Chlorine dioxide and chlorine effectiveness to prevent *Escherichia coli* O157:H7 and *Salmonella* cross-contamination on fresh-cut Red Chard

Alejandro Tomás-Callejas [a], Francisco López-Gálvez [b], Adrian Sbodio [c], Francisco Artés [a], Francisco Artés-Hernández [a], Trevor V. Suslow [c,*]

[a] *Postharvest and Refrigeration Group, Department of Food Engineering, Technical University of Cartagena, Paseo Alfonso XIII 48, Cartagena, Murcia 30203, Spain*
[b] *Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, P.O. Box 164, Espinardo, Murcia 30100, Spain*
[c] *Department of Plant Sciences, University of California, One Shields Avenue, Davis, CA 95616, USA*

ABSTRACT

Washing procedures applied to fresh produce have the potential to reduce contamination from the surface of the product. However, the wash water may also serve as a source of contamination or has great potential to result in cross-contamination. The objective of this study was to evaluate process handling cross-contamination potential and pathogen removal of initially low numbers of attached cells of *Salmonella* and *Escherichia coli* O157:H7 during the washing-disinfection, rinsing, and de-watering steps of fresh-cut Red Chard baby leaves as affected by NaClO and ClO_2. The efficacy and stability of liquid ClO_2 applied to the water as the disinfectant treatment was also evaluated. Non-inoculated leaves were mixed with inoculated leaves (about 3–5% of total weight) and processed as a unit. After processing, no confirmed colonies on selective media were recovered from the non-inoculated leaves and qPCR was used for detection below the limit of quantitative recovery. ClO_2 substantially prevented *E. coli* O157:H7 cross-contamination but was not effective for the inoculated *Salmonella*. Large populations of *Salmonella* were recovered from centrifugation discharge effluent water whereas no colonies were detected from water in contact with inoculated leaves collected from preceding washing unit operations. At an industrial level, this represents a potential risk of cross-contamination to product and equipment at the step immediately prior to packaging. These results suggest that the centrifugation effluent water could be used as a potential sample point to evaluate lot contamination and cross-contamination in the processing chain, even at low levels of pathogens, as were used in this study, undetectable by conventional sampling methods.

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1. Introduction

It is well known that a diet rich in fruit and vegetables can promote health benefits, including the prevention of chronic diseases such as cancer or cardiovascular diseases (Liu, 2003; Steinmetz & Potter, 1996). A deterrent to consumption and realization of these health benefits, fresh produce consumed raw has become widely recognized as a vehicle for transmitting infectious diseases (Harris et al., 2003; Leistner & Gould, 2002). Enteric diseases linked to consumption of fresh produce have dramatically increased in the last several decades (Morris, 2011; Scallan et al., 2011). *Escherichia coli* O157:H7 has been associated with multiple outbreaks linked to the consumption of fresh and fresh-cut leafy vegetables (CDC, 2006; CDPH, 2004; CDPH, 2005). Recent *Salmonella* outbreaks associated to leafy vegetables in Europe and the US have been also reported (Hanning, Nutt, & Ricke, 2009; Raybaudi-Massilia, Mosqueda-Melgar, & Martín-Belloso, 2009).

The specific operations involved in preparation of fresh-cut vegetables can facilitate attachment and stimulate microbial growth, which includes diverse complexes of spoilage microbiota and bacterial pathogens harmful to human health (Sapers, Gorny, & Yousef, 2006, 634 p.). Therefore, fresh-cut produce must be managed in primary production phases and elaborated for marketing following strict control procedures for reducing overall quality loss and assuring its safety to consumers (Artés, Gómez, Aguayo, Escalona, & Artés-Hernández, 2009).

Immature or baby leafy vegetables are typically consumed raw. Hence, washing and disinfection is a key step that contributes to effectively reducing microbial load across the supply chain (Artés et al., 2009; Beuchat, 2000). Chlorine (sodium hypochlorite

−NaClO−) in various approved agricultural and food grade formulations are the most widely used disinfectant in the fresh-cut industry. However, there is a concern about the use of NaClO by the fresh-cut industry due to its low effectiveness in the presence of organic matter, and the formation of known or potentially carcinogenic or mutagenic by-products such as chloroform and haloacetic acids (Hrudey, 2009; Nieuwenhuijsen, Toledano, & Elliot, 2000; Ölmez & Kretzschmar, 2009; Richardson, Plewab, Wagnerb, Schoenycy, & DeMarinid, 2007).

