Effectiveness of Stable Ozone Microbubble Water on Reducing Bacteria on the Surface of Selected Leafy Vegetables

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Effectiveness of a novel developed sanitizer, stable ozone microbubble-containing water (ozone microbubble water; OMBW), on reducing 13 kinds of bacteria was evaluated *in vitro*. The effectiveness of dip inoculated *Escherichia coli* O157:H7 or naturally attached bacteria on four kinds of leafy vegetables was also evaluated. For the purpose of comparison, the effectiveness of gaseous ozone (GO), sodium hypochlorite solution (NaClO) and distilled water (DW) were also evaluated. A 5.0 to 7.4 log reduction of viable cells was observed in an *in vitro* study. In contrast, a 0.8 to 1.2 or 0.9 to 1.8 log CFU/g reduction in *E. coli* viable cells was observed after washing with OMBW or NaClO, respectively, for all tested leafy vegetables. No significant difference was observed in the effectiveness of OMBW, ozonated water (OW) and DW. No reduction of viable cells was observed after exposure of leaves to GO. Similar experimental results were observed with the naturally attached bacteria. No difference in color and appearance was observed with DW and other sanitizing treatments. These results suggest that the effectiveness of OMBW in surface decontamination of leafy vegetables is similar to that of OW.

Keywords: ozone microbubble water, ozonated water, *E. coli* O157:H7, surface decontamination

Introduction

Consumption of fresh produce has been suggested to increase public health because fresh vegetables and fruits are thought to be an important part of a healthy diet and are a source of much-needed vitamins, minerals and fiber. Typically, most fruits, vegetables, and ready-to-eat (RTE) foods sold in markets rarely carry pathogenic bacteria. However, there is always the possibility of contamination with food borne pathogens, such as enterohemorrhagic *Escherichia coli* O157:H7, during any stage of product handling from production at the farms to the point of sale (Hanning *et al.*, 2009; Matthews 2006). Naturally contaminated bacteria, such as *Bacillus* sp., *Enterobacter* sp., *Erwinia* sp., *Flavobacterium* sp. and *Pseudomonas* sp., among others, are related to the spoilage of fresh produce during transportation and storage (Bari *et al.*, 2005; Barth *et al.*, 2009; Heard 2002). Thus, the

effectiveness of many kinds of surface-washing sanitizers have been evaluated on fresh produce (Gil *et al.*, 2009).

Sodium hypochlorite (NaClO) is commonly used for surface decontamination of fresh produce to reduce the risk of food poisoning or spoilage because of its broad spectrum of antimicrobial activity, ease of application and low cost (Sapers, 2006). This compound is usually used for surface decontamination of foods at levels of 50–200 mg/L free chlorine and with a typical contact time of less than 5 min (Rico *et al.*, 2007). However, chlorine is highly corrosive and may damage stainless steel equipment after prolonged exposure. Furthermore, its rapid depletion in the presence of a high organic load and concerns over the potential carcinogenicity of compounds resulting from the reaction of the active hypochlorite with nitrogen-containing compounds have prompted the consideration of alternative disinfectants (Wei *et al.*, 1985; Buschini *et al.*, 2004). An acidified sodium chlorite solution produced by mixing sodium chlorite (NaClO₂) and acid in water has been proposed as a candidate alterna-

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tive sanitizer (Inatsu *et al.*, 2010b; Elano *et al.*, 2010; Nei *et al.*, 2009; Stopforth *et al.*, 2008).

Ozone (O₃) is a strong antimicrobial compound with a high oxidative activity. In addition, it spontaneously decomposes into a non-toxic product (Rico *et al.*, 2007). In contrast to hypochlorous acid (HClO), ozone does not effectively penetrate the cell; however, it causes damage to the cell wall structure on the outside of the cell and eliminates the exposed bacteria (Cho *et al.*, 2010). Theoretically, the maximum concentration of dissolved ozone gas in water at 20°C and 0.10 MPa is 570 mg/L, and only a limited portion (10–50 mg/L) of the generated ozone exists due to the evaporation loss from water and its short half-life (1–10 min) (Dhillon *et al.*, 2009). Ozonated water (OW) exhibits more than a 5.0 log CFU/mL bactericidal activity against Gram-positive and -negative foodborne pathogens in liquid cultures (Restaino *et al.*, 1995). The effectiveness of the OW to control the growth of surface-attached *E. coli* O157:H7 or natural flora on vegetables has been investigated (Beltran *et al.*, 2005; Singh *et al.*, 2002; Zhang *et al.*, 2005; Koseki and Isobe, 2006).

