Original paper

Effects of Chlorine-Based Antimicrobial Treatments on the Microbiological Qualities of Selected Leafy Vegetables and Wash Water

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The disinfection efficacy of sodium hypochlorite (NaClO) and chlorine dioxide (ClO₂) was evaluated singly or in combination with micro-bubbling, ultra-sonication, or mild heat (50°C) against microorganisms on lettuce, spinach and Chinese cabbage. There were no significant differences in reductions of either intrinsic microflora or inoculated *Escherichia coli* O157:H7 between 5 and 10 min of treatment time, between 50, 100, 200 ppm NaClO, or between 25 and 50 ppm ClO₂. However, mild heat (50°C) significantly enhanced efficacy, with maximum log reductions of 2.91 in total plate count, 4.10 in total coliform count, and 2.13 – 2.53 in *E. coli* O157:H7. Heat was more effective when applied before or with chlorination than after. The concentration and efficacy of the chlorine wash water gradually decreased with reuse, allowing cross-contamination when the initial concentration was less than 50 ppm. These results can be used to guide the fresh-cut vegetable industry in designing chlorine-based treatments for better control of safety and quality.

Keywords: *Escherichia coli* O157:H7, leafy vegetable, mild heat, chlorine-based sanitizer, cross-contamination

Introduction

The popularity of fresh and fresh-cut vegetables has increased in line with consumer perceptions that such products are healthy, tasty, convenient, and fresh (Garrett *et al.*, 2003). Fruits and vegetables supply antioxidants, which are increasingly recognized as alleviating a number of degenerative processes, including cardiovascular disease, cancer, and aging (Kaur and Kapoor, 2001). Simultaneously, the preference for such foods has also increased consumers' risk of contracting microbial foodborne illnesses. This is attributed to the fact that vegetables can transport pathogenic bacteria from contaminated compost or irrigation water and from workers handling the produce on the farm (De Roever, 1998; Oliveira *et al.*, 2011). Harris *et al.* (2003) identified *Clostridium botulinum*, *Escherichia coli* O157:H7, *Salmonella* spp., *Shigella* spp., and *Listeria monocytogenes* as common pathogenic bacteria in produce-associated outbreaks. Outbreaks of *E. coli* O157:H7

infection have been related to the consumption of fresh produce, such as lettuce, carrots, spinach, alfalfa sprouts, radish sprouts, berries, and grapes (Abadias *et al.*, 2012; Beuchat, 1996; Huang and Chen 2011). Recently, there was a large outbreak of hemolytic uremic syndrome caused by Shiga toxin-producing *E. coli* O104:H4 associated with sprouts in Germany (Buchholz *et al.*, 2011), and an outbreak of *E. coli* O157:H7 infection due to the consumption of lightly pickled Chinese cabbage in Japan (Ministry of Health, Labor and Welfare, Japan, 2012).

Currently, farmers are provided with guidance or protocols to ensure food safety at the farm level and with provisions or practices in every area. These practices are geared towards prevention of hazards from entering into the food chain; however, this may not be sufficient. Thus, it is imperative to establish effective post-harvest sanitation processes. Washing is the most important step in the processing of vegetables. Although washing produce with tap

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water may remove soil and other debris, it cannot be relied upon to remove microorganisms, and may result in cross-contamination of food preparation surfaces, utensils, and other food items (Beuchat *et al.*, 2001; Brackett, 1992). Washing with disinfectant is useful for reducing microbial levels on fresh-cut vegetables (Pirovani *et al.*, 2004). Chlorination, whether in the form of chlorine gas, sodium hypochlorite (NaClO), calcium hypochlorite ($\text{Ca}(\text{OCl})_2$), or chlorine dioxide (ClO_2), is less expensive than ultraviolet irradiation, ozone, and peroxyacetic acid treatments (Garrett *et al.*, 2003). However, the efficacy of sodium hypochlorite is limited by the concentration of free or available chlorine, the presence of organic matter, pH, temperature, and porous and roughened sample surfaces (Beuchat, 1998; Bermúdez-Aguirre and Barbosa-Cánovas, 2013; Parish *et al.*, 2003; Sapers, 2001). Chlorine dioxide, in contrast, has a higher oxidation capacity than sodium hypochlorite and does not easily form potentially carcinogenic by-products (Keskinen *et al.*, 2009; Stevens, 1982).