Chlorine dioxide (ClO_2) has been utilized as a water treatment for several decades but has attracted increased interest over the past 10 years as an alternative to NaClO for the fresh and fresh-cut produce industry (Artés et al., 2009; Gómez-López, Rajkovic, Ragaert, Smigic, & Devlieghere, 2009). ClO_2 used as a disinfectant has several accepted or perceived advantages over NaClO, including higher oxidant capacity (Benarde et al., 1967), effectiveness over a broad range of pH (Parish et al., 2003), lower reactivity with organic matter (Gordon & Rosenblatt, 2005) and high effectiveness at low concentrations (Huang, Wang, Ren, & Ma, 1997). In addition, ClO_2 and its main by-product, chlorite (ClO_2^-), are classified as non-carcinogenic products (ATSDR, 2004; EPA, 2000; IARC, 1991). However, as with all chemicals used for process water and wastewater disinfection, ClO_2 has disadvantages associated with its use. ClO_2 is a very unstable substance; it is highly explosive as a concentrated gas (and therefore must be generated on-site). It decomposes readily when exposed to sunlight, as may occur in raw produce washing operations (Suslow, 1997).

Regardless of these practical challenges, the efficacy of ClO_2 in inactivating key food borne pathogens of concern among different commodities has been reported. Concentrations of 4–5 mg/L were effective to reduce *Salmonella* spp, *Escherichia coli* O157:H7 and *Listeria monocytogenes* inoculated onto cabbage, carrot, lettuce, strawberry and melon (Keskinen, Burke, & Annous, 2009; Mahmoud, Bhagat, & Linton, 2007; Mahmoud & Linton, 2008; Sy, Murray, Harrison, & Beuchat, 2005). ClO_2 can also decrease the viability of *Cryptosporidium parvum* oocysts (Peeters, Ares, Masschelein, Villacorta, & Debacker, 1989).

Washing procedures applied to fresh produce have the potential to reduce contamination from the surface of the product. However, the wash water may also cause cross-contamination within a lot and among sequentially washed lots. Due to economic and environmental factors, reconditioning and recycling of water has been a long employed practice for the industry and it is currently recommended by governmental institutions (USDA, 1999). The effective use of chemical sanitizers during the washing-disinfection step is a critical point for keeping quality and safety of fresh-cut produce.

Despite the wide use of antimicrobial chemicals added to water as processing aides, the survival of food borne pathogens on fresh-cut products and resultant illness and outbreaks remains a reality. The rapid detection of human pathogens at various points from preharvest to finished, packaged product has become a priority for the fresh-cut industry (Gómez, Pagnon, Egea-Cortines, Artés, & Weiss, 2010). Fresh-cut leafy vegetables are highly perishable, and hold times for pathogen test outcomes, consistent with conventional culture-based methods are unacceptable, even when sufficient large capacity refrigerated storage is available. Conventional methods of detecting pathogens in food samples are time-consuming; taking 3–5 days or longer for a confirmed positive result and intrinsic traits of some leafy vegetables can prevent or delay detection with rapid commercial detection kit formats (D'lima & Suslow, 2009). In addition, even a few surviving cells, at numbers below typical limits of detection are still a cause for concern. In the case of *E. coli* O157:H7, for example, evidence from epidemiological studies suggests that the infectious dose may be as few as 10 cells per serving (Jinneman et al., 1995). In recognition of this risk to consumers, research efforts have been focused on the development of more rapid, unequivocal and sensitive pathogen detection methods. Several commercially developed and approved or certified rapid methods for the detection of low levels of *E. coli* O157:H7 and *Salmonella* in fresh-cut leafy vegetables are available. The Qualicon BAX® Salmonella system and the Assurance GDS® O157:H7 are PCR-based systems and currently approved by the AOAC as official methods for detection of *Salmonella* and *E. coli* O157:H7 in selected foods (Feldsini et al., 2005; Silbernagel et al., 2003). Despite these advances, the optimal sampling locations for assessing pathogen risk exposure and control efficiencies during fresh-cut processing of salad blends have yet to be fully elucidated.