Recently, a novel commercially available sanitizer, i.e., a stable ozone microbubble-containing water (ozone microbubble water; OMBW), was purchased and applied to food hygiene control and the elongation of shelf life for foods (Nakamura and Shiina 2010); however, only limited scientific studies have been conducted on OMBW. In general, the diameter of the bubbles are mechanically produced on a 100-µm scale by using the flow pump or supersonic method (Xu *et al.*, 2008). However, at this size, the dispended bubbles are not stable and can be easily crushed, or they can surface. Recently, Takahashi *et al.* (2003) developed stable microbubbles of less than 10 µm in diameter (Takahashi *et al.*, 2003; Takahashi, 2005). One of the benefits of smaller bubbles in water is that they can remain stably dispended in water for longer periods of time (i.e., over several months). The purpose of this study was to evaluate the effectiveness of OMBW on reducing the viable cell count of surface-attached bacteria on leafy vegetables or *in vitro*.

Material and Methods

Bactericidal effectiveness of ozone microbubble water *in vitro* The experimental methods were the same as previously published (Inatsu *et al.*, 2010a), except for small modifications. The ozone microbubble water (OMBW) was purchased from Nano Industry Development Co. Ltd. (Miyagi, Japan) and was used within a month. The 13 bacterial strains analyzed in this study are listed in Table 1. The JCM and ATCC strains were purchased from Japan Collection of Microorganism and American Type Culture Collection, respectively. Four *E. coli* O157:H7 strains (CR-3, MN-

28, MY-29 and DT-66) were isolated from calf feces. The organisms were grown in brain heart infusion (BHI: Nissui Pharmaceutical Co. Ltd, Tokyo) agar plates or on de Man, Rogosa and Sharpe (MRS: Becton and Dickinson, USA) agar plates for lactic acid bacteria. Fresh cultures in the early stationary growth phase were prepared with BHI or MRS broth and were used for the assays after being washed twice in phosphate buffered saline (PBS). The washed cells were exposed to OMBW or PBS for 3 min. After treatment, viable cells were measured using the surface spread or pour plate method. The cells were then incubated for 24 to 48 h at 35°C or 30°C for *Listeria monocytogenes* and lactic acid bacteria, respectively. This experiment was repeated at least five times to confirm reproducibility. The statistical differences of log reduction between each of the tested strains were analyzed by Tukey-Kramer's multiple comparison tests.

Bactericidal effectiveness of ozone microbubble water on vegetable leaves The experimental methods were the same as previously described (Elano *et al.*, 2010; Nei *et al.*, 2009; Inatsu *et al.*, 2010b) except for small modifications. OW was generated with a flow-type electric ozone generator (Do-30, Kobe Steel, Ltd., Kobe, Japan) and was used within 5 min. The concentration of dissolved or dispended ozone was measured based on the indigo method (Kondo *et al.*, 2005). A sterile, distilled water solution (DW) was used for the control study. The gaseous ozone (GO) was generated by a discharge ionization machine (Ozonizer EO-1, Navi Engineering Co. Ltd., Yokohama, Japan).

Lettuce (*Lactuca sativa*), Chinese cabbage (*Brassica rapa* ssp. *Chinensis*), spinach (*Spinacia oleracea*) and cabbage (*Brassica oleracea*) were purchased from supermarkets in Tsukuba city, Japan, between February and August 2009. Each of the leafy vegetables (650 g) was cut into 3 × 3 cm square pieces and mixed well in a plastic bag. For the inoculation study, each of the vegetables was dipped into a mixture of four spontaneous rifampicin resistance *E. coli* O157:H7 strains (i.e., CR-3, MN-28, MY-29 and DT-66) (7.2 to 7.7 log CFU/mL) and dried for 1 h. The inoculated or non-inoculated vegetables (150 g) were submerged into 1.50 L of washing solution in a 3-L plastic beaker for 3 min with gentle mixing by a glass rod. The washing water was removed using a salad spinner for 1 min, and the viable cells of the samples were enumerated by the pour plate method after 1 min of subjection to a stomacher machine (Gunze industry, MC-D type 400D). The residual bactericidal activity of the washing solution was also measured by exposing PBS-washed *E. coli* cells for 3 min (Inatsu *et al.*, 2010a; Elano *et al.*, 2010). Instead of the washing treatment, exposing the vegetables to 100 mg/L of GO in plastic bags was also performed. A standard agar plate and desoxycholate agar