The efficacy of chlorination can be enhanced by combination with other treatments. Lewis Ivey and Miller (2005) described the sanitation of sprout seeds by sequential treatments with hot water ($47-51^\circ\text{C}$) and hypochlorite solutions. Ono *et al.* (2005) reported that combining sugar ester treatment with weakly acidic hypochlorous water (pH 6.0, 100 mg/L available chlorine) for sanitizing shredded vegetables was more effective in the reduction of bacterial populations (by $2.1-4.1 \log \text{cfu/g}$) than using weakly acidic hypochlorous water alone. Soli *et al.* (2010) reported the efficacy of combinations of weakly acidic hypochlorous water, pretreatment with sucrose fatty acid ester, micro-bubbling, and mild heating for sanitizing lettuce, which reduced viable counts by $3-4 \log$. Huang *et al.* (2006) described the efficacy of chlorinated water combined with ultra-sonication against *E. coli* O157:H7 in strawberries and broccoli. However, few of these studies have examined the efficacy of combining chlorination with physical treatments. Therefore, this study was conducted to evaluate and compare hypochlorite and chlorine dioxide, individually and combined with micro-bubbling, ultra-sonication, or mild heat, on microbial reduction in leafy vegetables. Cross-contamination when the chlorine solution is continuously used for washing vegetables was also investigated.

Materials and Methods

Microbial culture preparation As test inoculum, we used a mix of four rifampicin-resistant strains (CR-3, MN-28, MY-29, DT-66) of enterohemorrhagic *E. coli* O157:H7 obtained from the National Food Research Institute (Tsukuba, Japan), and which were isolated from bovine feces. The strains were transferred individually from nutrient agar slants (Eiken Chemical Co., Ltd., Tokyo, Japan) into 10 mL of brain–heart infusion broth (Eiken Chemical Co., Ltd) supplemented with 50 ppm rifampicin (Nakalai Tesque, Inc., Kyoto, Japan), and incubated at 35°C for 24 h. Each strain was subcultured again before being used as inoculum.

Cells were harvested by centrifuging in a Kubota 6500 high-speed cooling centrifuge (Kubota Corp., Tokyo, Japan) at $4,293 \times g$ for 10 min at 4°C . The pelleted cells were twice resuspended in 10 mL of sterile phosphate-buffered saline (PBS, pH 7.2) and centrifuged as before. The cells were then suspended in 10 mL of PBS. Equal volumes of each cell suspension were mixed and diluted to a final concentration of $\sim 7 \log \text{cfu/mL}$.

Preparation of leafy vegetables and microbial inoculation Lettuce, spinach, and Chinese cabbage were purchased from a local grocery store in Higashi Hiroshima City, Japan. Two or three outer leaves of the lettuce and Chinese cabbage were removed and discarded, and the spinach roots were removed. The leaves were washed three times under running tap water for about 5 seconds each time to remove soil and organic matter on the surface, and cut into squares of approximately $3 \text{ cm} \times 3 \text{ cm}$ with a knife sanitized with 75% ethanol. The samples were kept at 4°C for not more than 2 h before experiment. Both inoculated and non-inoculated leaves were used. For inoculation, 200 g of freshly prepared leaves were immersed in 2.5 L of the above cell suspension for 3 min. The inoculated samples were collected and placed in a sterile stainless container lined with paper towels, covered, and stored overnight at 4°C .

Preparation of chlorine-based sanitizers and vegetable sanitizing The chlorine wash solutions were prepared by adding sodium hypochlorite (Wako Pure Chemical Co., Ltd., Tokyo, Japan) to tap water to achieve free chlorine concentrations of 50, 100, 200, 500 ppm. The free chlorine concentration was measured by a spectrophotometric method based on *N,N*-diethyl-*p*-phenylenediamine in a Hanna Instruments HI95701 Free Chlorine ISM photometer (Woonsocket, RI, USA). Aqueous ClO_2 was prepared by mixing 90 mL of sterile distilled water with 5 mL each of reagent A (chiefly NaClO_2) and reagent B (chiefly HCl) according to the product manual (Kurosaki, Fukuoka, Japan). Before dilution for use, the mixture was placed in a sealed container and refrigerated (4°C) for 1 h to liberate aqueous ClO_2 at a designed concentration of 3,000 ppm.