Baby leafy vegetables have grown in popularity as a base ingredient for fresh-cut mixed salads. Baby Red Chard (*Beta vulgaris* cv. cicla) is one of the most commonly consumed which adds a sweet with slightly bitter flavor undertones to mixed salads.

The objective of the current work was to evaluate process handling cross-contamination potential and pathogen removal of initially low numbers of attached cells of *Salmonella* and *E. coli* O157:H7 during the washing-disinfection, rinsing, and de-watering steps of fresh-cut Red Chard (*Beta vulgaris* cv. cicla). To determine this, a model wash-process system was designed to evaluate efficacy and stability of liquid ClO_2 applied to the water as the disinfectant treatment.

2. Materials and methods

2.1. Liquid chlorine dioxide stability

A synthetic processing water to simulate commercial processing water conditions was created. To simulate the background oxidative demand of processing water, plant material with adhered soil was taken from University of California (Davis, CA) research farm and submerged in tap water. Dilutions were prepared and adjusted to create synthetic process water with turbidity values of 22 and 160 FAU (Formazin Turbidity Unit ≅ Nephelometric Turbidity Unit). These assays were carried out by adding ClO_2 (Aquapulse Systems Inc., San Luis Obispo, CA, USA) by serial dilutions in 100 mL of synthetic water to reach a final concentration of 3 mg ClO_2/L confirmed by using a colorimeter as described below. Changes in oxidation−reduction potential (ORP) and residual ClO_2 concentration (mg/L) during a 2 min interval at two temperatures (10 and 25 °C) and two water turbidities (22 and 160 FAU) were monitored. All physicochemical parameters were determined using standard protocols. Specifications of the instruments used are as follows: ORP-sensor (Thermo Fisher Scientific Inc., Waltham, MA, USA) for ORP; portable colorimeter for turbidity (DR/850, Hach Company, Loveland, CO, USA) and ClO_2 residual (Pocket Colorimeter™ II, Hach Company, Loveland, CO, USA). The samples of 160 FAU water were filtered by using a 0.45 μm filter before measuring the ClO_2 residual to avoid interference among suspended solids and the ClO_2 colorimeter. All analyses were made in triplicate.

2.2. Plant material

Red Chard (*Beta vulgaris* cv. cicla) leaves were grown under certified organic management in Salinas Valley (California, US) and hand harvested at a commercial development stage as immature or 'baby' leaves. Immediately after harvesting, the leaves were placed in pre-cooled, insulated chests (coolers) and transported to the Mann Lab (University of California, Davis) where they were held at 5 °C until further processing within 18 h.

2.3. Bacterial cultures and inoculum preparation

Attenuated strains of *E. coli* O157:H7 (PTVS155) and *S. enterica* sv. Typhimurium (PTVS177) were used in this study (Table 1). Spontaneous mutant strains resistant to 80 mg/L of rifampicin were derived for each test organism and used to minimize interference with other bacteria during quantitative and qualitative detection and determinative confirmation tests (Beuchat et al., 2001). Both strains were grown separately overnight in 9 mL of Tryptic Soy Broth (TSB) (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin at 37 °C. After incubation, cultures were centrifuged at $4000 \times g$ for 10 min. The pellet was re-suspended and washed twice with Butterfield's phosphate buffer (Whatman Inc., Piscataway, NJ, USA). The final cell pellet was suspended in Butterfield's phosphate buffer to obtain an initial cell density (OD_{600}) of approximately 0.7, which corresponds to a concentration of 10^8 CFU/mL. The final concentration was confirmed by serial dilution plating on Tryptic Soy Agar (TSA) (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin (TSA-rif). All experimentation was conducted in the Suslow Biosafety Level 2 Laboratory of the L. Mann Postharvest Laboratory at University of California, Davis.