was used to enumerate the aerobic plate count (APC) and coliform bacteria, respectively. A 50 mg/L rifampicin solution containing trypticase soy agar (TSA) and sorbitol MacConkey agar (SMAC) were used for the recovery of *E. coli* O157:H7. All media used in this experiment were purchased from Nissui Pharmaceutical Co. Ltd.

The leafy vegetables described above were washed in four batches for each of the washing solutions. These experiments were repeated six times on different days using different lots of vegetables purchased from different shops. The experimental results ($n = 24$) were subjected to statistical analysis by Turkey-Kramer's multiple comparison tests. We also analyzed the results of the four types of vegetables by analysis of variance (ANOVA) with the Fisher method.

Sensory evaluation The appearance and color of the sanitizer-treated vegetables were compared to water-washed vegetables by the three-point discrimination test (triangle

sensory test) (Stone and Sidel, 2004). Twenty well-trained panelists (9 males and 11 females) from the National Food Research Institute were asked to identify a sample that they thought to be able to distinguish from among three samples. More than 11 correct answers from 20 panelists showed the existence of a statistically significant difference ($P < 0.05$) between sanitizer-treated samples and the control.

Results and Discussion

To evaluate the bactericidal effectiveness of the ozone microbubble water (OMBW) sanitizer, we performed *in vitro* experiments on 13 kinds of bacteria (Table 1). A reduction of 5.0 to 7.4 log CFU/mL of viable cells was observed after 3 min of exposure to this sanitizer (5.44 mg/L O_3) at 25°C. *Pseudomonas aeruginosa* and *Staphylococcus aureus* showed statistically lower ($P < 0.05$) sensitivities compared to the other tested strains using this sanitizer. A similar ten-

Table 1. Bactericidal activity of ozone microbubble water *in vitro*.

Strains		Viable cell counts (log reduction) (log CFU/mL)	
<i>Escherichia coli</i> CR-3 (O157:H7)	PBS	8.7 ± 0.1	(7.4 ± 0.1) ^A
	OMBW	1.3 ± 0.0	
<i>Salmonella</i> Enteritidis JCM1652	PBS	8.6 ± 0.1	(6.8 ± 0.8) ^A
	OMBW	1.9 ± 0.8	
<i>Klebsiella pneumoniae</i> JCM1662 ^T	PBS	8.7 ± 0.1	(7.2 ± 0.6) ^A
	OMBW	1.5 ± 0.6	
<i>Citrobacter freundii</i> JCM1657 ^T	PBS	8.8 ± 0.2	(6.7 ± 1.0) ^A
	OMBW	2.1 ± 0.9	
<i>Cronobacter sakazakii</i> JCM1233 ^T	PBS	8.6 ± 0.1	(6.0 ± 0.9) ^A
	OMBW	2.6 ± 0.9	
<i>Pseudomonas aeruginosa</i> JCM5962 ^T	PBS	8.4 ± 0.3	(5.0 ± 0.6) ^B
	OMBW	3.3 ± 0.5	
<i>Listeria monocytogenes</i> ATCC13932	PBS	8.4 ± 0.1	(7.1 ± 0.1) ^A
	OMBW	1.3 ± 0.0	
<i>Staphylococcus aureus</i> JCM2413	PBS	8.6 ± 0.2	(5.5 ± 0.6) ^B
	OMBW	3.0 ± 0.5	
<i>Enterococcus faecalis</i> JCM8726 ^T	PBS	7.7 ± 0.5	(6.4 ± 0.5) ^A
	OMBW	1.3 ± 0.0	
<i>Leuconostoc mesenteroides</i> JCM6124 ^T	PBS	8.2 ± 0.2	(6.1 ± 1.0) ^A
	OMBW	2.1 ± 0.9	
<i>Lactobacillus plantarum</i> JCM1149 ^T	PBS	8.3 ± 0.3	(5.5 ± 0.9) ^{AB}
	OMBW	2.9 ± 0.9	
<i>Pediococcus pentosaceus</i> JCM5890 ^T	PBS	7.7 ± 1.0	(5.0 ± 1.5) ^{AB}
	OMBW	2.7 ± 1.1	
<i>Lactococcus lactis</i> JCM5805 ^T	PBS	7.6 ± 0.2	(5.5 ± 0.9) ^A
	OMBW	2.0 ± 0.8	

