学位論文

Antimicrobial Activity and Stability of Weakly Acidified Chlorous Acid Water

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The antimicrobial activity of weakly acidified chlorous acid water (WACAW) against *Staphylococcus aureus*, non-pathogenic *Escherichia coli*, enterohemorrhagic *E. coli* (EHEC O157:H7), *Candida albicans*, and spore-forming *Bacillus* and *Paenibacillus* species was evaluated in vitro. The antiviral activity was also examined using feline calicivirus (FCV). Diluted WACAW (>100 ppm) effectively reduced the number of non-spore-forming bacteria (>4 $\log_{10}$ CFU reductions) within 5 min. Treatment with this sanitizer at 400 ppm for 30 min achieved>5 $\log_{10}$ CFU reductions in spore-forming *Bacillus* and *Paenibacillus* species while an equivalent concentration of sodium hypochlorite (NaClO) resulted in only a 0.98 and 2.72 $\log_{10}$ CFU reduction, respectively. The effect of this sanitizer against FCV was equivalent to that of NaClO. Immersion in WACAW (400 ppm) achieved >4 and 2.26 $\log_{10}$ CFU reductions in *Campylobacter jejuni* and EHEC, respectively, on artificially contaminated broiler carcass pieces. Finally, the antimicrobial activity of this sanitizer was shown to be maintained for at least 28 d when in contact with nonwoven fabric (100% cotton). This study showed that pH control of chlorous acid is expected to modify its antimicrobial activity and stability. WACAW is expected to have applications in various settings such as the food processing and healthcare industries.

Key words : Chlorous acid water / Weak acidification / Broiler carcasses / Stability in fabrics.

INTRODUCTION

Chlorine has been used for many years to treat drinking water and to sanitize food processing equipment and environmental surfaces (Beuchat, 1998; Wei et al., 1985). Chlorine-based disinfectants show a wide spectrum of antimicrobial activity, and can inactivate bacterial endospores and alcohol-resistant non-enveloped viruses such as poliovirus and human norovirus (HNoV). Of the chlorine-based sanitizers, sodium hypochlorite (NaClO) shows strong oxidizing properties and is widely used for sanitation in various situations, such as for healthcare purposes and decontamination in food processing settings due to its efficient antimicrobial activity and low cost. In clinical settings, high concentrations (≥1,000 ppm) of NaClO are generally recommended against pathogens that are difficult to inactivate by other disinfectants, including *Clostridium difficile* spores and HNoV (Cohen et al., 2013; MacCannell et al., 2011).

NaClO is currently one of the major sanitizers for public health applications to prevent cross-transmission of infectious agents via human body fluid spills and environmental surfaces. However, the antimicrobial activity of NaClO is known to be greatly affected by the

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pH of the treatment solution and can be rapidly diminished upon contact with organic matter (Wei et al., 1985). Several studies suggested a relatively poor efficiency of NaClO when it was applied to commodities such as seeds and fresh-cut produce (Beuchat et al. 2004; Nthenge et al., 2007). In addition, higher concentrations of NaClO (e.g. 10,000 ppm) were reported to be insufficient for reducing the number of microorganisms in body fluid spills, especially blood (Chitnis et al., 2004; Coates, 1988). Another concern about NaClO is that there are several reports of carcinogen production resulting from its reaction with some organic matter (Richardson, 1998a; Richardson, 1998b). Therefore, the development of alternative chlorine-based sanitizers that are relatively resistant to organic matter is desired for sanitizing environments rich in biological materials.

Another form of chlorine that is based on sodium chlorite ($NaClO_2$) has been applied for sanitization of foods or environmental surfaces. This sanitizer, "acidified sodium chlorite" (ASC; Ecolab Inc., Minnesota, USA), has been approved by the Food and Drug Administration of the United States for use on poultry, red meats, seafood, and raw agricultural commodities (Anonymous, 1998; Federal Register, 1999). ASC is a broad-spectrum antiseptic (Castillo et al., 1999; Hajmeer et al., 2004) that can be easily prepared by mixing a $NaClO_2$ solution with organic acids that are generally recognized to be safe, such as citric acid and phosphate. Although $NaClO_2$ alone shows only weak antimicrobial activity at neutral or alkaline conditions, upon strong acidification antimicrobial chlorite ($HClO_2$) is generated (Kobayashi et al., 1989). $HClO_2$, which is a strongly oxidizing acid seen only in solution, is thought to be capable of penetrating bacterial cell walls and disrupting protein synthesis. In ASC, $HClO_2$ is thought to be generated from the conversion of the chlorite ion into its acid form under acidic conditions, although under strongly acidic pH $HClO_2$ is rapidly converted to chlorine dioxide, ClO_2 (Gordon et al., 1972). Therefore, the antimicrobial properties of ASC are likely mainly attributable to ClO_2. Although ASC is a powerful and rapid-acting sanitizer, it is a short-lived antiseptic that must be prepared at the time of use.

In 2013, the Ministry of Health, Labour and Welfare of Japan approved chlorous acid water as a food additive. In response to this approval, the chlorite-based sanitizer was developed and marketed. This sanitizer contains chlorous acid water as the principle antimicrobial component and is buffered at weakly acidic conditions (pH6.0) with phosphate and citrate. Unlike ASC, the pH control around weakly acidic conditions may decrease ClO_2 production and stabilize $HClO_2$ levels for long periods of time. In this study, we evaluated the antimicrobial efficacy of this weakly acidified chlorous acid water (WACAW) and its stability in contact with organic matter. The results indicate that WACAW is suitable for environmental sanitation in healthcare and food processing applications.