Fifty grams of each sample were immersed in 3 L of freshly prepared aqueous NaClO or ClO_2 of 50, 100, 200, or 500 ppm with gentle agitation with a glass rod. After exposure for 0, 1, 5, or 10 min, the samples were drained in a salad spinner. Next, 10 g of sample was aseptically withdrawn and immediately transferred to a Stomacher bag containing 90 mL PBS with 1% sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, Sigma-Aldrich Co., Tokyo, Japan) to neutralize the residual chlorine, and then homogenized for microbiological analysis.

Physical treatments The efficacy of adjuvant physical treatments was also investigated in this study. For mild heat treatment, samples were immersed in a water bath (Yamato Scientific Co., Ltd., Tokyo, Japan) at 50°C . Micro-bubbling was conducted using a Nafuron Bubbler (As One Corp., Osaka, Japan) connected to an LMP 180 air pump (Welch Gardner Denver,

Saitama, Japan); the pore size was 5–100 µm and the bubble generating pressure was set at 9.8 kPa. Ultra-sonication was conducted in a Honda W-338 ultrasonic tank (Honda Electronics Co., Ltd., Toyohashi City, Aichi, Japan) with a bolt-clamped Langevin transducer and a Dynashock wave generator with a maximum power output of 600 W. The frequencies are switched between 28, 45, and 100 kHz every 1 ms. In the combined treatments, 100 ppm NaClO, 50 ppm ClO₂, and 5 min treatment time were chosen. The combination of NaClO or ClO₂ and physical treatments was conducted as simultaneous treatment and sequential treatment. In the simultaneous treatment, combined treatments were simultaneously applied for 5 min. In the sequential treatment, combined treatments were sequentially applied, with each treatment lasting 5 min.

Microbiological analyses The sanitized samples were homogenized with PBS for 2 min before ten-fold serial dilution with PBS. For non-inoculated samples, total plate counts (TPC) were determined on standard methods agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and total coliform counts (TCC) were determined on desoxycholate agar (Nissui Pharmaceutical Co., Ltd). For inoculated samples, *E. coli* O157:H7 levels were determined on tryptic soy agar (TSA, Eiken Chemical Co., Ltd) and sorbitol MacConkey agar (SMAC, Eiken Chemical Co., Ltd), both supplemented with 50 ppm rifampicin (TSA-rif, SMAC-rif). All plates were incubated at 35°C for 24–48 h before colony counting. Microbial levels are expressed as log cfu/g.

Microbial cross-contamination from contaminated wash water To evaluate the effect of chlorine solution on preventing the transfer or cross-contamination of *E. coli* O157:H7 to subsequent batches, we washed 10 lots of 50 g of inoculated spinach in 3 L of the same wash solution (which initially contained 0, 10, 20, 50 or 100 ppm of NaClO) for 5 min each, and finally 50 g of non-inoculated spinach in the solution. After each treatment, the wash solutions were collected for the measurement of the residual free chlorine concentration. The microbial level in the washed sample was also determined.

To determine the presence of any *E. coli* O157:H7 below the detection level of the plating technique in the final batch of washed non-inoculated spinach, 25 g of vegetable sample was transferred to 100 mL of tryptic soy broth (Eiken Chemical Co., Ltd) containing 50 ppm rifampicin and incubated at 35°C for 24 h. A loopful of this pre-enriched culture broth was then inoculated onto SMAC-rif supplemented with 0.05 ppm cefixime and 0.5 ppm potassium tellurite (Merck, Darmstadt, Germany), and incubated overnight for colony count determinations. One hundred milliliters of the wash solution that was below the detection level was passed through a membrane filter (0.45 µm, Toyo Roshi, Ltd., Tokyo, Japan). The filters were then placed onto SMAC-rif, incubated at 35°C for 24–48 h, and *E. coli* O157:H7 colonies were subsequently counted.

