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Effect of sequential presoaking and chlorine dioxide treatment on the inactivation of pathogenic *Escherichia coli* and *Salmonella* spp. on sprout seeds

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Abstract

This study was conducted to evaluate the effect of sequential presoaking and chlorine dioxide (ClO_2) on the reduction of pathogenic *Escherichia coli* and *Salmonella* spp. in alfalfa. When unsoaked and presoaked alfalfa were exposed to 200 ppm ClO_2 for 15 min, the population of *E. coli* and *Salmonella* spp. on presoaked seeds reduced more than those on unsoaked seeds by 2.07 and 1.43 log CFU g^{-1} ($p < 0.05$), respectively. To determine the optimal concentration and treatment time to reduce pathogenic *E. coli* and *Salmonella* spp. in alfalfa seeds immersed in water for 5 h, presoaked seeds were exposed to four different concentrations of ClO_2 (50, 100, 150, and 200 ppm) for 15, 30, 45, and 60 min. The most effective condition to eliminate *E. coli* and *Salmonella* spp. from alfalfa seeds was sequential immersion in water for 5 h and 200 ppm ClO_2 treatment for 1 h. After the optimal condition was applied to eight kinds of sprout seeds, the pathogens were completely inactivated in all seeds, except radish seeds. Growth of pathogenic *E. coli* and *Salmonella* spp. during sprouting after ClO_2 treatment of alfalfa seeds was also completely inactivated. However, the germination rate of seeds did not significantly decrease after ClO_2 treatment. In addition, ClO_2 residues were not present in any sprout during 3 days of cultivation. These results demonstrated that sequential presoaking and 200 ppm ClO_2 treatment is the optimal seed disinfection treatment to prevent foodborne diseases associated with sprout consumption.

Keywords: Chlorine dioxide, Pre-soaking, Pathogenic *Escherichia coli*, *Salmonella* spp., Sprout seed

Introduction

Sprouted seed is gaining popularity globally due to its health-promoting biocapacity, including the prevention of arteriosclerosis, alleviation of osteoporosis, and reduced risk of cardiovascular diseases [1, 2]. However, seed and sprout also have been recognized as a possible causative vehicle involved in a number of foodborne diseases and illnesses. In particular, alfalfa, mung bean, radish, and clover seeds or sprouts were reported as an important reservoir causing multi-state oubtreaks of *Escherichia coli* O157:H7 and various *Salmonella* serotype infections in Canada, Japan, United Kingdom, and the United States [3–5]. Sprouted seeds were demonstrated to be linked with at least 33 outbreaks between 1998 and 2010 in the United States, concomitantly with 1330 suspected patients [6]. Consumption of radish sprouts contaminated with Shiga toxin producing *Escherichia coli* O104:H4 (STEC) accounted for approximately 4 haemolytic uraemic syndromes (HUS) and 3 STEC cases in Germany,

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2011 [7]. An epidemiological and molecular genotyping study showed that a total of 45 cases of *Salmonella* Weltevreden infection originated from alfalfa sprouts in Sweden, Finland, and Denmark [8].

The optimal temperature and high amounts of moisture under sprouting or germination conditions may contribute to the rapid growth of either resident microflora or pathogenic bacteria [9]. Then, soaking seed sprouts in 20,000 ppm calcium hypochlorite prior to their cultivation is recommended by U.S. Food and Drug Administration [3]. However, previous studies have demonstrated that chlorine-based sanitizers are ineffective for removing or eliminating *E. coli* O157:H7 and *Salmonella* spp. inoculated on seed sprouts [10–12]. In a study conducted by Taormina et al. [9], the efficacy of chlorine-based sanitizers on the decontamination of *E. coli* O157:H7 and *Salmonella* spp. in seeds or sprouts is well-documented, and the authors highlighted that chlorine, NaOCl and $Ca(OCl)_2$ at 100–2000 ppm resulted in less than 10^{1-3} CFU/g reductions. In order to overcome this limitation, heating [1], packaging [13], ozone [14], organic acid [10[, ozonated water [15], electrolyzed oxidizing water [16], and ultraviolet irradiation [17] have been applied to develop an effective alternative for inactivating pathogenic bacteria on seed or sprout products. Nevertheless, the localization of pathogens in some cracks, scars or damaged areas on the surface can contribute to the decreased effectiveness of these sanitizing methods [18]. The potential internalization of *E. coli* O157:H7 and *Salmonella* spp. into the inferior tissue may minimize the contact with the organisms and sanitizers. Moreover, sequential presoaking in water and hydrostatic pressure (HHP; 600 MPa) reduced the number of *E. coli* O157:H7 and *Salmonella* spp. in seeds below the detection limits, but the germination potential of seeds also was decreased substantially [19]. Hence, there is an urgent need to develop an effective decontamination strategy, which does not affect the seed-viability. Chlorine dioxide is a derivative chlorine compound, thus it has not only inherits the properties of chlorine but also got the superior properties such as 10 time more soluble in water then chlorine, no taste and odour [20]. It can be use as an alternative disinfectant to chlorine. Therefore, the purpose of the present study was to evaluate the effect of presoaking seeds in water on the inactivation of pathogenic *E. coli* and *Salmonella* spp. on sprout seeds using ClO_2. The optimal conditions for sequential presoaking and ClO_2 treatment to *E. coli* and *Salmonella* spp. without substantially reducing the viability of seeds were established.