MATERIALS AND METHODS

Microorganisms and culture conditions

The bacterial strains used in this study were *Staphylococcus aureus* IFO12732, *Escherichia coli* IFO3927, enterohemorrhagic *E. coli* (EHEC) O157:H7 Sakai strain and *Campylobacter jejuni* JCM2013. *Candida albicans* NBRC1594 was used as a representative yeast strain. Overnight liquid cultures of these strains at 37℃ in Brain Heart Infusion (BHI, Eiken Chemical Co. Ltd., Tokyo, for *S. aureus, C. jejuni* and *C. albicans*) or lysogeny broth (LB for *E. coli*) were centrifuged, washed with saline, and finally suspended in saline to a cell number that produced 10^7-10^8 colony-forming units (CFU)/ml. These suspensions were used as inocula to evaluate the antimicrobial activities of the test sanitizers.

Two spore-forming *Bacillus* and *Paenibacillus* species (strains KU1 and KU2, respectively) that were isolated from contaminated vinyl isolators in the animal facility of Kagawa University were also included. They were identified at the genus level by analyzing the respective 16S rDNA sequences. Spore inocula was prepared by culturing *Bacillus* and *Paenibacillus* isolates on LB agar plates for several days and collecting the resulting colonies with a sterile loop for suspension in saline. Spore-formation capacity was confirmed by staining with malachite green. The suspension was then centrifuged and the pellet was washed twice with saline before suspension to 10^7 CFU/ml of spores for use as spore inocula.

Feline calicivirus (FCV) F4 strain and the Crandell Rees feline kidney (CRFK) cell line were kind gifts from Dr. Katayama (National Institute of Infectious Diseases, Japan). Confluent CRFK cell monolayers cultured in 90 mm dishes in minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) were infected with FCV until most of the cells detached from the plate. The collected culture was centrifuged to remove cell debris and the supernatant was divided into aliquots, which were stored at -80℃ until use as a viral inoculum.

Preparation of test reagents

Sodium hypochlorite solution (NaClO; 6%) was purchased from Nankai Chemical Co., Ltd. (Osaka, Japan). The test solutions of WACAW was prepared by

diluting Autolock Super® (>1.0% as $HClO_2$, Honbu Sankei Co. Ltd., Osaka, Japan) with deionized water. The chlorine level in this sanitizer was measured as $NaClO_2$ using a previously described method based on iodometric titration (Ingram et al., 2004). The original sanitizers were kept at 4℃ in the dark. Test reagents were diluted just before examination.

In vitro bactericidal test

Glycerol stocks maintained at -80℃ for each bacterium or yeast were inoculated onto BHI or LB (for *E. coli*) agar plates. Single colonies of each strain were cultured overnight in BHI or LB (for *E. coli*) broth at 37℃. The cultures were then centrifuged and washed once with saline before resuspension in saline at cell densities of 10^7-10^8 CFU/mL. Each cell suspension (1 mL) was mixed with 8 ml of distilled water, 1 ml of 10 × sanitizer or distilled water (control) and incubated at 25℃. One milliliter of each mixture was periodically sampled and transferred to 9 mL of 0.5 M sodium thiosulfate solution for neutralization. The neutralized samples were further diluted with appropriate amounts of saline. One milliliter of each dilution was transferred to a 90 mm diameter sterile plastic dish and 20-25 mL of sterilized medium [desoxycholate agar (Nissui Pharmaceutical Co. Ltd., Tokyo) for *E. coli*, mannitol salt agar (Nissui) for *S. aureus*, or BHI (Eiken) for *C. albicans*, *Paenibacillus* and *Bacillus* species)] was poured into the dishes, gently mixed, solidified, and incubated at 37℃ for 48 h to enumerate the surviving test microorganisms.

Efficacy of WACAW on FCV inactivation

Antiviral activities of the test sanitizers against FCV were assessed by a standard plaque assay. The viral titer of stock FCV was 2×10^6 plaque-forming units (PFU)/mL. The FCV solution (0.5 mL) was mixed with 4.0 mL distilled water and 0.5 mL 10 × sanitizer (final concentrations: 200 ppm, 400 ppm or 1,000 ppm). In tests to assess the effect of organic matter on the antiviral activities of the sanitizers, bovine serum albumin (Wako Chemical Co. Ltd., Tokyo) was added to a final concentration of 0.05%. These mixtures were incubated at 25℃ in a water bath. The samples (0.3 mL) were collected periodically (5 min and 10 min after treatment) and neutralized with 2.7 mL 0.5 M sodium thiosulfate. The neutralized solutions (0.5 mL each) were poured onto a CRFK confluent culture monolayer [grown in 6 well plates containing MEM plus 5% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and washed with phosphate-buffered saline (PBS) before treatment] and rocked for 1 h at room temperature. After removing the viral solution, the cells were overlaid with 0.5% methylcellulose in MEM plus 0.5% FBS. The treated cells were incubated in a CO_2 incubator at 37℃ under a 5% CO_2 atmosphere. After 20-24 h, the cells were fixed and stained with 0.05% crystal violet in a 10% formaldehyde solution for 4 h. The cells were then washed vigorously with tap water. The clear plaques were enumerated and the PFU/mL of the surviving FCV was calculated.

Efficacy of eliminating food-borne pathogens from the surface of broiler carcasses

Broiler carcasses were purchased from a local market in Takamatsu city, Kagawa, Japan. Pieces of broiler carcasses (100-200 g) were placed in a stomacher bag and uniformly sprayed with 3 ml of EHEC O157:H7 (10^8 CFU/mL) bacterial suspension or *C. jejuni* (10^8 CFU/mL). After being dried on a clean bench, the carcass pieces were dipped into 10 volumes (v/w) of sanitizers (100, 200, 300 or 400 ppm) for 30 min and then transferred to 1,000 ml distilled deionized water to remove the test sanitizers. The test pieces were then removed from the water and placed on a stainless net to remove residual water. Samples (5-10 g) were cut out randomly from two sites, transferred to stomacher bags to which 10 volumes (v/w) of saline were added. The samples were homogenized at maximum speed for 5 min in a laboratory stomacher (As One Co. Ltd., Osaka, Japan). The homogenized samples were appropriately diluted with saline and the surviving bacterial cells were enumerated using MacConkey agar (Nissui) for EHEC O157:H7 or CCDA agar (Merck, Germany) for *C. jejuni*. After 48-h cultivation at 37℃, EHEC colonies growing on the plates were counted. For *C. jejuni*, the bacterial counts were measured after incubation at 42℃ for 48 h under microaerophilic conditions (5% CO_2) using the GasPack system (Mitsubishi Gas Chemical Co. Ltd., Tokyo).

