

Dry heat treatment alone and sequential treatment with ClO_2 gas and dry heat generally produced sublethally injured cells of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa or radish seeds. However, sequential treatment with ClO_2 gas and dry heat (70 or 80 °C) for 5 h did not produce sublethally injured cells of those pathogens on alfalfa or radish seeds.

3.2. Germination rate of seeds after sequential treatment with ClO_2 gas and dry heat

The germination rates of alfalfa and radish seeds after sequential treatments with ClO_2 gas and dry heat are shown in Figs. 1 and 2. The germination rates of alfalfa seeds treated with dry heat alone (70 and 80 °C) or sequential treatment with ClO_2 gas and dry heat (70 and 80 °C) were not significantly ($p > 0.05$) different from those of untreated seeds. Also, the germination rates of radish seeds after all treatments except for sequential treatment with ClO_2 gas and dry heat (80 °C) for 5 h were not significantly ($p > 0.05$) different from those of untreated seeds. The germination rate of radish seeds sequentially treated with ClO_2 gas and dry heat at 80 °C for 5 h decreased to 86.4% which was significantly ($p < 0.05$) different from that of untreated seeds.

4. Discussion

The FDA recommends achieving a 5-log reduction of pathogens on seeds used for sprout production (NACMCF, 1999). In the present study, 150 ppmv of ClO_2 gas treatment for 1 h resulted in 1.22–1.61 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds, and 0.82 to 1.16 log reductions on radish seeds. Annous and Burke (2015) reported exposure to ClO_2 gas (2 mg/l) for 6 h resulted in only 1.5 log reduction of *Salmonella* Montevideo on mung bean seeds. This means that ClO_2 gas treatment alone may be inadequate for controlling foodborne pathogens on seeds. Also, in this study, *E. coli* O157:H7 and *S.* Typhimurium populations on alfalfa or radish seeds decreased by 1.43–2.39 CFU/g after dry heat treatment at 70 °C for 5 h, and 2.27 to 3.23 log CFU/g after dry heat treatment at 80 °C for 5 h. Although dry heat treatment at 80 °C for 5 h achieved the greatest reductions in levels of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds (3.08 and 3.23 log CFU/g, respectively), further reduction is required to meet the demands of the US FDA.

To achieve greater reductions in pathogen populations on seeds, combining two or more types of treatment, either simultaneously or in sequence, has been evaluated previously (Bang et al., 2011b, c; Bari et al., 2009; Kim, Kim, Bang, Beuchat, & Ryu, 2010; Neetoo & Chen, 2011; Neetoo et al., 2009). Annous and Burke (2015) evaluated the efficacy of the simultaneous application of ClO_2 gas and

Table 1
Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds sequentially treated with 150 ppmv ClO_2 gas and dry heat (70 °C).

Microorganism	Treatment	Medium	Log reduction (log CFU/g)			
			Dry heat time (h)			
			0 h	1 h	3 h	5 h
E. coli O157:H7	Dry heat alone	SMAC	−0.05 ± 0.07Aa[b]	1.93 ± 0.33Bb	2.12 ± 0.43Ba	2.39 ± 0.47Ba
		SPRAB	−0.07 ± 0.27Aa	0.98 ± 0.10Ba	1.47 ± 0.41BCa	1.80 ± 0.36Ca
	Dry heat + ClO_2 gas	SMAC	1.22 ± 0.39Ab	3.03 ± 0.15Bc	3.56 ± 0.32BCc	4.17 ± 0.49Cb
		SPRAB	1.04 ± 0.41Ab	2.79 ± 0.10Bc	2.86 ± 0.28Bb	3.84 ± 0.50Cb
S. Typhimurium	Dry heat alone	XLD	0.19 ± 0.19Aa	1.46 ± 0.47Bb	1.71 ± 0.33Bb	1.82 ± 0.36Bb
		OV-XLD	0.07 ± 0.54Aa	0.56 ± 0.42ABa	0.83 ± 0.24ABa	1.05 ± 0.49Ba
	Dry heat + ClO_2 gas	XLD	1.45 ± 0.58Ab	2.66 ± 0.41Bc	3.47 ± 0.21Cd	3.70 ± 0.21Cc
		OV-XLD	1.12 ± 0.42Ab	1.94 ± 0.23Bbc	2.77 ± 0.40Cc	3.31 ± 0.28Cc