Materials and methods

Preparation of inocula and inoculation of seeds

Preliminarily, three strains of *E. coli* were isolated from irrigation water, kohlrabi, and radish sprouts harvested in Republic of Korea, 2018 and demonstrated to harbor e*aeA*, *ipaH*, and *stx2* via PCR assays. Three kinds of *Salmonella* Typhimurium strains (ATCC 4931, ATCC 19585, and NCCP 13697) were used in this study. Planting the inocula on a medium containing rifampicin allowed the suppression of growth of seed commensals; thus, rifampicin resistance was induced in all those strains. To obtain spontaneous rifampicin resistance, all strains were cultured in Luria–Bertani broth (BD Difco™, Sparks, MD, USA) supplemented with 50 μg mL^{-1} rifampicin (Biosesang, Seongnam, Gyeonggi-do, South Korea) at 37 °C for 18 h at 180 rpm. Each strain was then streaked on tryptic soy agar (TSA; Oxoid, Basingstoke, UK) supplemented with 50 μg mL^{-1} rifampicin (TSA-R) and incubated at 37 °C for 24 h followed by subculturing under identical conditions to isolate rifampicin-resistant strains. Colonies were then inoculated into 7 mL tryptic soy broth (TSB; Oxoid, Basingstoke, UK) supplemented with 50 μg mL^{-1} rifampicin (TSB-R) and enriched at 37 °C for 16 h at 180 rpm. Each enriched culture was centrifuged at 4000 rpm at 4 °C for 15 min. Cells were washed twice with 10 mL phosphate-buffered saline (PBS; Oxoid) and resuspended in 10 mL PBS. Each culture cocktail of pathogenic *E. coli* and *Salmonella* spp. was checked and adjusted at O.D. 600 by 1 ($\sim 10^9$ CFU mL^{-1}). Then, these cell suspensions were serially diluted at 1:10 until they reach 10^5 CFU mL^{-1} with PBS.

Nine different kinds of sprout seeds (Dongwoobio Co., Ltd., Gyeonggi-do, South Korea), including alfalfa (*Medicago sativa*), radish (*Radishes raphanistrum* subsp. *sativus*), red kohlrabi (*Brassica oleracea*), tatsoi (*Brassica rapa*), kohlrabi (*B. oleracea*), crimson clover (*Trifolium incarnatum* L.), cabbage (*B. oleracea*), red radish (*R. raphanistrum* subsp. *sativus*), and rapeseed (*Brassica napus* L.), were used in this study. All seeds used in the study were purchased from an agricultural material market in Jeollabuk-do, Republic of Korea and stored at 4 °C. The seeds were previously screened to ensure that no presumptive *E. coli* or *Salmonella*-like colonies were recovered from uninoculated samples. Each seed sample (450 g) was placed in 450 mL of each mixed cell suspension and gently agitated for 10 min. The cell suspension was drained and the seeds were placed on an aluminum foil and dried in a laminar flow hood at 22 ± 1 °C for 18 h before use in downstream experiments.

Preparation of ClO_2 solution

The ClO_2 solution was prepared immediately before use. Aqueous ClO_2 (50, 100, 150, and 200 ppm) was prepared by adding 1, 2, 3, and 4 g ClO_2 (Vibrex; Agranco, South Korea) in 1 L distilled water, respectively. In addition, NaOCl (Yuhan Co., Ltd., Gyeonggido, South Korea) was used as control in this study. NaOCl (200 ppm) was prepared by diluting 1.6 mL of 12.5% NaOCl to 1 L sterile distilled water. The concentration of free chlorine in NaOCl and ClO_2 solutions was determined using quick chlorine test kits (high-range chlorine dioxide; La Motte, USA) and chlorine detector (1200 colorimeter; La Motte), respectively.

Evaluate the synergic effect of presoaking on the inactivation of pathogenic *E. coli* and *Salmonella* spp. on alfalfa seeds using ClO_2

To determine the synergic effect of presoaking on the inactivation of pathogenic *E. coli* and *Salmonella* spp. on alfalfa seeds using ClO_2, ClO_2 was applied to unsoaked and presoaked seeds. Briefly, 10 g of each seed sample inoculated with pathogenic *E. coli* and *Salmonella* spp. were placed in 50 mL sterile plastic tube. Before ClO_2 treatment, seeds were immersed in 40 mL distilled water at room temperature for 5 h for soaking treatment, and then distilled water was drained. Then, 40 mL of 200 ppm NaOCl and four different concentrations of ClO_2 (50, 100, 150, and 200 ppm) were dispensed into a 50 mL plastic tube of the above samples, respectively. Each sample was treated for 15 min and the sanitizer was drained. Each treated seed was placed into a sterile plastic bag with 90 mL DE neutralizing broth (BD Difco™, Sparks, MD, USA) to neutralize the effect of the disinfectant. Bacteria were detached using a stomacher (Bagmixer 400VW; Interscience®, Paris, France) for 1 min at speed 7. Homogenates (1 mL) were serially diluted in 9 mL of 0.1% peptone water, and 0.2 mL of each dilution was spread on TSA-R. Plates were incubated at 37 °C for 24 h, and the colonies were counted manually.