Statistical analysis All experiments were performed in

triplicate. To test for differences among treatments, all data were subjected to one-way ANOVA and Tukey's multiple range test (SPSS 17.0, IBM) at a 5% level of significance.

Results and Discussion

Effects of chlorine disinfectant concentration and treatment time Washing with NaClO at 0, 50, 100, 200 and 500 ppm for 5 min significantly reduced TPC, TCC, and *E. coli* O157:H7 levels on leafy vegetables when compared with the unwashed control (Fig. 1). An approximately 2.0 log CFU/g reduction in microbial level was observed. However, the effectiveness of reducing TPC did not differ significantly when washing spinach and Chinese cabbage with 50 and/or 500 ppm of NaClO for 5 min. On the other hand, 1.0 log CFU/g reduction of TCC was observed in spinach and Chinese cabbage with 50 and/or 500 ppm of NaClO for 5 min. No significant differences were observed in washing lettuce and Chinese cabbage with 200 and 500 ppm of NaClO for 5 min. However, significant reduction (2.0 log CFU/g) of *E. coli* O157:H7 was observed in the inoculated lettuce and Chinese cabbage samples.

TPC in lettuce, spinach and Chinese cabbage were significantly reduced (2.0 log CFU/g) with NaClO treatment at 100 ppm for 10 min and these reductions were significant even after 1 min. (Fig. 2). Moreover, further significant reductions were observed after 5 min of washing, but not after 10 min of washing of lettuce and spinach samples. In addition, significant reduction of TCC and *E. coli* O157:H7 was observed in lettuce, spinach and Chinese cabbage while washing with 100 ppm NaClO for 1 min, and additional reductions of 1.0 log CFU/g were achieved with longer exposure time. These results are similar to those obtained by Rahman *et al.* (2011), who reported that 100 ppm chlorine treatment for 3 min showed superior reduction of TPC than 1 min in carrot samples; however, 5 min exposure was not significantly better. Likewise, 100 ppm NaClO treatment for 3 min reduced microbial counts in cabbage and a non-significant difference was observed for 10 min exposure (Rahman *et al.*, 2010).

The maximum reductions of TPC, TCC, and *E. coli* O157:H7 with 200 ppm ClO₂ washing for 5 min was recorded as 2.38, 2.05 and 1.60 CFU/g, respectively (Fig. 3). In contrast to the consistent reductions in TPC and TCC with increasing ClO₂ concentration, *E. coli* O157:H7 counts were not reduced significantly while 25 to 200 ppm ClO₂ was used. The apparent ineffectiveness of both chlorine treatments at higher concentrations and longer treatment times may be attributed to the protection rendered by the microtopography of the vegetables; microorganisms can find protection in plant tissues such as stomata, crevices, and cuts, and even in self-assembled biofilms (Stevens, 1982; Durak *et al.*, 2012; Lin *et al.*, 2002; López-Gálvez *et al.*, 2009; Luo *et al.*, 2011; Morris *et al.*, 1997; Seo and Frank 1999; Takeuchi and Frank, 2001; Takeuchi *et al.*, 2000).

Efficacy of combining chlorine disinfectants with physical

Fig. 1. Effect of NaClO concentrations on the microbiological quality of leafy vegetables treated for 5 min. (A: total plate counts; B: total coliform counts; C: *E. coli* O157:H7). Reported values are the mean of 3 replicates; error bars represent standard deviation. Control does not involve any form of washing procedure. Levels within the same microbial group and leafy vegetable with varying letters are significantly different ($p < 0.05$).

treatments Neither micro-bubbling nor ultra-sonication significantly reduced the microbial levels relative to tap water treatment of any vegetables; the observed reductions were not greater than 1 CFU/g (Table 1). Washing with 100 ppm NaClO resulted in the following reductions: TPC, 0.73 – 0.95 log CFU/g; TCC, 0.99 – 1.26 log CFU/g; and *E. coli* O157:H7, 1.14 – 1.22 log CFU/g on non-selective medium, and 1.15 – 1.36 log CFU/g on selective medium. On the other hand, washing with 50 ppm ClO₂ showed the following reductions: TPC, 1.04 – 1.43 log CFU/g,