Cells washed with phosphate buffered saline (PBS) were exposed to ozone micro bubble water (OMBW) or PBS for 3 min. The average and standard deviation of the logarithmic number of enumerated viable cells are shown ($n > 6$). The different letters on the shoulder of the log reduction value indicate significant difference ($P < 0.05$).

dependency in tolerance difference to ozonated water (OW) treatment *in vitro* was also reported by Restaino *et al.* (1995). The different tolerances to ozone (microbubble) water exposure seen among tested strains may reflect differences in cellular responses used to deal with the oxidative stress caused by the ozone, or the amount of enzymes decomposed by the ozone.

To confirm the efficacy of the decontamination of surface-attached bacteria on four kinds of leafy vegetables, we conducted washing experiments of native or *E. coli* O157:H7 inoculated samples. The average level of contamination of aerobic mesophilic bacteria and coliform bacteria on the four kinds of vegetables before washing were 4.7 (cabbage) to 6.5 (spinach) log CFU/g and 3.6 (cabbage) to 5.9 (spinach) log CFU/g, respectively (Table 2). Among the four tested vegetables, Chinese cabbage and spinach exhibited higher viable bacterial counts compared to lettuce and cabbage. Washing the vegetables with DW for 3 min at 25°C reduced the viable

aerobic bacteria and the coliform bacteria by less than 1.0 log CFU/g for each of the tested vegetables. The use of OW (4.64 mg/L O₃) or OMBW (5.44 mg/L O₃) instead of DW did not show any additional bactericidal effectiveness for all tested vegetables. Washing the vegetables with a 100 mg/L NaClO solution reduced the naturally contaminating bacteria by approximately 2.0 log CFU/g in all tested vegetables. However, exposure to 100 mg/L GO for 3 min did not reduce the viable bacterial cell count on the tested vegetables, except for the aerobic mesophilic bacteria in cabbage. No significant differences ($P > 0.05$) in the effectiveness of viable bacterial cell reduction were observed between each of the four types of vegetables. The color and appearance of all tested vegetables after each treatment did not differ significantly compared to the water-washed samples (Table 3).

Similar results were obtained in the case of artificially inoculated *E. coli* O157:H7 on the surface of vegetables (Table

Table 2. Bactericidal effectiveness of sanitizers on naturally attached bacteria on leafy vegetables.

Viable cell counts of aerobic methophilic bacteria (log CFU/g)						
	Before washing	Distilled water	Sodium hypochlorite	Ozone micro bubble water	Ozonated water	Gaseous ozone
Lettuce	5.1 ± 0.4 ^A	4.4 ± 0.7 ^B	3.3 ± 0.7 ^C	4.2 ± 0.6 ^B	4.3 ± 0.4 ^B	5.1 ± 0.6 ^{AB}
Chinese cabbage	6.4 ± 0.6 ^A	5.9 ± 0.5 ^A	4.4 ± 0.8 ^C	5.2 ± 0.4 ^B	5.4 ± 0.2 ^B	5.8 ± 0.6 ^{AB}
Spinach	6.5 ± 0.4 ^A	5.9 ± 0.3 ^B	4.7 ± 0.6 ^C	5.6 ± 0.5 ^B	5.5 ± 0.4 ^{BC}	6.1 ± 0.4 ^{AB}
Cabbage	4.7 ± 1.2 ^A	4.0 ± 1.2 ^{AC}	2.8 ± 0.7 ^{BC}	3.0 ± 0.7 ^{BC}	3.3 ± 0.8 ^{BC}	3.1 ± 0.9 ^{BC}