Optimization of ClO_2 treatment condition in presoaked alfalfa seeds

To determine the most optimal concentration and treatment time to reduce pathogenic *E. coli* and *Salmonella* spp. in alfalfa seeds, inoculated seeds were exposed to four different concentrations of ClO_2 (50, 100, 150, and 200 ppm) for 15, 30, 45, and 60 min. Briefly, 10 g of the inoculated seeds were immersed in 40 mL distilled water for 5 h, followed by draining the water before ClO_2 treatment. Then, 40 mL of the four different concentrations of ClO_2 were added into 5 h presoaked seeds inoculated with pathogenic *E. coli* or *Salmonella* spp. individually. After each sample was treated for 15, 30, 45, and 60 min, the sanitizer was drained. Each treated seed unit was placed into a sterile plastic bag with 90 mL DE neutralizing broth (BD Difco™) to neutralize the effect of the disinfectant. Bacterial analysis was performed using the aforementioned method.

Growth of pathogenic *E. coli* and *Salmonella* spp. during sprouting after ClO_2 treatment of presoaked seeds

To estimate the microbiological risks of sprouts produced from alfalfa seeds treated with ClO_2, populations of pathogenic *E. coli* and *Salmonella* spp. in sprouts during cultivation were determined. The treatment steps have done follow above method, and the each treated seed was placed on a dish covered with Whatman paper No. 2 (Advantec Toyo Kaisha, Tokyo, Japan), 4 mL sterile distilled water was added, and sterile distilled water was then periodically provided to maintain the amount of moisture required for sprouting. Seeds were incubated at 25 °C for 3 days. Each sample was withdrawn at 3, 6, 9, 18, 24, 48, and 72 h. And bacterial population were also done using the aforementioned method.

For evaluation the present or absence of those pathogenes. the selective agar medium were used. Briefly, for pathogenic *E. coli*, after enrichment step in EC broth, the enriched sample was streaked onto an eosin methylene blue (EMB) agar (Oxoid) plate using a sterile disposable loop and incubated at 37 °C for 24 h. The typical morphology of *E. coli* developed green metal color on EMB plate. For the detection of *Salmonella* spp., after enrichment step in buffe peptone water (Oxoid), the enriched sample (0.1 mL) was inoculated into 10 mL Rappaport–Vassiliadis (Oxoid) enrichment broth before incubation at 42 °C for 24 h. A loopful of each enrichment culture was then streaked onto XLT-4 (Oxoid) agar. After incubation at 37 °C for 24 h, black colonies were picked for confirmation. Suspected colonies of *E. coli* and *Salmonella* spp. were identified by VITEK, which automatically identifies foodborne pathogens on a biochemical basis.

Application of optimal ClO_2 treatment conditions to various seeds

To verify whether the ClO_2 treatment condition optimized in alfalfa seeds is applicable to other sprout seeds, the performance of optimized condition was assessed using eight kinds of sprout seeds, including radish, red kohlrabi, tatsoi, kohlrabi, crimson clover, cabbage, red radish, and rapeseed. Eight kinds of seeds were inoculated with pathogenic *E. coli* and *Salmonella* spp. at initial levels of 2.0 and 4.0 log CFU g^{-1} using the aforementioned method. The optimal method of 5 h presoaking and major treatment (200 ppm ClO_2 for 60 min) was also applied to treat all kinds of seeds. Then, each set of