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after treatment. Populations of *E. coli* O157:H7 before treatment were 6.03 and 6.11 log CFU/g on SMAC and SPRAB, respectively. Populations of *S.* Typhimurium before treatment were 6.29 and 6.40 log CFU/g on XLD and OV-XLD, respectively.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). For each pathogen species, means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table 2
Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds sequentially treated with 150 ppmv ClO_2 gas and dry heat (80 °C).

Microorganism	Treatment	Medium	Log reduction (log CFU/g)			
			Dry heat time (h)			
			0 h	1 h	3 h	5 h
E. coli O157:H7	Dry heat alone	SMAC	0.20 ± 0.26Aa[b]	2.23 ± 0.48Bb	2.51 ± 0.13BCb	3.08 ± 0.35Cb
		SPRAB	0.13 ± 0.15Aa	1.38 ± 0.16Ba	1.45 ± 0.32BCa	1.76 ± 0.02Ca
	Dry heat + ClO_2 gas	SMAC	1.58 ± 0.52Ab	4.51 ± 0.18Bc	5.25 ± 0.08Cd	>5.32Dc
		SPRAB	1.37 ± 0.18Ab	4.00 ± 0.26Bc	4.65 ± 0.46Cc	>5.34Dc
S. Typhimurium	Dry heat alone	XLD	0.38 ± 0.28Aab	1.78 ± 0.23Bb	2.71 ± 0.25Cb	3.23 ± 0.22Db
		OV-XLD	0.03 ± 0.39Aa	1.27 ± 0.14Ba	1.56 ± 0.35BCa	2.15 ± 0.40Ca
	Dry heat + ClO_2 gas	XLD	1.61 ± 0.42Ac	4.46 ± 0.15Bd	5.03 ± 0.15Cd	>5.29Dc
		OV-XLD	1.04 ± 0.31Abc	3.68 ± 0.29Bc	4.19 ± 0.07Cc	>5.32Dc

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after treatment. Populations of *E. coli* O157:H7 before treatment were 6.32 and 6.34 log CFU/g on SMAC and SPRAB, respectively. Populations of *S.* Typhimurium before treatment were 6.29 and 6.32 log CFU/g on XLD and OV-XLD, respectively.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). For each pathogen species, means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table 3
Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on radish seeds sequentially treated with 150 ppmv ClO_2 gas and dry heat (70 °C).

Microorganism	Treatment	Medium	Log reduction (log CFU/g)			
			Dry heat time (h)			
			0 h	1 h	3 h	5 h
E. coli O157:H7	Dry heat alone	SMAC	0.08 ± 0.12Aa[b]	0.94 ± 0.41Ba	1.27 ± 0.50BCab	1.72 ± 0.28Cb
		SPRAB	0.06 ± 0.07Aa	0.77 ± 0.24Ba	0.97 ± 0.57Ba	1.11 ± 0.12Ba
	Dry heat + ClO_2 gas	SMAC	0.82 ± 0.31Ab	2.18 ± 0.05Bc	2.55 ± 0.34Bc	3.33 ± 0.24Cc
		SPRAB	0.33 ± 0.15Aa	1.51 ± 0.32Bb	1.83 ± 0.10Bbc	2.87 ± 0.26Cc
S. Typhimurium	Dry heat alone	XLD	0.01 ± 0.16Aa	0.85 ± 0.20Ba	1.11 ± 0.46BCab	1.43 ± 0.25Cb
		OV-XLD	0.17 ± 0.41Aa	0.51 ± 0.39ABa	0.62 ± 0.25ABa	0.94 ± 0.24Ba
	Dry heat + ClO_2 gas	XLD	0.90 ± 0.24Ab	2.03 ± 0.09Bc	2.52 ± 0.08Cc	2.69 ± 0.13Cc
		OV-XLD	0.47 ± 0.30Aab	1.40 ± 0.25Bb	1.66 ± 0.28BCb	2.20 ± 0.32Cc

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after treatment. Populations of *E. coli* O157:H7 before treatment were 6.56 and 6.62 log CFU/g on SMAC and SPRAB, respectively. Populations of *S.* Typhimurium before treatment were 6.97 and 7.02 log CFU/g on XLD and OV-XLD, respectively.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). For each pathogen species, means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table 4
Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on radish seeds sequentially treated with 150 ppmv ClO_2 gas and dry heat (80 °C).