Stability test on unwoven fabric

To test the stability of NaClO and WACAW on nonwoven fabric, we checked the antimicrobial activity of solutions recovered from wipes moistened with these sanitizers. Nonwoven fabrics (350 g, 30 cm × 40 cm, Hashimoto-Cloth Co. Ltd., Shiga, Japan) were moistened with four volumes (v/w) of the test disinfectants (1,000 and 6,000 ppm each of NaClO and WACAW) and preserved in tightly sealed plastic containers. The test solution was periodically wrung out from the five pieces of wipe (on day 0, 3, 7, 14, 21 and 28 after moistening) and the bactericidal activities of the recovered disinfectants were assessed using non-pathogenic *E. coli* and *S. aureus* as described above.

Statistical analysis

Quantitative microbiological measurements were transformed to $\log_{10}$ prior to conducting an analysis. All experiments were performed at least three times. The data were analyzed by analysis of variance (ANOVA) and Tukey's test (StatFlex ver.6, Artec Co., Ltd., Tokyo) to assess the significance. p values less than 0.05 were considered to be significant.

RESULTS

Antimicrobial activity of WACAW against bacteria and yeast

The results of a killing test against the selected bacteria and yeast are shown in Table 1. NaClO at all concentrations tested (50, 100, and 400 ppm) and WACAW at more than 100 ppm achieved a >4 $\log_{10}$ reduction within 5 min in all non-spore-forming bacteria (*S. aureus* IFO12732, *E. coli* IFO3927 and EHEC O157:H7 Sakai) and a yeast (*C. albicans* NBRC1594). At a lower concentration (50 ppm), the killing activity of WACAW against *S. aureus* and *C. albicans* seemed weaker than that of NaClO with a short contact time. For spore-forming bacteria, 400 ppm NaClO reduced *Paenibacillus* and *Bacillus* species by 2.72 ± 0.84 and 0.98±0.36 $\log_{10}$CFU/mL after 30 min contact, respectively (Table 2). In contrast, WACAW achieved a >5 $\log_{10}$ reduction in *Paenibacillus* and *Bacillus* species following treatment with 400 ppm for 30 min. Compared to NaClO, WACAW over 200 ppm showed a superior capability to reduce *Paenibacillus* and *Bacillus* spores with a 30-min exposure.

Antiviral activity of WACAW

To assess the efficacy of WACAW on HNoV inactivation in vitro, we investigated the antiviral activity of this sanitizer against FCV, a HNoV surrogate, by

TABLE 1. Comparison of the killing activity of WACAW and NaClO towards bacteria and yeast

Treatment	Reduction numbers ($\log_{10}$CFU/mL)*			
	S. aureus	*E. coli*	EHEC O157:H7	*C. albicans*
WACAW				
50 ppm	3.84 ± 0.27[a]	>4.00[a]	>4.00[a]	2.83 ± 0.20[a]
100 ppm	>4.00[a]	>4.00[a]	>4.00[a]	>4.00[b]
200 ppm	>4.00[a]	>4.00[a]	>4.00[a]	>4.00[b]
NaClO				
50 ppm	>4.00[a]	>4.00[a]	>4.00[a]	>4.00[b]
100 ppm	>4.00[a]	>4.00[a]	>4.00[a]	>4.00[b]
200 ppm	>4.00[a]	>4.00[a]	>4.00[a]	>4.00[b]

*Initial cell density of each strain in the test mixture was 10^6 CFU/mL. The reduction number was calculated by subtracting the viable count after each treatment from that after treatment with deionized water (control). Within the same column, means ± standard deviation followed by different letters were significantly different ($p<0.05$). Data are reported as $\log_{10}$CFU/mL.

TABLE 2. Comparison of the killing activity of WACAW and NaClO against spore-forming bacteria

Treatment	Reduction numbers ($\log_{10}$CFU/mL)*					
	Paenibacillus sp.			*Bacillus* sp.		
	5 min	10 min	30 min	5 min	10 min	30 min
NaClO						
100 ppm	0.90 ± 0.23[a]	1.28 ± 0.64[a]	1.16 ± 0.64[a]	0.11 ± 0.10[a]	0.58 ± 0.14[a]	0.47 ± 0.43[a]
200 ppm	0.86 ± 0.16[a]	1.30 ± 0.71[a]	2.52 ± 0.58[a]	0.50 ± 0.46[a]	0.80 ± 0.20[a]	1.14 ± 0.12[a]
400 ppm	2.08 ± 0.31[b]	2.12 ± 0.19[a,b]	2.72 ± 0.84[a]	1.05 ± 0.29[a,b]	0.82 ± 0.42[a]	0.98 ± 0.36[a]
WACAW						
100 ppm	0.83 ± 0.29[a]	0.96 ± 0.18[a]	1.40 ± 0.35[a]	1.31 ± 0.08[b]	0.90 ± 0.60[a]	1.00 ± 0.44[a]
200 ppm	1.51 ± 0.65[a]	2.24 ± 0.94[a,b]	4.68 ± 1.00[b]	1.98 ± 0.84[b,c]	3.01 ± 2.02[a,b]	3.98 ± 1.43[b]
400 ppm	2.16 ± 0.58[b]	3.75 ± 1.71[b]	5.26 ± 0.00[b]	2.85 ± 0.07[c]	4.56 ± 1.35[b]	5.41 ± 0.00[b]

*Initial cell density of each spore-forming isolate in the test mixture was 10^6 CFU/mL. The reduction number was calculated by subtracting the viable count after each treatment from that after treatment with deionized water (control). Within the same column, means ± standard deviation followed by different letters were significantly different ($p<0.05$). Data are reported as $\log_{10}$CFU/mL.