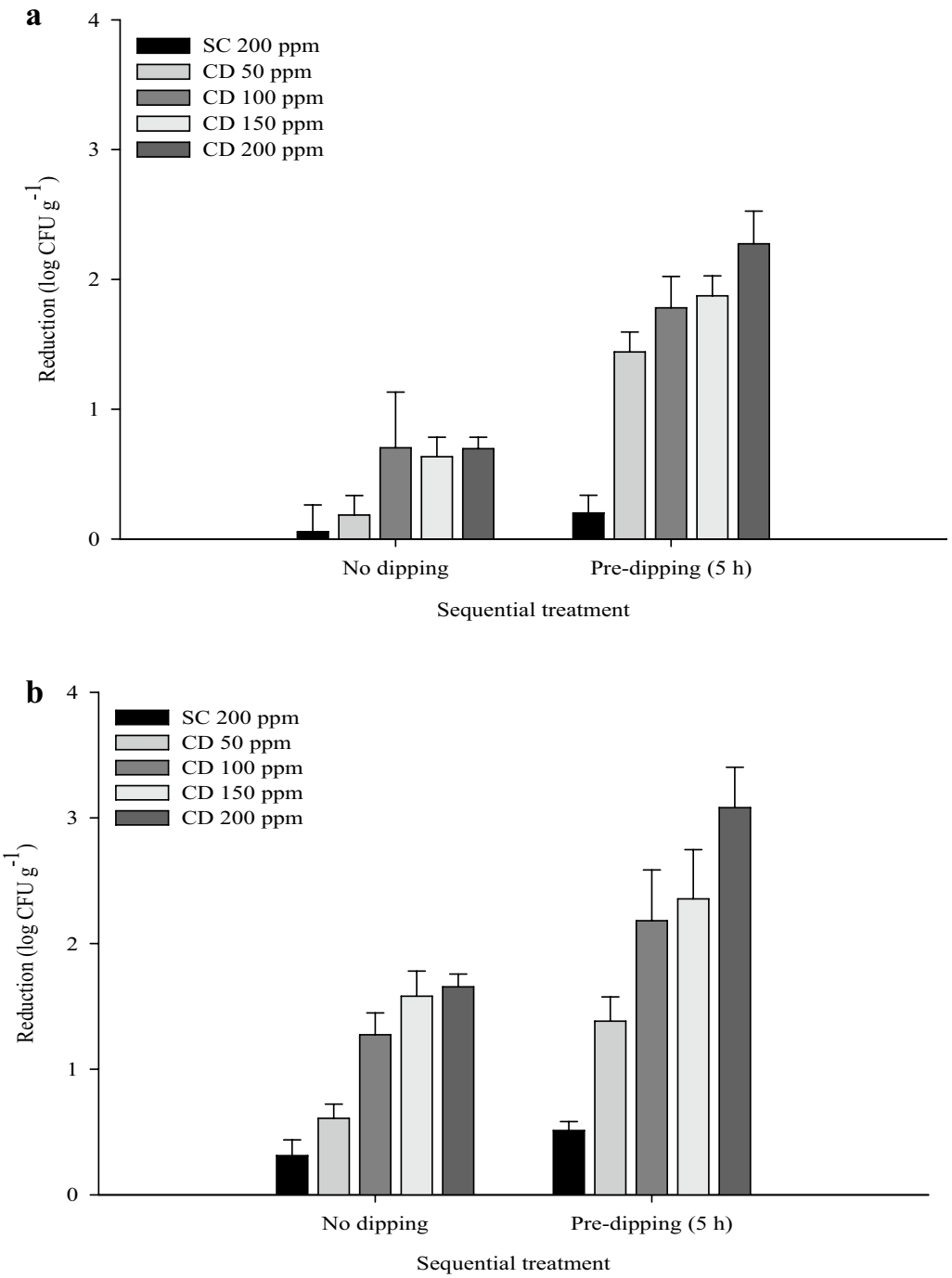

Fig. 1 Comparison between non-soaking and pre-soaking to the effect of ClO_2 treatment on the inactivation of pathogenic *E. coli* (**a**) and *Salmonella* spp. (**b**) in alfalfa seeds

seed was scattered on a plate with Whatman paper No. 2, 5 mL sterile distilled water was added, and sterile distilled water was periodically provided to maintain the amount of moisture required for sprouting. After cultivation for 3 days, each set of sprout was analyzed to determine the presence of pathogenic *E. coli* and *Salmonella* spp. in sprouts by the aforementioned method. Various sprout seeds treated with ClO_2 before cultivation were also analyzed for pathogenic *E. coli* and *Salmonella* spp.

Determination of seed germination rate

To investigate the effect of ClO_2 treatment on seed germination, treated or untreated seeds after soaking ($n = 100$) were placed on two plates of Petridish (90 mm in diameter) covered with Whatman paper No. 2. 4 to 6 mL of distilled water was added to each Petri dish, and distilled water was periodically provided to maintain the moisture content required for sprouting. The seeds were incubated at 25 °C for 3 days. Only seeds with a protruding hypocotyl were counted as a sprout every day, and ruptured or swollen seeds were not counted. The germination rate was determined as the proportion of sprout seeds to the total number of seeds. Experiments were performed in triplicate.

Determination of residue of ClO_2

The concentration of residual ClO_2 in seeds after sterilization was performed by iodometric analysis. Briefly, pulverized seeds were quickly placed into 50 mL polyethylene tubes containing 20 mL phosphate buffer followed by 2 g KI. After shaking, the sample was allowed to stand for 10 min under dark conditions, and the solution was titrated with 0.1 N $Na_2S_2O_3$.

Statistical analysis

This study was repeated twice with triplicate samples per replication. For statistical analysis, microbiological data were transformed to log CFU g^{-1} and analyzed using SAS software (SAS Institute, Inc., USA). All comparisons of means were performed using Tukey's multiple range test at alpha = 0.05.

Results

Evaluate the synergic effect of presoaking on the inactivation of pathogenic *E. coli* and *Salmonella* spp. on alfalfa seeds using ClO_2

Figure 1 shows the reduced populations of pathogenic *E. coli* and *Salmonella* spp. in unsoaked and presoaked alfalfa seeds after ClO_2 treatment. There were significant differences ($p < 0.05$) in the reduced number of bacteria among types of condition of seeds, pathogens, and sanitizers. After ClO_2 treatment, the reduced pathogen numbers in presoaked seeds were higher than those in unsoaked seeds. Furthermore, the reduced number of pathogenic *E. coli* was lower ($p < 0.05$) than those of *Salmonella* spp. However, there were no