Fig. 2. Effect of 100 ppm NaClO treatment time on the microbiological quality of leafy vegetables. (A: total plate counts; B: total coliform counts; C: *E. coli* O157:H7). Reported values are the mean of 3 replicates; error bars represent standard deviation. Control does not involve any form of washing procedure. Levels within the same microbial group and leafy vegetable with varying letters are significantly different ($p < 0.05$).

TCC, 1.32 – 1.78 log CFU/g, and *E. coli* O157:H7, 1.38 – 1.56 log CFU/g on non-selective medium, and 1.23 – 1.62 log CFU/g on selective medium. Simultaneous micro-bubbling or ultra-sonication with chlorine water did not significantly reduce the microbial levels compared to chlorination alone. The ineffectiveness of sonication in improving the efficacy of chlorine water has also been reported (Inatsu *et al.*, 2005a).

Mild heat treatment at 50°C for 5 min resulted in significant reductions in all microbial levels in all vegetables. However, efficacy varied with the microbe and vegetable type. In general, this treatment was less effective in reducing *E. coli* than the natural microflora. On the other hand, the combination of mild heat treatment and chlorination showed the highest reduction: TPC, 2.91 log CFU/g; TCC, 4.10 log CFU/g; *E. coli*, 2.13 log CFU/g on TSA, and 2.53 log CFU/g on SMAC. Combination treatment was

Fig. 3. Effect of ClO₂ concentrations on the microbiological quality of lettuce treated for 5 min. (A: total plate counts; B: total coliform counts; C: *E. coli* O157:H7). Reported values are the mean of 3 replicates; error bars represent standard deviation. Control does not involve any form of washing procedure. Levels within the same microbial group with varying letters are significantly different ($p < 0.05$).

more effective when heating was applied before or with chlorination than after. It is possible that heating caused injury to the bacteria and increased the sensitivity to chlorine disinfectant. Klaiber *et al.* (2005) reported that washing cut carrots with warm chlorinated water (200 ppm, 50°C) reduced aerobic mesophilic bacteria by 2.3 log cycles. Inatsu *et al.* (2005a) reported that acidified sodium chlorite was significantly more effective against *E. coli* O157:H7 on Chinese cabbage at 50°C than at 25°C or 4°C. Kondo *et al.* (2006) reported that 200 ppm NaClO at 50°C for 1 min reduced *E. coli* O157:H7 on lettuce by 1.5 log cycles. Moreover,

the combination of chlorine disinfectant and mild heat not only enhanced the bactericidal effect, but also improved the quality of vegetables. For example, Murata *et al.* (2004) reported that the organoleptic quality of cut lettuce subjected to heat shock was significantly better than that of the untreated control. Also, Roura *et al.* (2008) reported that heat shock in a 50°C water-bath for 2 min prevented browning of fresh-cut lettuce by reducing phenylalanine ammonia lyase activity.

In most of the treatments, higher numbers of *E. coli* O157:H7 were recovered using non-selective (TSA-rif) than using selective (SMAC-rif) medium. The same study reported the poor performance of selective medium in recovering *E. coli* O157:H7 from chemically treated vegetable samples (Inatsu *et al.*, 2005a). The use of media with less selective components could facilitate the resuscitation of injured cells, resulting in higher cell counts (Gündüz *et al.*, 2009; Huang and Chen, 2011; Lee and Kang, 2009; Wang *et al.*, 1996).

Effect of sanitizer solution reuse on free chlorine concentration and cross-contamination Recycling of NaClO solution gradually decreased free chlorine concentrations, with greater reductions in solutions with a lower initial concentration (Table 2): from an initial 100 ppm, the free chlorine concentration dropped to 44.83 ppm at the 10th cycle; from an initial 50 ppm to 12.30 ppm; and from an initial 10 or 20 ppm to < 0.20 ppm.