TABLE 3. Comparison of the killing activity of WACAW and NaClO against feline calicivirus

Treatment	Reduction in FCV numbers (log_{10}PFU/mL)*			
	BSA (−)		BSA (+)	
	5 min	10 min	5 min	10 min
WACAW				
200 ppm	3.16 ± 0.36[a]	3.14 ± 0.36[a]	0.82 ± 0.12[a]	2.20 ± 0.49[a]
400 ppm	>4.00[b]	>4.00[b]	2.95 ± 0.38[b,c]	3.04 ± 0.57[a,b]
1,000 ppm	>4.00[b]	>4.00[b]	>4.00[c,d]	>4.00[b]
NaClO				
200 ppm	>4.00[b]	>4.00[b]	1.49 ± 0.71[a,e]	0.43 ± 0.45[c]
400 ppm	>4.00[b]	>4.00[b]	2.55 ± 0.60[b,e]	2.91 ± 0.41[a]
1,000 ppm	>4.00[b]	>4.00[b]	>4.00[c,d]	>4.00[b]

*Initial FCV number in the test mixture was 2×10^5 PFU/mL. The reduction number was calculated by subtracting the plaque count after each treatment from that after treatment with deionized water (control).Within the same column, means ± standard deviation followed by different letters were significantly different ($p<0.05$). Data are reported as log_{10}PFU/mL. BSA (-) and BSA (+) indicate the examinations without and with 0.05% bovine serum albumin, respectively, in the test mixtures at the final concentration.

means of a standard plaque assay. To examine the effect of organic matter on the antiviral activity of the test sanitizers, test solutions containing 0.05% bovine serum albumin (BSA) were also included. As shown in Table 3, WACAW achieved >3 log_{10}PFU/mL reduction at all concentrations tested (200, 400 and 1,000 ppm) in the absence of BSA. Although BSA affected the antiviral efficacy of both NaClO and WACAW, these sanitizers still sufficiently reduced the FCV number (>4 log_{10}PFU/mL) in the presence of BSA when a 1,000 ppm concentration was applied. Overall, the antiviral activity of NaClO and WACAW against FCV was similar.

Efficacy of WACAW against food-borne pathogens in broiler carcasses

We next examined the efficacy of WACAW on the decontamination of food surfaces. After broiler carcass pieces artificially contaminated by *C. jejuni* or EHEC O157:H7 were dipped into WACAW or NaClO for 30 min, the residual numbers of these foodborne pathogens were determined. As shown in Table 4, WACAW achieved a >3 log_{10}CFU/mL reduction in *C. jejuni* when a 300 ppm and 400 ppm concentration was used. For EHEC O157:H7, a >2 log_{10}CFU/mL reduction was obtained with 200-400 ppm WACAW. In contrast, 400 ppm NaClO reduced both pathogens by <1 log_{10}CFU/mL (0.81±0.26 for *C. jejuni* and 0.91±0.08 for EHEC O157:H7). These data indicate that WACAW is an effective sanitizer for food decontamination purposes, provided that a sufficient contact time is allowed.

TABLE 4. Efficacy of WACAW to eliminate *Campylobacter* or EHEC O157:H7 in broiler carcasses

Treatment	Reduction numbers (log_{10}CFU/g)*	
	C. jejuni	EHEC O157:H7
WACAW		
100 ppm	1.42 ± 0.06[a]	1.18 ± 0.49[a]
200 ppm	2.66 ± 0.01[b]	2.13 ± 0.08[b]
300 ppm	3.77 ± 0.34[c]	2.13 ± 0.08[b]
400 ppm	3.98 ± 0.68[c]	2.33 ± 0.17[b]
NaClO		
400 ppm	0.81 ± 0.26[d]	0.91 ± 0.08[a]

*100-200 g pieces of broiler carcasses were uniformly sprayed with 3 ml bacterial suspension of *C. jejuni* JCM2013 or EHEC O157:H7 Sakai (10^8 CFU/mL each). After drying, the carcass pieces were dipped into WACAW or NaClO at the indicated concentrations for 30 min. After washing the pieces by dipping them in 1,000 mL distilled water, 5-10 g of the samples were cut out and homogenized. The surviving bacteria were enumerated by the plate culture method. Within the same column, means ± standard deviation followed by different letters were significantly different ($p<0.05$). Data are reported as log_{10}CFU/mL.

Potential of WACAW-moistened wipes for chlorine-based environmental sanitation

From the data described above, WACAW appears to gradually release its antimicrobial component ($HClO_2$) and maintain its antimicrobial activity even after contact with organic matter. This characteristic prompted the evaluation of the possibility to preserve this sanitizer in the form a wet wipe, similar to alcohol wipes in current use. To evaluate the effectiveness of WACAW-moistened wipes as a sanitation tool in healthcare