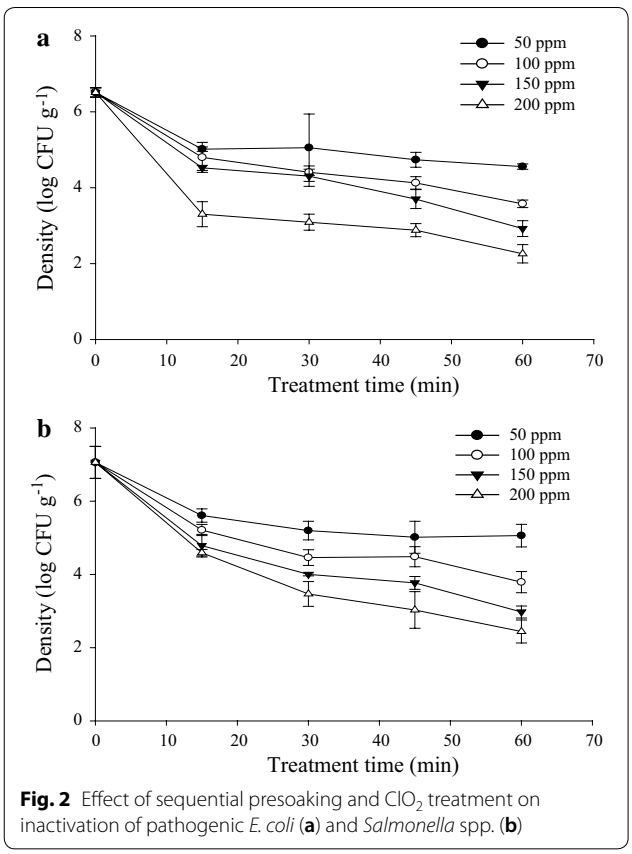

Fig. 2 Effect of sequential presoaking and ClO_2 treatment on inactivation of pathogenic *E. coli* (**a**) and *Salmonella* spp. (**b**)

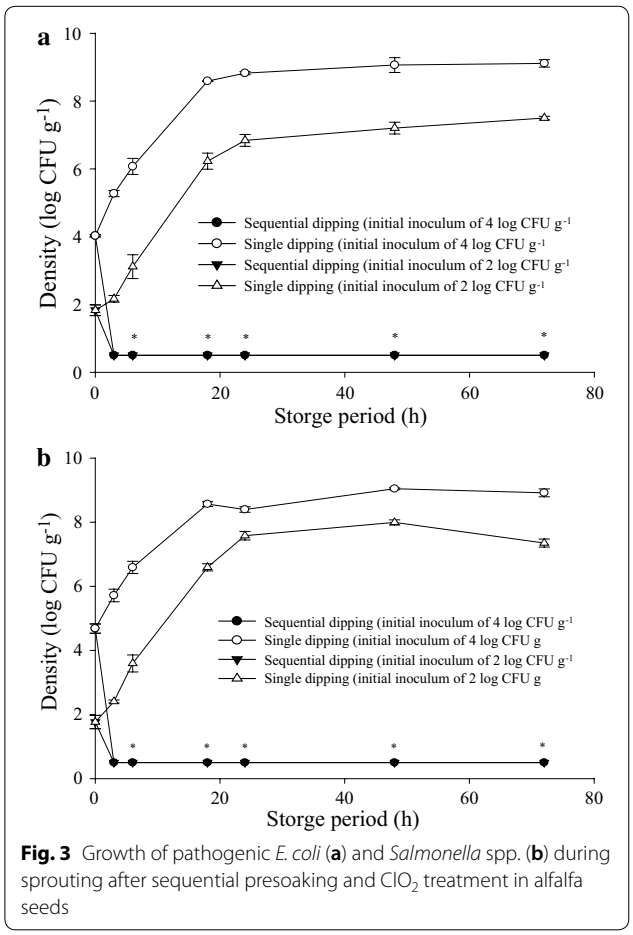

Fig. 3 Growth of pathogenic *E. coli* (**a**) and *Salmonella* spp. (**b**) during sprouting after sequential presoaking and ClO_2 treatment in alfalfa seeds

significant reductions ($p > 0.05$) in pathogen numbers in seeds treated with 200 ppm NaOCl used as control. As the concentration of ClO_2 increased from 50 to 200 ppm in presoaked seeds, the pathogen numbers decreased further. When alfalfa seeds were exposed to 50, 100, 150, and 200 ppm ClO_2 for 15 min, the numbers of pathogenic *E. coli* in presoaked seeds were reduced more ($p < 0.05$) than those in unsoaked seeds by 1.25, 1.07, 1.63, and 2.07 log CFU g^{-1}, respectively. Sequential presoaking and 50, 100, 150, and 200 ppm ClO_2 for 15 min caused 1.38, 2.18, 2.35, and 3.08 log CFU g^{-1} reductions of *Salmonella* spp., respectively. However, ClO_2 treatment alone was less effective at reducing the population of *Salmonella* spp. in alfalfa seeds compared to sequential presoaking and ClO_2 treatment, with 0.61, 1.27, 1.58, and 1.65 log CFU g^{-1} reductions of *Salmonella* spp., respectively. Our results indicated that presoaking and then ClO_2 treatment have a synergic effect in reducing foodborne pathogens in alfalfa seeds compared to unsoaked.