Viable cell counts to coliform bacteria (log CFU/g)						
	Before washing	Distilled water	Sodium hypochlorite	Ozone micro bubble water	Ozonated water	Gaseous ozone
Lettuce	4.7 ± 0.6 ^A	4.2 ± 0.8 ^A	3.2 ± 0.5 ^B	3.8 ± 0.8 ^B	3.6 ± 0.8 ^B	4.6 ± 0.7 ^A
Chinese cabbage	5.0 ± 0.9 ^A	4.5 ± 0.8 ^A	3.5 ± 0.7 ^B	3.8 ± 0.6 ^{AB}	3.7 ± 0.6 ^{AB}	4.7 ± 0.4 ^A
Spinach	5.9 ± 0.5 ^A	5.3 ± 0.7 ^A	4.0 ± 1.0 ^C	5.2 ± 0.7 ^{AB}	4.8 ± 0.8 ^{ABC}	5.2 ± 0.5 ^A
Cabbage	3.6 ± 1.3 ^A	3.4 ± 1.2 ^A	2.7 ± 0.6 ^A	2.9 ± 0.9 ^A	2.7 ± 0.7 ^A	3.1 ± 1.0 ^A

Un-inoculated vegetables were washed with each of the solutions or exposed to gaseous ozone (GO) for 3 min. Viable cells were enumerated after removing water by gentle spinning. The average value and standard deviation of the results evaluated from six trials are shown ($n = 24$). The different letters on the shoulder for the same vegetables indicate significant difference ($P < 0.05$).

Table 3. Effect of sanitizer washings on the visible quality of the vegetables.

Number of correct answers from a panel of 20 people				
	Sodium hypochlorite	Ozone micro bubble water	Ozonated water	Gaseous ozone
Lettuce	10	9	7	6
Chinese cabbage	3	5	11	6
Spinach	10	9	9	6
Cabbage	9	3	6	6

Triangle tests were performed by using water-washed vegetables as a control. No significant difference in the color or appearance was observed among the three samples ($P > 0.05$).

4). Washing with DW, OW and OMBW reduced the surface-attached *E. coli* O157:H7 by approximately 0.5 to 1.0 log CFU/g, and after washing, no significant differences in viable bacterial cell count were observed between any of the tested vegetables. A 0.3 to 1.0 log CFU/g higher bactericidal effectiveness was observed when a 100 mg/L NaClO solution

was used for washing compared to water. No change in the *E. coli* O157:H7 population was observed after exposure to 100 mg/L GO. The results discussed above were not dependant on the kind of sanitizer used for the four kinds of vegetables.

The remaining bactericidal activity of the washing solution after a one-time use is shown in Table 5. The NaClO

Table 4. Bactericidal effectiveness of sanitizers on dip-inoculated *E. coli* O157:H7 cells on the surface of leafy vegetables..

	Viable cell counts enumerated by TSA-Rif agar medium (log CFU/g)					
	Before washing	Distilled water	Sodium hypochlorite	Ozone micro bubble water	Ozonated water	Gaseous ozone
Lettuce	6.5 ± 0.2 ^A	5.7 ± 0.2 ^B	4.9 ± 0.4 ^C	5.6 ± 0.2 ^B	5.5 ± 0.3 ^B	6.4 ± 0.1 ^A
Chinese cabbage	6.1 ± 0.3 ^A	5.6 ± 0.3 ^B	5.2 ± 0.3 ^{CD}	5.3 ± 0.2 ^{BD}	5.5 ± 0.2 ^B	6.1 ± 0.3 ^A
Spinach	6.0 ± 0.1 ^A	5.2 ± 0.1 ^B	4.9 ± 0.2 ^C	5.2 ± 0.1 ^B	5.3 ± 0.2 ^B	6.1 ± 0.1 ^A
Cabbage	5.5 ± 0.2 ^A	4.7 ± 0.3 ^B	3.7 ± 0.6 ^C	4.3 ± 0.3 ^{BD}	4.7 ± 0.3 ^B	5.0 ± 0.3 ^{BD}