TABLE 5. Stability of WACAW on fabric

Preservation period (days)	Reduction numbers (log_{10}CFU/mL)*			
	E. coli		*S. aureus*	
	5 min	10 min	5 min	10 min
NaClO (1,000 ppm)				
0	$7.18 \pm 0.00^{a,b}$	7.18 ± 0.00^{a}	6.98 ± 0.00^{a}	6.99 ± 0.10^{a}
1	$6.79 \pm 0.00^{a,b}$	6.79 ± 0.00^{b}	$6.32 \pm 0.00^{a,b}$	6.14 ± 0.00^{b}
3	6.58 ± 0.32^{b}	6.47 ± 0.00^{c}	$6.47 \pm 0.00^{a,b}$	$6.47 \pm 0.00^{a,b}$
7	0.00 ± 0.09^{d}	0.42 ± 0.16^{f}	$0.23 \pm 0.08^{d,e}$	0.14 ± 0.19^{d}
14	0.05 ± 0.08^{d}	0.06 ± 0.01^{g}	$0.10 \pm 0.01^{d,e}$	0.61 ± 0.11^{d}
21	0.34 ± 0.04^{d}	$0.66 \pm 0.04^{e,f}$	0.01 ± 0.05^{e}	0.01 ± 0.03^{d}
28	0.09 ± 0.04^{d}	0.11 ± 0.02^{g}	2.22 ± 0.00^{c}	$0.70 \pm 1.29^{c,d}$
WACAW (1,000 ppm)				
0	$7.18 \pm 0.00^{a,b}$	7.18 ± 0.00^{a}	6.98 ± 0.00^{a}	6.99 ± 0.00^{a}
1	$6.79 \pm 0.00^{a,b}$	6.79 ± 0.00^{b}	$6.32 \pm 0.00^{a,b}$	5.98 ± 0.28^{b}
3	$6.69 \pm 0.45^{a,b}$	6.47 ± 0.00^{c}	$6.47 \pm 0.00^{a,b}$	$6.47 \pm 0.00^{a,b}$
7	5.67 ± 0.00^{c}	$6.67 \pm 0.00^{b,c}$	$6.52 \pm 0.00^{a,b}$	$6.53 \pm 0.00^{a,b}$
14	0.31 ± 0.03^{d}	$0.60 \pm 0.08^{e,f}$	$0.04 \pm 0.04^{d,e}$	0.49 ± 0.06^{d}
21	0.00 ± 0.15^{d}	$0.25 \pm 0.13^{f,g}$	0.00 ± 0.05^{e}	0.12 ± 0.20^{d}
28	0.12 ± 0.01^{d}	0.11 ± 0.02^{g}	0.81 ± 1.23^{d}	0.00 ± 0.02^{d}
NaClO (6,000 ppm)				
0	$5.92 \pm 0.70^{b,c}$	$6.71 \pm 0.70^{b,c}$	5.95 ± 0.00^{b}	6.05 ± 0.00^{b}
1	5.18 ± 0.72^{c}	5.22 ± 0.22^{d}	$6.32 \pm 0.00^{a,b}$	6.14 ± 0.00^{b}
3	$6.76 \pm 0.00^{a,b}$	6.83 ± 0.00^{a}	$6.52 \pm 0.00^{a,b}$	$6.60 \pm 0.00^{a,b}$
7	0.48 ± 0.11^{d}	0.50 ± 0.07^{e}	0.06 ± 0.02^{c}	0.19 ± 0.21^{d}
14	0.00 ± 0.02^{d}	$0.00 \pm 0.00^{f,g}$	0.00 ± 0.20^{c}	0.00 ± 0.01^{d}
21	0.36 ± 0.10^{d}	$0.39 \pm 0.14^{f,g}$	0.33 ± 0.02^{c}	1.53 ± 0.27^{c}
28	0.00 ± 0.01^{d}	0.04 ± 0.05^{g}	0.26 ± 0.05^{c}	0.31 ± 0.05^{d}
WACAW (6,000 ppm)				
0	6.57 ± 0.28^{b}	$6.71 \pm 0.00^{b,c}$	5.95 ± 0.00^{b}	6.05 ± 0.00^{b}
1	$6.79 \pm 0.00^{a,b}$	6.79 ± 0.00^{b}	$6.32 \pm 0.00^{a,b}$	6.14 ± 0.00^{b}
3	7.34 ± 0.00^{a}	7.34 ± 0.00^{a}	7.10 ± 0.28^{a}	7.13 ± 0.00^{a}
7	$7.18 \pm 0.00^{a,b}$	7.28 ± 0.00^{a}	6.98 ± 0.00^{a}	6.99 ± 0.00^{a}
14	$7.18 \pm 0.00^{a,b}$	6.77 ± 0.00^{b}	$6.52 \pm 0.00^{a,b}$	$6.53 \pm 0.00^{a,b}$
21	$7.18 \pm 0.00^{a,b}$	6.77 ± 0.00^{b}	$6.34 \pm 0.00^{a,b}$	$6.30 \pm 0.00^{a,b}$
28	6.51 ± 0.00^{b}	$6.51 \pm 0.00^{b,c}$	$6.41 \pm 0.00^{a,b}$	$6.46 \pm 0.00^{a,b}$

*Initial cell density of each strain in the test mixture was 10^6-10^7 CFU/mL. Within the same column, means ± standard deviation followed by different letters were significantly different ($p<0.05$). Data are reported as log_{10}CFU/mL.

settings, antibacterial activities of NaClO solutions and WACAW in nonwoven fabric were compared for 28 d. The total chlorine levels of NaClO and WACAW were first adjusted to 1,000 ppm or 6,000 ppm. The total chlorine level of 1,000 ppm NaClO decreased to 50 ppm following 3-d preservation on a wipe while that of 6,000 ppm WACAW was maintained at 6,000 ppm for 28 d (data not shown). Consistently, NaClO of both 1,000 ppm and 6,000 ppm completely lost its antibacterial activity 7 d after preparation (Table 5). On the other hand, 1,000 ppm WACAW showed >5 log_{10} CFU reduction in *E. coli* and *S. aureus* until 7 d after preparation. In the case of 6,000 ppm WACAW, its bactericidal activities against *S. aureus* and *E. coli* were kept for at least 28 d in fabrics. All recovered solutions from 6,000 ppm WACAW-moistened fabrics reduced *E. coli* to under detection limits within 5 min and achieved sufficient reductions of *S. aureus* (5.95-7.10 log_{10} CFU reduction within 5 min and under the detection limit with a 10 min exposure). These data indicate that WACAW is more stable in fabric than NaClO.