2.4. Inoculation procedure

Red Chard leaves were submerged in phosphate buffer (pH 7.0) containing 10^5 CFU/mL for 1 min and were subsequently dewatered and dried overnight at 15 °C and 95% RH prior to processing to allow for presumptive cell attachment on the plant phyllosphere. Inoculated leaves were marked in the distal tip of the petiole with a colored tape for differentiation from non-inoculated leaves combined with treated leaves during processing.

2.5. Sample preparation and processing

Two sets of different experiments with Red Chard baby leaves, separately for *Salmonella* and *E. coli* O157:H7, were subjected to simulated minimally processing at room temperature (nominally 20 °C). In each experiment as a preparative step, non-inoculated leaves with defects such as yellowing, decay, harvest and transport damage, and bruising were discarded. Twenty inoculated and marked Red Chard leaves were mixed with 300 g (roughly 120 leaves) of non-inoculated leaves from the same lot and processed as a unit. Within each replicated experiment and each specific treatment variation, inoculated material represented the 3–5% of the total weight. Two different model washing systems were evaluated as follows:

A. Prewashing (1 min, spray-shower with municipal tap water), Disinfection Wash (1 min, sanitizing solution −aerated batch agitation wash), Rinsing (30 s, spray-shower with municipal tap water) and De-watering (30 s − manual centrifugation with 10 L foodservice spin dryer).

B. Prewashing (1 min, immersion into municipal tap water), Disinfection Wash (1 min, sanitizing solution − immersion), Rinsing (30 s, spray-shower with municipal tap water) and De-watering (30 s − manual centrifugation with 10 L foodservice spin dryer).

Prewashing, washing-disinfection and rinsing were performed in different tanks using a ratio of 5 L water/300 g product. The aerated agitation bath was created by passing compressed air through a 1.5×35 cm-long aquarium airstone. Two chemical disinfectant solutions: ClO_2 (3 mg/L) (Aquapulse Systems Inc. San Luis Obispo, CA, USA) and NaClO (6%) (The Clorox Company, Oakland, CA) (25 mg/L, adjusted to pH 6.5 with citric acid), were prepared immediately before application and added in the washing-disinfection tank. After processing, inoculated and non-inoculated leaves were collected, divided by type and analyzed separately. The experiment was repeated twice for each strain.

2.6. Bacterial detection, recovery and enumeration

2.6.1. Water
Three random water samples of 20 mL were collected from each washing step (prewashing, washing, rinsing and centrifugation) to evaluate the potential transfer of pathogens from inoculated leaves to the processing water. Samples were immediately neutralized using sodium thiosulphate ($Na_2S_2O_3$) (1N). The amount of $Na_2S_2O_3$ used was previously tested in the laboratory under anticipated experimental conditions and was added in excess to ensure rapid and complete ClO_2/NaClO neutralization. Recovery was done by 0.45 μm hydrophobic grid filtration using the Neogen ISO-GRID system (Neogen Corporation, Lansing, MI, USA). Membranes containing any viable captured bacterial cells were transferred to TSA-rif and incubated at 37 °C for 24 h. TSA-rif plates were also supplemented with 1 g/L of sodium pyruvate {$C_3H_3NaO_3$;(TSARP)} during preparation to facilitate resuscitation of sub-lethally injured cells (Knudsen, Yamamoto, & Harris, 2001). Following incubation, the grid squares containing presumptive positive colonies were counted to yield CFU/10 mL.

2.6.2. Red Chard
Inoculated and marked leaves ($n = 20$) were collected after processing and placed in 5 sterile bags (Whirl-Pak™, Nasco, Modesto, California, US) containing 4 leaves each and 0.1% sterile buffered peptone water (BPW) (BD Diagnostics, Sparks, MD, USA) added in a 1:2 w/v ratio. Samples were then vigorously massaged by hand for 1 min. Ten-fold dilutions were prepared with BPW and plated on TSARP for both *E. coli* O157:H7 and *S. enterica* recovery. Plates were incubated at 37 °C for 24 h, counted and results reported as log CFU/g. Five replicates for each strain; sanitizing type and washing system were performed.