	Viable cell counts enumerated by SMAC-Rif agar medium (log CFU/g)					
	Before washing	Distilled water	Sodium hypochlorite	Ozone micro bubble water	Ozonated water	Gaseous ozone
Lettuce	5.6 ± 0.2 ^A	4.7 ± 0.3 ^B	4.2 ± 0.6 ^C	4.9 ± 0.5 ^B	4.9 ± 0.5 ^B	5.9 ± 0.1 ^A
Chinese cabbage	5.1 ± 0.4 ^A	4.4 ± 0.4 ^A	4.1 ± 0.6 ^B	4.7 ± 0.3 ^{AC}	4.4 ± 0.5 ^{AB}	5.3 ± 0.6 ^{AC}
Spinach	5.5 ± 0.6 ^A	4.5 ± 0.2 ^B	4.4 ± 0.4 ^B	5.0 ± 0.4 ^{BC}	4.8 ± 0.1 ^{BC}	5.8 ± 0.2 ^A
Cabbage	4.7 ± 0.2 ^A	4.0 ± 0.5 ^B	2.9 ± 0.3 ^C	3.9 ± 0.5 ^B	3.8 ± 0.3 ^B	4.5 ± 0.5 ^{AB}

A cocktail of four strains of *Escherichia coli* O157:H7 was used to dip-inoculate vegetables, and the samples were washed as in Table 2. A high-recovery (Trypticase soy agar: TSA) and a low-recovery (Sorbitol MacConkey: SMAC) media supplemented with rifampicin (Rif) were used for enumeration. The average value and standard deviation of the results evaluated from four trials are shown (n = 12). The different letters on the shoulder for the same vegetables indicate significant difference (P < 0.05).

Table 5. Residual bactericidal activity of the washing solutions after use.

	Viable cell counts enumerated by TSA-Rif agar medium (log CFU/g)					
	Distilled Water		Sodium hypochlorite		Ozone micro bubble water	
	Before use	After use	Before use	After use	Before use	After use
Lettuce	8.5 ± 0.2 ^A	8.6 ± 0.1 ^A	1.3 ± 0.0 ^B	1.7 ± 0.9 ^B	2.0 ± 0.5 ^B	8.6 ± 0.1 ^A
Chinese cabbage	8.6 ± 0.1 ^A	8.6 ± 0.2 ^A	1.3 ± 0.0 ^B	1.3 ± 0.0 ^B	1.7 ± 0.8 ^B	8.5 ± 0.3 ^A
Spinach	8.6 ± 0.2 ^A	8.6 ± 0.3 ^A	1.6 ± 0.3 ^B	1.4 ± 0.2 ^B	1.2 ± 0.2 ^B	8.6 ± 0.1 ^A
Cabbage	8.5 ± 0.2 ^A	8.6 ± 0.2 ^A	1.7 ± 0.6 ^B	2.0 ± 0.9 ^B	1.8 ± 0.8 ^B	8.6 ± 0.1 ^A

	Viable cell counts enumerated by SMAC-Rif agar medium (log CFU/g)					
	Distilled Water		Sodium hypochlorite		Ozone micro bubble water	
	Before use	After use	Before use	After use	Before use	After use
Lettuce	8.3 ± 0.2 ^A	8.4 ± 0.3 ^A	1.3 ± 0.0 ^B	1.7 ± 0.8 ^B	1.7 ± 0.5 ^B	8.5 ± 0.2 ^A
Chinese cabbage	8.3 ± 0.1 ^A	8.3 ± 0.2 ^A	1.5 ± 0.2 ^B	1.7 ± 0.6 ^B	1.6 ± 0.6 ^B	8.2 ± 0.3 ^A
Spinach	8.4 ± 0.2 ^A	8.4 ± 0.3 ^A	1.3 ± 0.1 ^B	1.3 ± 0.1 ^B	1.6 ± 0.5 ^B	8.4 ± 0.1 ^A
Cabbage	8.3 ± 0.2 ^A	8.3 ± 0.2 ^A	1.5 ± 0.2 ^B	1.7 ± 0.6 ^B	1.7 ± 0.7 ^B	8.3 ± 0.1 ^A

E. coli O157:H7 cells washed with PBS were mixed with vegetable washing solutions before and after use. After 3 min, the oxidative compound was neutralized by the addition of sodium thiosulfate and the viable cells were measured by TSA and SMAC. The average value and standard deviation of viable cells evaluated in three independent experiments (n = 6) are shown. The different letter on the shoulder of the values indicate significant difference (P < 0.05).