DISCUSSION

Appropriate selection of a disinfectant is of great importance to reduce the risk of nosocomial and food-borne pathogen outbreaks. Chlorine is a powerful oxidizing agent that shows a wide antimicrobial spectrum and sporicidal activity when used at high concentrations. Therefore, chlorine-based sanitizers are

generally recommended to reduce the microbial load on environmental surfaces in hospitals and food processing facilities. NaClO (>1,000 ppm) is a primary disinfectant in healthcare settings to inactivate spores (e.g. *C. difficile*) and non-enveloped viruses (e.g. HNoV), which often cause outbreaks via transmission to humans following contact with contaminated environmental surfaces (Cohen et al., 2013; Doultree et al., 1999; Duizer et al., 2004; Girard et al., 2010; Park et al., 2007).

Sodium chlorite ($NaClO_2$) is also an oxidizing agent but its antimicrobial activity is weak compared to NaClO (Kobayashi et al., 1989). However, its strongly acidified form (pH2.3-2.9) called ASC shows rapid and powerful antimicrobial activity, and this compound has been applied for sanitation of various kinds of agricultural commodities and meats (Olaimat and Holley, 2012). ASC at 800 ppm was reported to achieve a 4.0 $\log_{10}$ CFU/g reduction of *Salmonella* on alfalfa seeds, which was 1.2 $\log_{10}$ unit higher than that seen for 20,000 ppm $Ca(ClO)_2$ (Liao, 2009). Thus, pH control is one strategy to increase the antimicrobial activity of $NaClO_2$. Acidification of $NaClO_2$ solutions produces chlorous acid ($HClO_2$), and this acid is capable of penetrating the bacterial membrane, and exerts its bactericidal effect by affecting protein functions inside cells (Kemp et al., 2000). However, $HClO_2$ is rapidly converted to chlorine dioxide (ClO_2) under strong acidic conditions as in ASC (Gordon et al., 1972); thus ClO_2 is considered to be a principle component of the strong antimicrobial activity observed for ASC. Because ClO_2 is easily released into the air, the antimicrobial activity of ASC decreases relatively quickly after preparation. Therefore, ASC must be prepared by mixing the $NaClO_2$ solution with "generally recognized as safe (GRAS)" organic acids such as citrate, phosphate and lactate immediately before use.

Recently, the usefulness of a stabilized oxychloro-sanitizer called Germin-8-or® for sanitation of sprouts was reported (Hora et al., 2007; Kumer et al., 2006). Adjustment of the pH of this $NaClO_2$-based sanitizer to 7.2 resulted in efficient reduction of *Salmonella* (>4 $\log_{10}$ CFU/g) on mung bean seeds, while only a limited reduction (1.2 $\log_{10}$ decrease) was obtained with 20,000 ppm $Ca(ClO)_2$ (Hora et al., 2007). Germin-8-or® appears to require long treatment times for efficient elimination of contaminated bacteria and a 8 to19 h treatment was reported to be necessary to achieve pathogen elimination on artificially contaminated seeds (Hora et al., 2007). This slow action is probably due to the low $HClO_2$ generation from the chlorite ion at neutral pH. However, to date few reports have examined the antimicrobial activity and stability of "weakly acidic" chlorous acid solution.

In this study, we evaluated the antimicrobial activity and stability of the novel chlorous-acid-based sanitizer WACAW (Autolock Super®), which has been recently marketed in Japan (Honbu Sankei Co., Ltd., Osaka, Japan). This is the first commercially available chlorous-acid-based sanitizer with a pH adjusted to be around the weakly acidic range (pH 6.0) that aims to increase the antimicrobial activity and stability in organic matter-rich environments such as that seen for ASC and Germin-8-or®, respectively. An in vitro antimicrobial test of WACAW showed efficacy that was equivalent or superior to NaClO for inactivating bacterial pathogens, yeast and *Bacillus* spores. In addition, a 5 min treatment with this sanitizer at more than 400 ppm was effective at inactivating FCV in the absence of BSA. Chlorine-based sanitizers such as NaClO and ClO_2 were previously reported to be ineffective for spore elimination unless the spore coat protein had been removed (Young and Setlow, 2003). At present, why WACAW is effective against spores is unclear, but one explanation might be that $HClO_2$ destabilizes the spore coat by preferentially reacting with disulfide bond-rich spore coat proteins such as those seen in *Bacillus subtilis* to enhance the penetration of the acid into the spore (Driks, 1999).

From the results shown in Tables 3 and 4, WACAW is unlikely to lose its antimicrobial activity as quickly as NaClO upon contact with organic matter, indicating the difference between the killing mechanisms of chlorite and hypochlorite. To assess the applicability of this sanitizer for decontaminating food surfaces, the broiler carcass pieces artificially contaminated with *C. jejuni* or EHEC O157:H7 were prepared. WACAW was shown to be effective in reducing (>2 $\log_{10}$CFU/mL reduction) foodborne pathogens on these carcasses when they were dipped into 200-400 ppm of this sanitizer for 30 min at 25℃, while NaClO was not effective even at 400 ppm. These results are consistent with previous work, in which the acidified form of $NaClO_2$ effectively reduced EHEC O157:H7 on fresh-cut cilantro where NaClO achieved only a 1-1.3 $\log_{10}$CFU/g reduction (Allende et al., 2009).