Microorganism	Treatment	Medium	Log reduction (log CFU/g)			
			Dry heat time (h)			
			0 h	1 h	3 h	5 h
E. coli O157:H7	Dry heat alone	SMAC	0.24 ± 0.16Aab[b]	1.09 ± 0.28Ba	1.81 ± 0.44Ca	2.49 ± 0.46Ca
		SPRAB	0.12 ± 0.14Aa	1.34 ± 0.30Ba	1.72 ± 0.37Ba	1.95 ± 0.47Ba
	Dry heat + ClO_2 gas	SMAC	0.91 ± 0.01Ac	3.34 ± 0.06Bb	4.12 ± 0.14Cc	4.38 ± 0.36Cb
		SPRAB	0.57 ± 0.29Ab	2.87 ± 0.31Bb	3.13 ± 0.44Bb	3.82 ± 0.31Bb
S. Typhimurium	Dry heat alone	XLD	0.06 ± 0.08Aa	1.00 ± 0.40Ba	1.54 ± 0.12Ca	2.27 ± 0.31Db
		OV-XLD	−0.08 ± 0.06Aa	0.77 ± 0.10Ba	1.11 ± 0.38BCa	1.48 ± 0.06Ca
	Dry heat + ClO_2 gas	XLD	1.16 ± 0.25Ac	3.41 ± 0.13Bc	3.69 ± 0.10BCc	4.11 ± 0.28Cc
		OV-XLD	0.77 ± 0.21Ab	2.63 ± 0.32Bb	2.77 ± 0.34BCb	3.60 ± 0.28Cc

[a] Log reduction = population (log CFU/g) before treatment − population (log CFU/g) after treatment. Populations of *E. coli* O157:H7 before treatment were 6.62 and 6.68 log CFU/g on SMAC and SPRAB, respectively. Populations of *S.* Typhimurium before treatment were 7.02 and 7.06 log CFU/g on XLD and OV-XLD, respectively.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). For each pathogen species, means with different lowercase letters within a column are significantly different ($p < 0.05$).

dry heat for reducing populations of *S.* Montevideo populations on mung bean seeds. Dry heat treatment (at 70 °C and 8% RH) for 4 h reduced *S.* Montevideo populations on mung bean seeds by 4.07 log CFU/g, and 4.77 log reduction was achieved when combination-treated with ClO_2 gas (3.5 mg/L). Only a marginal effect on microbial reduction, possibly due to low RH (8% RH), occurred during combination treatment of ClO_2 gas and dry heat. It is well known that the antimicrobial effect of ClO_2 gas increases with increasing RH (Han et al., 2001; Park & Kang, 2015). In the present study, sequential treatments with ClO_2 gas and dry heat were applied to inactivate *E. coli* O157:H7 and *S.* Typhimurium on alfalfa and radish seeds. Sequential treatments with ClO_2 gas and dry heat showed additive or synergistic effects on microbial reductions: the total microbial inactivation of the combined treatment was not significantly ($p > 0.05$) different from or significantly ($p < 0.05$) higher than the sum of individual treatments. It seems that ClO_2 gas treatment sublethally damaged pathogen cells, thus their sensitivity to the following dry heat treatment increased. Ryu and