Three replicates ($n = 3$) of 25 g of non-inoculated leaves were placed in sterile bags containing 50 mL of buffer supplemented with 80 mg/L of rifampicin to evaluate the potential transference of pathogens from inoculated material to non-inoculated leaves. BPW and mEHEC (Biocontrol Systems Inc., Bellevue, WA, USA) were used as broth enrichment media for *Salmonella* and *E. coli* O157:H7, respectively. Samples were treated for 30 s at medium speed in a Pulsifier (Microgen Bioproducts Ltd., Surrey, UK) and direct plated on TSARP plates. Then, enrichment cultures were further supplemented with BPW or mEHEC, supplemented with rifampicin as above, to reach a final volume of 225 mL. Broth enrichments were incubated for 24 h at 37 °C without shaking and subsequently plated on TSARP, as above, and also small aliquots were removed for PCR-based detection as described below. Results from plates were reported as log CFU/g.

Table 1
Characteristics of the *Escherichia coli* O157:H7 and the *S. enterica* sv. Typhimurium strains used in the study.

Strain	Source of information
PTVS 155	*E. coli* O157:H7. ATCC # 700728, does not possess *stx1* and *stx2* genes (BSL-1). PTVS 155 is a derivative selected for rifampicin resistance.
PTVS 177	*S. enterica sev.* Typhimurium. χ3895 phage type13a Plasmid: pSeV2⁺. Transformed rifampic resistant. From parent strain PTVS 150, originally made available by R. Curtis; Biodesign Institute, Arizona State University.

2.6.3. Inoculated bacterial isolate detection by PCR

Pathogen detection kits were used to evaluate the potential cross-contamination by *Salmonella* and *E. coli* O157:H7 from inoculated to non-inoculated plant material during processing, anticipated to be below the quantitative limit of enumeration. BAX® Salmonella (Dupont/Qualicon, Wilmington, DE) and GDS® O157:H7 (BioControl, Bellevue, WA) were used in this study as rapid detection kits. BAX® Salmonella is based on real-time detection of SYBR intercalation into double-stranded DNA during PCR amplification. Standard detection time includes 8 h of non-selective enrichment plus 3 h for processing and detection. For these experiments, a selective enrichment by the addition of rifampicin was used to enhance the recovery of applied isolates. Assurance GDS® O157:H7 is a probe-based real time PCR which is combined with an immuno-magnetic separation step prior to processing, amplification, and detection. Time to detection includes 8 h of semi-selective enrichment plus 75 min for amplification and detection. The specific technical directions provided with each kit were followed carefully with the exception of the use of rifampicin supplementation. Enriched samples were incubated according to provider specifications and results were used to determine presence/absence of the target pathogen. All analyses were made in triplicate.

2.7. Statistical analysis

Statistical analysis was carried out with the Statistical Analysis System (SAS) V. 9.2. (SAS Institute, Cary, NC, USA) software. Each treatment was repeated in triplicate. To assess significant differences among treatments, two-way analysis of variance using the GLM procedure and Tukey's multiple comparison test mean separation were utilized. Statistical significance was established when $p < 0.05$.

3. Results

3.1. Liquid chlorine dioxide stability

Changes in ORP and residual ClO_2 as affected by water temperature and turbidity are shown in (Fig. 1). Residual ClO_2 concentrations were monitored after its addition during a 2 min interval. The impact of turbidity was more pronounced than the effects of water temperature in ORP and residual ClO_2 values. From an initial ClO_2 concentration of 3 mg/L, residuals quickly decreased to 1 mg/L within the first 20 s when added to 160 FAU turbidity water regardless of the water temperature and subsequently remained quite constant during the remaining 2 min (Fig. 1A). A turbidity value of 22 FAU at a water temperature of 10 °C was less influential in affect on reducing the initial ClO_2 concentration during the monitored time. However, a slight decrease of residual ClO_2 for 25 °C water temperature within 2 min was observed. In general, lower reductions of residual ClO_2 were observed at 10 °C than at 25 °C as well as at 22 FAU than at 160 FAU. A ClO_2 concentration of 3 mg/L was associated with ORP values greater than 650 mV for water turbidity of 22 FAU at 10 or 25 °C. However, when water turbidity was 160 FAU, ORP values decreased throughout the time and dropped to values less than 500 mV within 10 s. For the ClO_2 concentration tested, ORP values were not significantly different irrespective of water temperature tested for each turbidity condition (Fig. 1B).