The mode of action against lipid membranes was previously reported to be different for NaClO and $NaClO_2$ in that NaClO rapidly reacted with membrane phospholipids and generated large amounts of chlorohydrins by electrophilic attack, while $NaClO_2$ did not generate chlorohydrins but instead induced peroxide generation that led to oxidative stress in bacteria mediated by oxygen radicals (Ingram et al., 2003). Kumer et al. (2007) concluded in their report that this mode of action is attributable to the slow reaction of stabilized $NaClO_2$, Germin-8-or®. $NaClO_2$ is known to preferentially react with glutathione, indicating

that the microbicidal activity of this agent is derived from the inactivation of disulfide bond-rich proteins present in the target microorganisms. Kumar et al. (2007) also demonstrated that the glutathione level was decreased by Germin-8-or®, and that alkaline phosphatase, which contains two disulfide bonds, was more susceptible to chlorite than other proteins that possess a single disulfide bond. Ingram et al. (2003) further reported that the microbicidal effect of $NaClO_2$ against *S. aureus* was lower than that against *E. coli*, and they attributed the relative chlorite resistance of *S. aureus* to the absence of glutathione in this organism. Similarly, WACAW was less effective against *S. aureus* than *E. coli* (Table 1). Therefore, the mode of action of WACAW is likely similar to that of $NaClO_2$ and Germin-8-or®. It has been reported that the elimination time required by Germin-8-or® for *Salmonella* on sprouted seeds was 19 h (Hora et al., 2007). Although a simple comparison of WACAW (pH6.0) with Germin-8-or® (pH7.2) is difficult, the weak acidification of $HClO_2$ as seen in WACAW seems to be successful in enhancing the reactivity of this sanitizer to microbial proteins.

Exposure to light, high temperature and especially contact with organic substances markedly reduces the sanitizing activity of chlorine. Therefore, preserving the sanitizing capacity of NaClO in wipes as with alcohol is difficult, even though NaClO is a main disinfectant for environmental sanitation in hospitals or nursing homes. In addition to microbicidal activity, stability and safety are other critical factors in developing sanitizers. In this regard, WACAW was shown to maintain bactericidal effect for at least 28 d on fabrics (100% cotton) while NaClO rapidly lost antimicrobial activity and the chlorine level of 1,000 ppm NaClO decreased from 1,000 ppm to 50 ppm after only 3 d (data not shown). The difference in action between NaClO and $NaClO_2$ described above is likely attributable to the differences in the stability on fabrics between NaClO and WACAW, wherein HClO may progressively attack electron-dense hydroxyl groups in cellulose fibers while $HClO_2$ seems to be relatively unresponsive to them. The stability of WACAW in fabrics might thus create an application for this compound in chlorine-based sanitation wipes similar to alcohol-based wipes that was previously difficult due to the instability of chlorine-based sanitizers in fabrics. In addition, contaminated fabrics and carpets have been reported to be sources of HNoV transmission (Dalling, 2004). Since few studies on the efficacy of contaminated fabric disinfection are available, it is therefore important to evaluate in future studies the effectiveness of WACAW in eliminating HNoV-contamination from fabrics such as curtains, towels and other cloth products.

In conclusion, this study evaluated the novel chlorous-acid-based sanitizer (WACAW). This sanitizer exhibited microbicidal activity against a wide range of microorganisms, including yeast and spore-forming bacteria. WACAW is also effective even following contact with organic matter-rich objects such as broiler carcasses and fabrics (100% cotton). This characteristic will prove valuable for applications of this sanitizer in healthcare or food processing settings. Strong acidification of $NaClO_2$ remarkably enhances its microbicidal capability but decreases the stability, which results in the rapid loss of antimicrobial power. The weak acidification shown in this study is likely to help maintain both the antimicrobial power and stability of $HClO_2$, although to obtain maximal advantages of $HClO_2$ precise determination of the optimal acidic range will be needed.

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REFERENCES

Allende, A., McEvoy, J., Tao, Y., and Luo, Y. (2009) Antimicrobial effect of acidified sodium chlorite, sodium chlorite, sodium hypochlorite, and citric acid on *Escherichia coli* O157:H7 and natural microflora of fresh-cut cilantro. *Food Control*, **20**, 230-234.

Anonymous (1998) Secondary direct food additives permitted in food for human consumption-acidified sodium chlorite solutions. Code of Federal Regulations, 21 CFR 173.325. Office of the Federal Register, U.S. Government Printing Office, Washington, D.C.

Beuchat L. R. (1998) Surface decontamination of fruits and vegetables eaten raw: a review. Geneva, Switzerland: World Health Organization, Food Safety Unit. Available from: WHO/FSF/FOS/98.2.

Beuchat, L. R., Adler, B. B., and Lang, M. M. (2004) Efficacy of chlorine and a peroxyacetic acid sanitizer in killing *Listeria monocytogenes* on iceberg and romaine lettuce using simulated commercial processing conditions. *J. Food Prot.*, **67**, 1238-1242.

Castillo, A., Lucia, L. M., Kemp, G. K., and Acuff, G. R. (1999) Reduction of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium on beef carcass surfaces using acidified sodium chlorite. *J. Food Prot.*, **62**, 580-584.

Chitnis, V., Chitnis, S., Patil, S., and Chitnis, D. (2004) Practical limitations of disinfection of body fluid spills with 10,000 ppm sodium hypochlorite (NaOCl). *Am. J. Infect. Control*, **32**, 306-308.

Coates, D. (1988) Comparison of sodium hypochlorite and sodium dichloroisocyanurate disinfectant: neutralization by serum. *J. Hosp. Infect.*, **11**,60-67.

Cohen, S. H., Gerding, D. N., Johnson, S., Ciaran, P. K., Loo, V. G., McDonald, L. C., Pepin, J., and Wilcox, M. H.