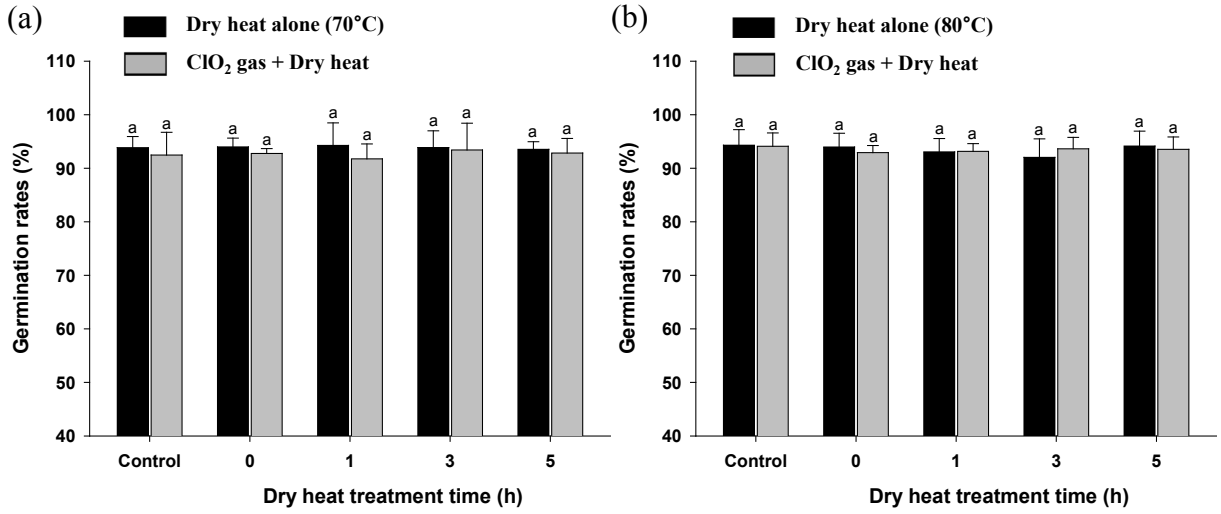

Fig. 1. Effect of sequential treatment of 150 ppmv ClO2 gas and 70 °C (a) and 80 °C (b) dry heat on germination of alfalfa seeds.

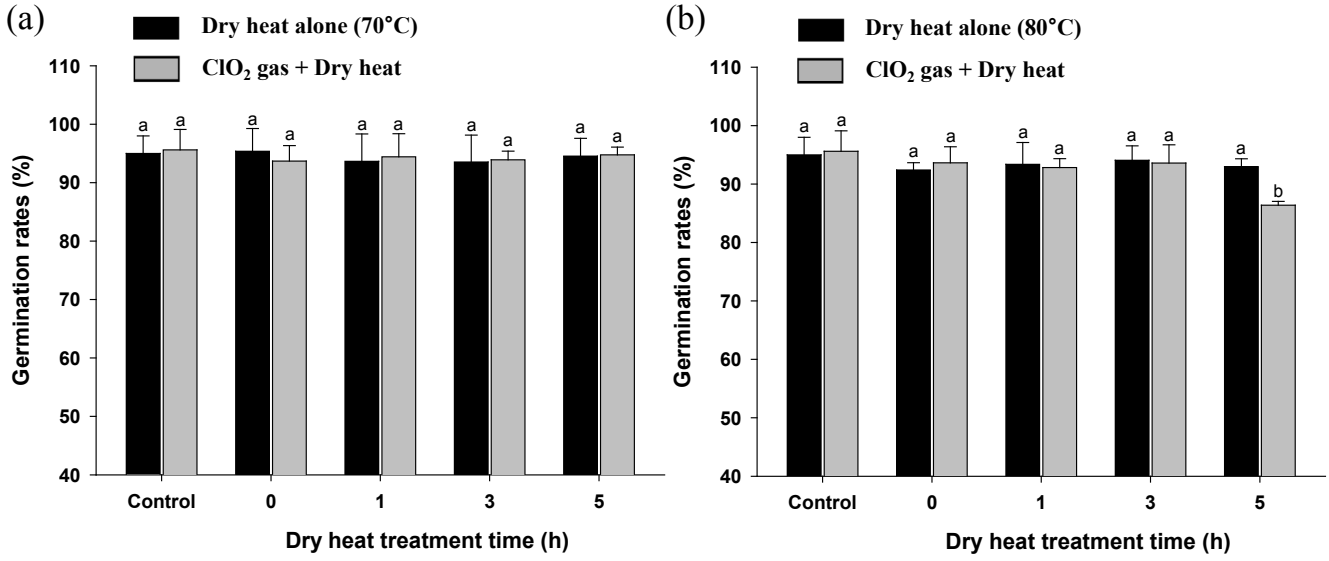

Fig. 2. Effect of sequential treatment of 150 ppmv ClO2 gas and 70 °C (a) and 80 °C (b) dry heat on germination of radish seeds.