After the first wash cycle, PBS showed the lowest efficacy and 100 ppm NaClO showed the highest (Fig. 4). Repeated use resulted in decreased efficacy in reducing *E. coli* O157:H7. As described above, 10 ppm NaClO was less effective in reducing *E. coli* O157:H7 on vegetables. Sodium hypochlorite loses its effectiveness on reacting with organic compounds (Inatsu *et al.*, 2005b; Rodgers *et al.*, 2004; Warriner *et al.*, 2003). After using it to wash fresh-cut lettuce inoculated with a high organic load, Baert *et al.* (2009) reported reductions from an initial free chlorine concentration of 200 ppm to 112 ppm and from 20 ppm to 0.5 ppm. In addition, Luo *et al.* (2011) explained that when the initial chlorine concentration is low, most chlorine present in the wash solution is consumed in such reactions, leaving little or none for disinfection. The factors responsible for reducing the bactericidal effect of chlorine during vegetable washing includes vegetable type and wash water ratio, the amount of surface organic matter, vegetable preparation (e.g., shredded or pieces), and inoculation methods (Kondo *et al.*, 2006; Beuchat, 1999; Beuchat *et al.*, 2004; Francis and O'Beirne, 2004; Lang *et al.*, 2004; Nthenge *et al.*, 2007; Singh *et al.*, 2002). In our study, the amount of organic matter capable of reacting with the free chlorine was minimized by washing the vegetables before treatments. The bacterial cells were also washed twice to reduce extraneous organic matter before vegetable inoculation.

The microbial cross-contamination of non-inoculated vegetables (Table 2) was also investigated. With re-use of NaClO solutions, viable *E. coli* O157:H7 cell levels increased in solutions

Table 1. Microbial log reductions in leafy vegetables following single and combination antimicrobial treatments.