Optimization of ClO_2 treatment in presoaked alfalfa seeds

The inactivation of the numbers of pathogenic *E. coli* and *Salmonella* spp. in alfalfa seeds was significantly affected by concentration and treatment time. As ClO_2 concentration and treatment time increased, the numbers of pathogenic *E. coli* and *Salmonella* spp. were decreased. Aqueous ClO_2 (50, 100, 150, and 200 ppm) treatment for 1 h after soaking seeds for 5 h caused 1.95, 3.00, 2.93, and 4.00 log CFU g^{-1} reductions

Table 1 Presence of pathogenic *E. coli* and *Salmonella* spp. on various seeds and sprouts after application of the optimized ClO_2 treatment condition

Seeds	Initial level on the seed							
	Present of *E. coli* on seed		Present of *E. coli* on sprout		Present of *Salmonella* spp. on seed		Present of *Salmonella* on sprout	
	2 log CFU g^{-1}	4 log CFU g^{-1}	2 log CFU g^{-1}	4 log CFU g^{-1}	2 log CFU g^{-1}	4 log CFU g^{-1}	2 log CFU g^{-1}	4 log CFU g^{-1}
Radish	3/3*	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Red radish	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Crimson clover	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Kohlrabi	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Red Kohlrabi	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Rape seed	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Tatsoi	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Cabbage	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

* Present of *E. coli* or *Salmonella* spp. on seed or sprout = the number of positive samples/the number of tested samples

Table 2 Germination rate of various seeds after sequential presoaking and ClO_2 treatment

Seeds	Germination rate (%)					
	1 day		2 days		3 days	
	Untreated	ClO_2 treated	Untreated	ClO_2 treated	Untreated	ClO_2 treated
Radish	87.7[a]	85.3[a]	94.0[a]	93.7[a]	97.0[a]	97.0[a]
Red Radish	88.0[a]	86.3[a]	94.0[a]	93.3[a]	96.7[a]	97.7[a]
Crimson clover	73.7[a]	57.7[b]	82.7[a]	85.0[a]	84.0[a]	87.7[a]
Kohlrabi	71.7[a]	80.7[a]	96.3[a]	92.0[a]	98.0[a]	96.3[a]
Red Kohlrabi	85.3[a]	87.7[a]	96.0[a]	93.0[a]	97.0[a]	93.7[a]
Rape seed	76.0[a]	78.0[a]	90.7[a]	87.7[a]	94.0[a]	91.7[a]
Tatsoi	50.3[b]	88.0[a]	97.7[a]	96.0[a]	99.0[a]	98.3[a]
Cabbage	76.0[a]	79.0[a]	90.7[a]	84.7[a]	92.0[a]	93.0[a]
Alfalfa	87.0[b]	95.0[a]	93.0[b]	97.0[a]	97.0[a]	98.0[a]

The same letters in each row are not significantly different from the germination rate in the comparison between untreated and ClO_2-treated groups on the same day with $p < 0.05$ according to Tukey's test

of pathogenic *E. coli* in alfalfa seeds, respectively ($p < 0.05$). When presoaked alfalfa seeds were exposed to 200 ppm ClO_2 for 15, 30, 45, and 60 min, the population of pathogenic *E. coli* was significantly decreased by 2.20, 3.00, 3.40, and 4.00 log CFU g^{-1} compared to untreated samples, respectively ($p < 0.05$). The inactivation of *Salmonella* spp. in alfalfa seeds after ClO_2 treatment showed a similar pattern as those of pathogenic *E. coli*. The number of *Salmonella* spp. in alfalfa seeds was reduced by 2.00, 3.27, 4.09, and 4.26 log CFU g^{-1} after treatment with 50, 100, 150, and 200 ppm ClO_2 for 1 h. At the level of 200 ppm, *Salmonella* spp. in alfalfa seeds exposed for 1 h were inactivated faster compared to those exposed to 30 min. These results demonstrated that sequential immersion in water for 5 h and 200 ppm ClO_2 treatment for 1 h was the optimal condition to eliminate *E. coli* and *Salmonella* spp. from alfalfa seeds (Fig. 2).

Growth of pathogenic *E. coli* and *Salmonella* spp. during sprouting after ClO_2 treatment of alfalfa seeds

Figure 3 shows the growth of pathogenic *E. coli* and *Salmonella* spp. during sprouting after treatment of presoaked alfalfa seeds. Pathogenic *E. coli* and *Salmonella* spp. were completely inactivated in presoaked alfalfa seeds after treatment of 200 ppm ClO_2 for 1 h, regardless of the inoculation level of pathogens. When these pathogens were completely inactivated in alfalfa seeds, the pathogens were not detected after sprouting. However, when seeds contaminated with pathogenic *E. coli* at 2.0 and 4.0 log CFU g^{-1} were not treated with ClO_2, subsequent sprouting resulted in populations of *E. coli* at 6.0 and 8.0 log CFU g^{-1}, respectively. *Salmonella* spp. in alfalfa seeds were also increased to 7–8 log CFU g^{-1} after 3 days of sprouting. These results emphasized the importance of the elimination of foodborne pathogens on sprout seeds to prevent foodborne diseases associated with sprout consumption. In addition, 200 ppm ClO_2 treatment for 1 h after presoaking for 5 h can enhance the safety of alfalfa sprouts.