Beuchat (2005) reported that ClO_2 treatment reduced heat resistance of *Bacillus cereus* spores by damaging them. Heat resistance of *E. coli* O157:H7 was reduced after exposure to aqueous ClO_2 treatment (Kim et al., 2010).

Bang et al. (2011a) evaluated the lethality of sequential treatments with aqueous ClO_2 and dry heat on microbial populations on radish seeds. *E. coli* O157:H7 on radish seeds treated with 500 μg/ml aqueous ClO_2 for 5 min and subsequently heated at 60 °C (at 23% RH) for 48 h decreased by more than 4.80 log CFU/g. However, the germination percentage decreased from 91.2 to 68.7% after this sequential treatment. Dry heat treatment could damage aqueous ClO_2-treated radish seeds which have high moisture content, resulting in a reduced germination rate. To preserve seed viability, a drying interval between aqueous ClO_2 and dry heat treatment has been introduced (Bang et al., 2011c). Sequential treatments with aqueous ClO_2 (500 μg/ml) for 5 min, drying (45 °C, 23% RH) for 24 h, and subsequent heating at 70 °C for 48 h eliminated *E. coli* O157:H7 on radish seeds without decreasing the germination rate. Although this sequential treatment effectively inactivated pathogens on seeds without negatively impacting seed germination, a long drying time is required. Conversely, a long drying interval is not required between ClO_2 gas and dry heat treatment because ClO_2 gas treatment does not greatly increase moisture content of seeds as does aqueous ClO_2. In the present study, when inoculated alfalfa and radish seeds were dried in a laminar flow hood for 18 h, their moisture contents were 6.76 and 6.97%, respectively. After ClO_2 gas treatment for 1 h at 90% RH, those levels increased slightly to 7.76 and 8.11%, respectively.

Single as well as sequential treatment with ClO_2 gas and dry heat was more effective in reducing *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds than those pathogens on radish seeds. Differences in pathogen reduction levels may be due to differences in surface characteristics of seeds. Bari et al. (2009) reported that dry heat treatment at 50 °C for 17 h could reduce numbers of *E. coli* O157:H7 on alfalfa seeds to below the detection limit, while a 24 h treatment was required for radish seeds. Fransisca and Feng (2012) found that several sanitizers including $Ca(OCl)_2$ and malic acid reduced *E. coli* O157:H7 87-23 more on radish seeds than on alfalfa

seeds. The R_a (arithmetic mean roughness) value of radish seeds (6.08 μm) was higher than that of alfalfa seeds (0.56 μm), and generally a negative correlation existed between the R_a values of seeds and microbial reduction by sanitizer treatment (Fransisca & Feng, 2012). This means the more rough the seed surface, the less pathogen reduction occurred in ClO_2 gas treatment. Also, Park and Kang (2017) reported the R_a and R_q (root mean squared roughness) values of produce surfaces were negatively correlated with the log reductions of foodborne pathogens after ClO_2 gas treatment. These results are in agreement with the results of this study.

5. Conclusion

In conclusion, greater microbial reductions were achieved by sequential treatment with ClO_2 gas and dry heat with short treatment times than those achieved by each single treatment. In the case of alfalfa seeds, 5-log reductions of *E. coli* O157:H7 and *S.* Typhimurium were achieved without decreasing the germination rate. Also, seed type could affect the antimicrobial efficacy of sequential treatment with ClO_2 gas and dry heat. Although ClO_2 gas and dry heat sequential treatment failed to completely inactivate *E. coli* O157:H7 and *S.* Typhimurium on alfalfa and radish seeds, these treatments might be a potential commercial intervention that could be optimized.

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