Treatments ²	Log reductions of test leafy vegetable ¹									
	Lettuce			Spinach			Chinese cabbage			
	<i>E. coli</i> O157:H7			<i>E. coli</i> O157:H7			<i>E. coli</i> O157:H7			
	TPC	TCC	SMAC	TPC	TCC	SMAC	TPC	TCC	TSA	SMAC
Tap water	0.22 ^a	0.38 ^a	0.68 ^{a,x}	0.25 ^a	0.63 ^a	0.92 ^{a,x}	0.34 ^a	0.44 ^a	0.59 ^{a,x}	0.52 ^{a,x}
NaClO (100 ppm)	0.95 ^b	0.99 ^b	1.14 ^{b,x}	0.78 ^b	1.26 ^b	1.22 ^{b,x}	0.73 ^b	1.12 ^b	1.18 ^{b,c,x}	1.36 ^{b,x}
ClO ₂ (50 ppm)	1.23 ^b	1.32 ^c	1.38 ^{b,x}	1.04 ^{bc}	1.78 ^c	1.44 ^{cde,x}	1.43 ^{cd}	1.64 ^c	1.56 ^{cde,x}	1.62 ^{bc,x}
Micro-bubbling	0.41 ^a	0.41 ^a	0.71 ^{a,x}	0.31 ^a	0.56 ^a	0.84 ^{a,x}	0.35 ^a	0.29 ^a	0.62 ^{ac,x}	0.50 ^{ax}
Micro-bubbling + NaClO	1.02 ^b	0.85 ^b	0.21 ^{b,x}	0.81 ^b	1.31 ^b	1.12 ^{b,x}	0.81 ^b	1.16 ^b	1.20 ^{b,c,x}	1.36 ^{b,x}
Micro-bubbling + ClO ₂	1.24 ^b	1.28 ^c	1.42 ^{bc,x}	1.10 ^{bc}	1.80 ^c	1.35 ^{cde,x}	1.42 ^{cd}	1.56 ^c	1.62 ^{de,x}	1.66 ^{bc,x}
Ultra-sonication	0.38 ^a	0.42 ^a	0.82 ^{a,x}	0.35 ^a	0.71 ^a	1.01 ^{a,x}	0.41 ^a	0.46 ^a	0.71 ^{ax}	0.62 ^{ax}
Ultra-sonication + NaClO	1.10 ^b	1.12 ^{bcd}	1.21 ^{b,x}	0.82 ^b	1.32 ^b	1.32 ^{bd,x}	0.81 ^b	1.21 ^b	1.21 ^{bc,x}	1.41 ^{bc,x}
Ultra-sonication + ClO ₂	1.32 ^b	1.41 ^{cd}	1.41 ^{bc,x}	1.21 ^c	1.82 ^c	1.41 ^{de,x}	1.41 ^{cd}	1.61 ^c	1.63 ^{de,x}	1.61 ^{bc,x}
50°C	1.09 ^b	2.31 ^e	1.19 ^{b,x}	1.24 ^c	2.60 ^d	1.11 ^{b,x}	1.57 ^{cd}	2.71 ^d	1.00 ^{ab,x}	1.33 ^{bc,x}
50°C → NaClO	1.92 ^c	3.80 ^f	1.45 ^{ce,x}	2.65 ^d	3.50 ^e	1.53 ^{eg,x}	2.73 ^e	3.72 ^e	1.57 ^{eg,x}	1.84 ^{ef,x}
50°C → ClO ₂	2.39 ^c	3.91 ^f	1.71 ^{de,x}	2.76 ^d	3.60 ^e	1.94 ^{fhg,x}	2.75 ^e	3.77 ^e	2.11 ^{fx}	2.47 ^{de,y}
NaClO → 50°C	1.32 ^b	3.35 ^f	1.31 ^{bc,x}	1.56 ^e	2.91 ^e	1.31 ^{bc,x}	1.56 ^d	3.41 ^e	1.20 ^{b,x}	1.72 ^{cy}
ClO ₂ → 50°C	1.42 ^b	3.56 ^f	1.48 ^{cx}	1.63 ^e	3.31 ^e	1.43 ^{bc,x}	1.62 ^d	3.45 ^e	1.64 ^{cx,x}	1.86 ^{cy}
50°C + NaClO	1.94 ^c	4.00 ^f	1.61 ^{ex}	2.74 ^d	4.10 ^e	1.91 ^{gx}	2.84 ^e	3.90 ^e	1.81 ^{gx}	2.11 ^{dy}
50°C + ClO ₂	2.45 ^c	4.00 ^f	1.91 ^{fx}	2.77 ^d	4.10 ^e	2.13 ^{hx}	2.91 ^e	3.90 ^e	2.12 ^{fx}	2.53 ^{dy}

¹Values represent an averages of 3 values from independently conducted experiments, SD ranges: (Lettuce: TPC: 0.09 – 0.38, TCC: 0.00 – 0.51, *E. coli* TSA: 0.07 – 0.24, *E. coli* SMAC: 0.04 – 0.28); (Spinach: TPC: 0.02 – 0.39, TCC: 0.00 – 0.75, *E. coli* SMAC: 0.04 – 0.27); (Chinese cabbage: TPC: 0.11 – 0.41, TCC: 0.00 – 0.46, *E. coli* TSA: 0.13 – 0.42, *E. coli* SMAC: 0.16 – 0.42).

²Individual treatments lasted for 5 min. Combinations of treatments denoted with ‘+’ signs were simultaneously applied for 5 min. Combinations of treatments denoted with ‘→’ signs were sequentially applied, with each treatment lasting 5 min.

^{a,d,e}Microbiological levels in the same column, in a specific leafy vegetable, followed by different letters are significantly different ($P < 0.05$).

^{x,y}*E. coli* levels enumerated on TSA and SMAC in the same row, in a specific leafy vegetable, followed by different letters are significantly different ($P < 0.05$).

Table 2. Effect of wash water reuse for vegetable washing on free chlorine concentration, *E. coli* O157:H7 in wash solution, and cross-contamination.