(2013) Clinical Practice Guidelines for *Clostridium difficile* Infection in Adults: 2010 Update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). *Infect. Control Hosp. Epidemiol.*, **31**, 423-455.

Dalling, J. (2004) A review of environmental contamination during outbreaks of Norwalk-like virus. *Br. J. Infect. Control*, **5**, 9-13.

Doultree, J. C., Druce, J. D., Birch, C. J., Bowden, D. S., and Marshall, J. A. (1999) Inactivation of feline calicivirus, a Norwalk virus surrogate. *J. Hosp. Infect.*, **41**, 51-57.

Driks, A. (1999) *Bacillus subtilis* spore coat. *Microbiol. Mol. Biol. Rev.*, **63**, 1-20.

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F., and Koopmans M. (2004) Inactivation of caliciviruses. *Appl. Environ. Microbiol.*, **70**, 4538-4543.

Federal register (1999) Secondary direct food additives permitted in food for human consumption; final rule. *Fed. Regist.*, **64**, 49981-49982.

Girard, M., Ngazoa, S., Mattison, K., and Jean, J. (2010) Attachment of noroviruses to stainless steel and their inactivation using household disinfectants. *J. Food Prot.*, **73**, 400-404.

Gordon, G., Kieffer, R. G., and Rosenblatt, D. H. (1972) The chemistry of chlorine dioxide. In *Progress in inorganic chemistry* **15** (Lippard, S. J., ed.), pp.15201-15286. John Wiley & Sons, New York.

Hajmeer, M. N., Marsden, J. L., Fung, D. Y. C., and Kemp, G. K. (2004) Water, sodium chloride, and acidified sodium chlorite effects on *Escherichia coli* O157:H7 and *Staphylococcus aureus* on beef briskets. *Meat Sci.*, **68**, 277-283.

Hora, R., Kumar, M., Kostrzynska, M., Dixon, M. A., and Warriner, K. (2007) Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on artificially or naturally contaminated mung beans (*Vigna radiate* L) using a stabilized oxychloro-based sanitizer. *Lett. in Appl. Microbiol.*, **44**, 188-193.

Ingram, P. R., Homer, N. Z. M., Smith, R. A., Pitt, A. R., Wilson, C. G., Olejnik, O., and Spickett, C. M. (2003) The interaction of sodium chlorite with phospholipids and glutathione: a comparison of effects in vitro, in mammalian and in microbial cells. *Arch. Biochem. Biophys.*, **410**, 121-133.

Ingram, P. R., Pitt, A. R., Wilson, C. G., Olejnik, O., and Spickett, C. M. (2004) A comparison of the effects of ocular preservatives on mammalian and microbial ATP and glutathione levels. *Free Radic. Res.*, **38**, 739-750.

Kemp, G. K., Aldrich, M. L., and Waldroup, A. L. (2000) Acidified sodium chlorite antimicrobial treatment of broiler carcasses. *J. Food Protect.*, **63**, 1087-1092.

Kobayashi, M., Akiyama, S., Iwashita, M., and Suzuki, A. (1989) A study of the bactericidal effect of a sodium chlorite preparation. *J. Food Hyg. Soc. Jap.*, **30**, 367-374.

Kumar, M., Hora, R., Kostrzynska, M., Waites, W. M., and Warriner, K. (2006) Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on mung beans, alfalfa, and other seed types destined for sprout production by using an oxychloro-based sanitizer. *J. Food Prot.*, **69**, 1571-1578.

Kumar, M., Hora, R., Kostrzynska, M., and Warriner K. (2007) Mode of *Salmonella* and *Escherichia coli* O157:H7 inactivation by a stabilized oxychloro-based sanitizer. *J. Appl. Microbiol.*, **102**, 1427-1436.

Liao, C. H. (2009) Acidified sodium chlorite as an alternative to chlorine for elimination of *Salmonella* on alfalfa seeds. *J. Food Sci.*, **74**, 159-164.

MacCannell, T., Umscheid, C. A., Agarwal, R. K., Lee, I., Kuntz, G., Stevenson, K. B., and the Healthcare Infection Control Practice Advisory Committee (HICPAC) (2011). Guideline for the prevention and control of norovirus gastroenteritis outbreaks in healthcare settings. http://www.cdc.gov/hicpac/pdf/norovirus/Norovirus-Guideline-2011.pdf.

Nthenge, A. K., Weese, J. S., Carter, M., Wei, C., and Huang, T. (2007) Efficacy of gamma radiation and aqueous chlorine on Escherichia coli O157:H7 in hydroponically grown lettuce plants. *J. Food Prot.*, **70**, 748-752.

Olaimat, A. N., and Holley, R. A. (2012) Factors influencing the microbial safety of fresh produce: A review. *Food Microbiol.*, **32**, 1-19.

Park, G. W., Boston, D. M., Kase, J. A., Sampson, M. N., and Sobsey, M. D. (2007) Evaluation of liquid- and fog-based application of Sterilox hypochlorous acid solution for surface inactivation of human norovirus. *Appl. Environ. Microbiol.*, **73**, 4463-4468.

Richardson, S. D. (1998a) Drinking water disinfection by-product. In *the Encyclopedia of Environmental Analysis and Remediation* 3 (Meyers, R. A. ed.), pp.1398-1421, John Wiley & Sons, New York.

Richardson, S. D., Thurston, A. D., Caughran, T. V., Collette, T. W., Patterson, K. S., and Lykins, B.W. (1998b) Chemical by-products of chlorine and alternative disinfectants. *Food Technol.*, **52**, 58-61.

Wei, C. I., Cook, D. L., and Kirk, J. R. (1985) Use of chlorine compounds in the food industry. *Food Technol.*, **39**, 107-115.

Young, S. B., and Setlow, P. (2003) Mechanisms of killing of *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. *J. Appl. Microbiol.*, **95**, 719-727.