Application of optimized ClO_2 treatment condition to various seeds

Table 1 shows the results of the application of optimized ClO_2 treatment conditions to eight kinds of seeds. Pathogenic *E. coli* and *Salmonella* spp. were not detected in all kinds of seeds, except radish seeds, treated with 200 ppm ClO_2 for 1 h after soaking for 5 h. After sprouting for 3 days, these pathogens were not also detected from each sprout when each seed was treated with optimized ClO_2 treatment condition. However, the optimized ClO_2 treatment condition for alfalfa seeds does not have an effect on the inactivation of pathogenic *E. coli* and *Salmonella* spp. on radish seeds. Thus, pathogenic *E. coli* and *Salmonella* spp. were detected after sprouting for 3 days. These results indicated that the optimal condition for alfalfa seeds was also applicable for the inactivation of pathogenic *E. coli* and *Salmonella* spp. in various seeds, except radish seeds.

Determination of seed germination rate

Seed viability was evaluated by monitoring the germination percentage during 3 days. The germination rates of nine kinds of untreated and treated seeds are shown in Table 2. The mean germination rates of nine kinds of seeds on the third day of germination ranged from 84% to 98%.

Overall, the germination rates of nine kinds of sprout seeds with 200 ppm ClO_2 for 1 h after soaking for 5 h

Table 3 Residue of ClO_2 on various seeds after sequential presoaking and ClO_2 treatment

Seed name	The residual of ClO_2 in the seed after treatment (h)								
	0	1	2	4	8	12	24	48	72
Red radish	N.D^{a}	N.D^{a}	N.D^{a}	N.D^{a}	N.D^{a}	N.D^{a}	N.D^{a}	N.D^{a}	N.D^{a}
Crimson clover	23.1 ± 4.4^{a}	N.D^{b}	N.D^{b}	N.D b	N.D^{b}	N.D^{b}	N.D^{b}	N.D^{b}	N.D^{b}
Red Kohlrabi	20.0 ± 4.0^{a}	N.D^{b}	N.D^{b}	N.D^{b}	N.D^{b}	N.D^{b}	N.D^{b}	N.D^{b}	N.D^{b}
Radish	59.4 ± 11.5^{a}	30.3 ± 9.7^{b}	9.7 ± 3.5^{c}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}
Tatsoi	71.5 ± 7.2^{a}	42.4 ± 3.1^{b}	24.5 ± 1.9^{c}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}
Kohlrabi	110.8 ± 18.0^{a}	88.8 ± 16.9^{a}	57.1 ± 13.1^{b}	35.0 ± 7.9^{c}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}
Rape seed	112.2 ± 17.4^{a}	74.3 ± 18.5^{b}	50.6 ± 14.4^{b}	21.3 ± 7.7^{c}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}
Cabbage	111.7 ± 13.9^{a}	92.8 ± 14.7^{a}	67.8 ± 15.6^{b}	10.2 ± 0.6^{c}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}	N.D^{d}
Alfalfa	196.1 ± 24.5^{a}	151.8 ± 10.9^{b}	115.4 ± 12.9^{c}	58.5 ± 18.2^{d}	34.9 ± 1.7^{d}	N.D^{e}	N.D^{e}	N.D^{e}	N.D^{e}

N.D not detected

The same lowercase letters in each row are not significantly different from the residue of ClO_2 in the seeds after sequential presoaking and ClO_2 treatment with $p < 0.05$ according to Tukey's test

were not significantly different from those of untreated seeds. Moreover, the germination rates of tatsoi and alfalfa seeds on the first day of germination after treatment with ClO_2 were higher than those of untreated seeds by 37.7% and 8.0%, respectively. These results implied that ClO_2 treatment after soaking does not have a significantly adverse effect on the seed germination rate.

Determination of residue of ClO_2

As ClO_2 effectively inhibited the growth of pathogenic *E. coli* and *Salmonella* spp., it was necessary to confirm if ClO_2 remained in the sprouts. The residue of ClO_2 in nine kinds of sprouts for 3 days was determined (Table 3). The mean of ClO_2 residues in various seeds ranged from 0 to 196 mg kg^{-1} after treatment. There were significant differences ($p < 0.05$) in ClO_2 residues among types of seeds. The highest ClO_2 residue was from alfalfa seeds and the lowest was from red radish seeds ($p < 0.05$). However, ClO_2 was not presented in any sprout after 3 days of cultivation.

Discussion

In the present study, 200 ppm ClO_2 treatment for 1 h resulted in 1.22 to 1.61 log CFU g^{-1} reductions of pathogenic *E. coli* and *Salmonella* spp. in unsoaked alfalfa seeds. A higher reduction of *E. coli* and *Salmonella* spp. resulted from presoaked alfalfa seeds compared to unsoaked seeds in the present study (2.07 and 1.47 log CFU g^{-1} reduced more than unsoaked, respectively). Taormina et al. [9] also reported that exposure to 200 ppm ClO_2 for 3 to 10 min resulted in only 1.0 to 2.0 log reduction of *E. coli* O157:H7 in alfalfa seeds. This means that ClO_2 treatment alone may be inadequate for controlling foodborne pathogens on seeds. Taormina et al. [9] concluded that this is due to cracks and crevices harboring pathogens on alfalfa seed surfaces, where these chemicals could not sufficiently contact the pathogens. Thus, we investigated the synergic effect of presoaking and ClO_2 treatment on the reduction of those bacteria in alfalfa seeds. Previous authors also agreed with us on the effectiveness of presoaked to the main treatment in their study [19].