Initial NaClO (ppm)	Wash water and vegetable quality parameters	Number of times of wash solution use					
		1st	2nd	4th	6th	8th	10th
0	Free chlorine concentration (ppm)	0.16	0.09	0.11	0.00	0.00	0.00
	<i>E. coli</i> O157:H7 level in wash solution (log cfu/mL)	3.30	4.33	4.77	4.97	5.07	5.30
	<i>E. coli</i> O157:H7 level transferred to uninoculated spinach (log cfu/g) ¹	-	-	-	-	-	4.13
10	Free chlorine concentration	6.87	4.10	0.75	0.21	0.16	0.11
	<i>E. coli</i> O157:H7 level in wash solution	<1.00	<1.00 ^a	<1.00 ^a	3.40	3.63	3.97
	<i>E. coli</i> O157:H7 level transferred to uninoculated spinach	-	-	-	-	-	2.50
20	Free chlorine concentration	16.20	13.13	5.80	1.44	0.27	0.17
	<i>E. coli</i> O157:H7 level in wash solution	<1.00	<1.00	<1.00 ^a	<1.00 ^a	2.37	3.23
	<i>E. coli</i> O157:H7 level transferred to uninoculated spinach	-	-	-	-	-	1.90
50	Free chlorine concentration	43.90	39.60	32.50	24.50	16.80	12.30
	<i>E. coli</i> O157:H7 level in wash solution	<1.00	<1.00	<1.00	<1.00	<1.00 ^a	<1.00 ^a
	<i>E. coli</i> O157:H7 level transferred to uninoculated spinach	-	-	-	-	-	<1.00 ^b
100	Free chlorine concentration	92.50	90.67	84.50	74.00	62.00	44.83
	<i>E. coli</i> O157:H7 level in wash solution	<1.00	<1.00	<1.00	<1.00	<1.00 ^a	<1.00 ^a
	<i>E. coli</i> O157:H7 level transferred to uninoculated spinach	-	-	-	-	-	<1.00 ^b

¹Occurrences of cross contamination of *E. coli* O157:H7 from reused wash water to uninoculated spinach were measured after the 10th use of the wash water.^aNot detected in both direct plating and membrane plating after 100 mL filtration^bNot detected in both direct plating and selective enrichment isolation in 25 g samples

Fig. 4. Effect of wash water recycling on treatment efficacy against *E. coli* O157:H7 in spinach. Reported values are the mean of 3 replicates; error bars represent standard deviation. Levels with varying letters are significantly different ($p < 0.05$).

with initial concentrations of 10 and 20 ppm NaClO. Cell survival was evident in the 10 ppm NaClO solution after the 6th use cycle (0.21 ppm residual free chlorine) and in the 20 ppm solution after the 8th use cycle (0.27 ppm). After the 10th use cycle, cell survival reached 3.97 log cfu/mL in 10 ppm solution and 3.23 log cfu/mL in 20 ppm NaClO solution. Cross-contamination of non-inoculated spinach was evident with re-use of the 20 ppm (initial conc.) NaClO solution, and the cell counts were recorded as 1.90 log CFU/g. In contrast, washing solutions with higher initial NaClO concentrations (50 and 100 ppm) remained effective even after the 10th use cycle, and no cross-contamination was observed. This illustrates how microorganisms can survive for extended periods in wash water, and that cross-contamination can occur when sanitizer levels fall below the bactericidal concentrations (Chang and Fang, 2007). Furthermore, repeated use of wash water increases the turbidity and chemical oxygen demand of the wash water, reducing the vegetable quality (Baert *et al.*, 2009).

In conclusion, our results confirm that washing vegetables with common chlorine sanitizers at 50 to 200 ppm for as short as 1 min can decontaminate both intrinsic and introduced microorganisms, although 5 min is more reliable. An initial concentration of ≥ 50 ppm NaClO can prevent the cross-contamination of microorganisms from the wash solution to vegetables. Concentrations made little difference to the bactericidal effects of chlorine. However, the bactericidal effect of the initial concentration must be validated to avoid the risk of cross-contamination. The combination of chlorine-based disinfectant and mild heat (50°C) showed the highest reduction of microorganisms in vegetables. Although several factors can limit the efficacy of chlorine-based sanitizers, and while harmful chlorinated organic by-products can result from their use, the merits of chlorine-based sanitizers justify their use in the fresh produce industry. Thus, the findings of this study will help fresh-cut vegetable processors to

improve the microbial safety and quality of their products.

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