solution exhibited an 8.0 log CFU/mL reduction in inoculated *E. coli* O157:H7 before and after its use. Conversely, the OMBW completely lost its bactericidal activity after one use. This result reflected the difference in chemical reactivity between ozone and hypochlorite. The chemical reactivity of ozone is higher than that of hypochlorite because the oxidation-reduction potential (ORP) of ozone and hypochlorite is 2.07 V and 1.63 V, respectively. In addition, chloramine compound produced by the chemical reaction between hypochlorite and food has weak bactericidal activity, in contrast to oxygen produced by the reaction between ozone and food.

As shown in Table 1, the OMBW was able to reduce free *E. coli* O157:H7 cells by 7.4 log CFU/mL. However, a reduction of less than 2.0 log CFU/g was achieved in the surface-attached bacteria of the vegetable. Zhang *et al.* (2005) reported a 0.8 to 1.7 log CFU/g reduction in naturally attached bacteria on the surface of freshly cut celery by OW (0.03 to 0.18 mg/L O₃). Dipping iceberg lettuce into 4 mg/L of ozone-containing water for 2 min reduced the surface-attached mesophilic micro-organisms by 1.7 log CFU/g (Akbas and Olmez, 2007). In other studies (Singh *et al.*, 2002; Koseki and Isobe, 2006), OW (less than 16.5 mg/L O₃) exhibited similar or weaker bactericidal effectiveness compared to the NaClO solution on the surface-attached *E. coli* 157:H7 cells on shredded lettuce or baby carrots. In addition, no meaningful relationship between the bactericidal effectiveness of OW and exposure time was observed in this study. These results can be, at least partially, explained by the decomposition of dissolved ozone by reacting with organic materials. Restaino *et al.* (1995) have reported the *in vitro* effectiveness results with the addition of 20 mg/L bovine serum albumin (BSA) in ozone water to reduce the dissolved effective ozone concentration. The oxidative activity of the OMBW was stable for at least 6 months in water at 4°C (data not shown). However, once the vegetable was mixed with ozone OMBW, it easily lost its bactericidal activity, which was similar to the ordinary OW (Table 5). According to the evaluation experiments performed in a commercial lettuce washing facility, removing attached organic material from the leaves of vegetables by pre-washing was very important to prevent the reduction of dissolved ozone (Hassenberg *et al.*, 2007).

As shown in Table 4, 100 mg/L NaClO solution could reduce the attached *E. coli* O157:H7 cells on the surface of tested vegetables by 0.9 (Chinese cabbage) to 1.8 (Cabbage) log CFU/g; this value was rather high compared to the OMBW and OW. These results were in agreement with others studies (Sapers, 2006; Baert *et al.*, 2009; Gil *et al.*, 2009) and were explained by the difference in molecular reactivity of these sanitizers. In contrast to the limited reduction level of surface-attached viable bacterial cells on vegetables, a

higher bactericidal activity level was retained even after washing with NaClO (Tables 2, 4 and 5). This phenomenon may be, at least partially, explained by the restriction of mass transportation of the active compound around the area close to the surface of the vegetable (Elano *et al.*, 2010). Briefly, the active compounds near the surface of vegetables rapidly react with organic materials and are used up. The supply of additional active compounds is thought to be restricted because of the viscosity of the fluid near the surface; mass transport is subjected to the concentration gradient of the active compound.

Conclusion

The effectiveness of OMBW to reduce viable bacterial cells of surface-attached bacteria on the four leafy vegetables tested was similar to ozonated water (OW). The bactericidal activity of this sanitizer was completely lost after washing of the vegetables. A 100 mg/L NaClO solution was shown to reduce surface-attached bacteria by approximately 2.0 log CFU/g; a 7.0 log CFU/mL reduction was achieved by using an *in vitro* washing solution. We concluded from these results that the use of OMBW or OW may provide only limited effectiveness for the decontamination of leafy vegetable surfaces and prevents cross-contamination via washing solutions compared to the use of the 100 mg/L NaClO solution. However, the high stability of OMBW during storage might facilitate transport and supply of the chlorine alternative sanitizer to improve hygiene conditions at processing lines or processing environments of small and/or local facilities.

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