To explain this issue, Delaquis et al. [21] performed a microscopic examination of alfalfa seeds and reported that although the seed surface is relatively smooth the stem scar is relatively porous with areas capable of harboring pathogens, thus affording protection to hidden bacterial cells. In addition to their topographic complexity, the surface of alfalfa seeds is covered with a waxy cuticle (cutin), lowering their water wettability. Charkowski et al. [22], however, mentioned that, by presoaking seeds in water for a certain period of time, water is believed to permeate the seed coat, causing bacteria trapped in cracks, crevices, or other discontinuities to be released, and the released bacteria are inactivated by ClO_2 easily.

This study also presented the optimum treatment condition of ClO_2 as a sanitizer agent after soaking of alfalfa seeds. As ClO_2 concentration and treatment time increased, significant differences ($p < 0.05$) were observed in the inactivation levels of pathogenic *E. coli* and *Salmonella* spp. When presoaked alfalfa seeds were exposed to 200 ppm ClO_2 for 1 h, the populations of pathogenic *E. coli* and *Salmonella* spp. was significantly decreased by 4.00 and 4.26 log CFU g^{-1}, respectively ($p < 0.05$). In addition, these pathogens were completely inactivated in alfalfa seeds inoculated with 4 log CFUg-1 and the pathogens were not detected after sprouting. However, untreated seeds contained pathogens at low levels, such as 2 log CFU g^{-1}, and subsequent sprouting resulted in populations of *E. coli* and *Salmonella* spp. of 7.5 and 7.35 log CFU g^{-1} after 3 days sprouting, respectively. In a previous study, although the populations of *E. coli* O157:H7 and *Salmonella* spp. were reduced by more than 5 log CFU g^{-1} in alfalfa seeds using dryheat treatment, *E. coli* O157:H7 and *Salmonella* spp. increased to 5 to 7 log CFU g^{-1} after 3 days of sprouting. These studies emphasized the importance of the elimination of foodborne pathogens on sprout seeds to prevent or greatly minimize foodborne diseases associated with sprout consumption.

As the optimal condition was applied to various seeds, *E. coli* and *Salmonella* spp. were completely inactivated on all seeds inoculated individually with 2 and 4 log $CFUg^{-1}$, and these pathogens were not also detected after sprouting, except radish seeds. Differences in the pathogen reduction levels may be due to the differences in the surface characteristics and size of seeds. Bari et al. [23] reported that dry heat treatment at 50 °C for 17 h could reduce the numbers of *E. coli* O157:H7 in alfalfa seeds to below the detection limit, whereas 24 h treatment was required for radish seeds. Fransisca et al. [24] found that several sanitizers, including $Ca(OCl)_2$ and malic acid, reduced *E. coli* O157:H7 more in alfalfa seeds than in radish seeds. The Ra (arithmetic mean roughness) value of radish seeds (6.08 mm) was higher than that of alfalfa seeds (0.56 mm), and generally, a negative correlation existed between the Ra values of seeds and microbial reduction by sanitizer treatment [24]. It is supposed that the size of the seeds also affects the reduction of *E. coli* and *Salmonella* spp. Radish seeds are more than 2.2 mm but less than 1.0 mm, which is different from crimson clover seeds (data not shown). The larger the size of seeds, the more areas capable of harboring pathogens occurred in seeds. Thus, chemicals could not sufficiently contact the pathogens in radish seeds with a rough surface and large size. Although the efficacy of ClO_2 treatment against a number of viral and bacterial pathogens

and/or rot organisms is not generally questioned, germination rate and chemical residues are concerns due to ClO_2 treatment. It is important to monitor the residual levels of ClO_2. In the present experiments, there were no differences ($p > 0.05$) in germination between untreated and 200 ppm ClO_2-treated seeds, consistent with previous studies using gaseous or aqueous ClO_2. This results implied that the combination of presoaking and ClO_2 treatment does not have an effect on the germination of seeds.

In addition, the final product of sprouts may be safe because ClO_2 was not detected after sprouting for 3 days. Smith et al. [25] revealed that significant quantities of chlorate residues were formed from ClO_2 treatment of seeds, but these residues were removed during the sprouting process. Moreover, chlorate residues that possibly remained on seeds might have been transformed by bacterial action during the sprouting process as chlorate residues are prone to biodegradation in biotic environments conducive to bacterial growth. This is in harmony with consumer preference for safe fresh produce [26].

In conclusion, most sprout farms in Korea use sanitizers before 5 h soaking. However, data presented here suggested that sequential 5 h presoaking and 200 ppm ClO_2 treatment for 1 h is more effective to eliminate *E. coli* and *Salmonella* spp. from sprout seeds without decreasing the germination rate. For industrial applications, the efficacy of the decontamination procedure developed in this study should be validated using commercial-scale sprout production practices.

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Authors' contributions
NBH performed the experiment, data analysis and interpretation, and wrote the fnal manuscript. WRP analyzed data and drafted the manuscript. BHY and DCS performed the experiment, data analysis. WIK and HJK contributed materials and editorials. SHH and SRK supervised the project and revised the fnal manuscript. All authors read and approved the final manuscript.

Availability of data and materials
Datasets used and/or analyzed during the current study that are not included in the manuscript are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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