3.2. Pathogen inactivation on inoculated leaves

Inoculated leaves represented 3–5% of total weight of minimally processed Red Chard. After washing the combined leaf mass, inoculated and marked leaves were selectively removed and analyzed separately. The antimicrobial effects of ClO_2 and NaClO on *E. coli* O157:H7 and *S. enterica* are presented in Table 2.

The initial population of *E. coli* O157:H7 on inoculated leaves before processing was 3.51 ± 0.13 log CFU/g. For the washing type A, log reductions after processing of 0.70 reaching a final load of 2.80 log CFU/g for both sanitizing solutions were achieved. Washing type B was apparently more effective for NaClO compared to washing type A and a reduction of 0.85 log CFU/g just after washing was observed.

Greater log reductions for *S. enterica* as compared to *E. coli* O157:H7 after processing were achieved. The initial *Salmonella* load was 4.27 log CFU/g. Significant differences in log reductions after washing among sanitizers within the same washing system were found. Washing with NaClO resulted in 1.50 and 1.12 log CFU/g reductions of *Salmonella* for the washing types A and B respectively. Reductions of 0.88 and 1.53 log CFU/g after washing with ClO_2 for the model systems A and B respectively were achieved.

3.3. Pathogen cross-contamination from inoculated leaves to processing water and to non-inoculated leaves

The potential cross-contamination from inoculated leaves to non-inoculated ones via processing water was studied (Table 3). After processing the Red Chard leaves, no confirmed colonies on selective media were recovered and, therefore, levels of the applied pathogen surrogate isolates were below the limit of quantitative detection (1.4 log CFU/g) regardless of the sanitizer and the
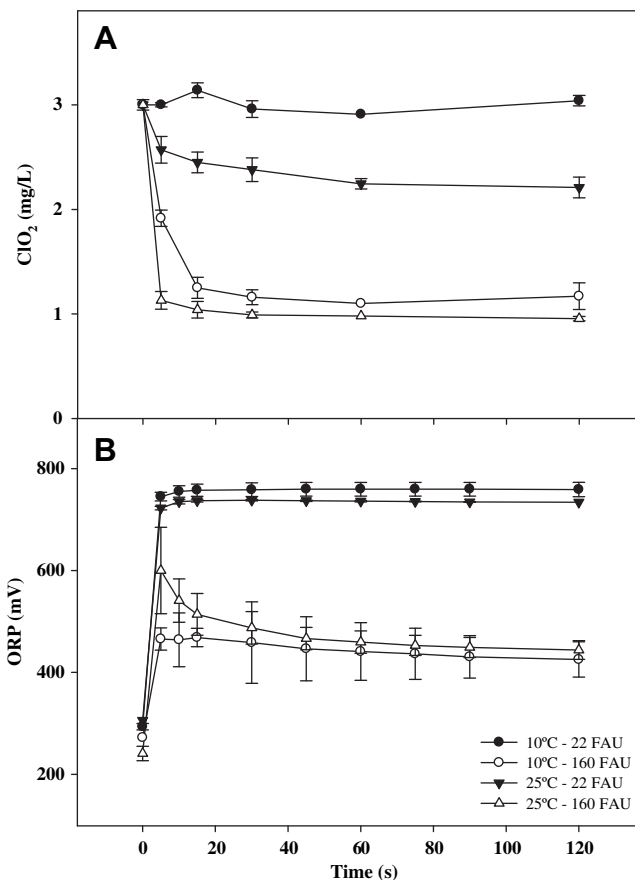

Fig. 1. Effect of water temperature (°C) and turbidity (FAU) on the residual ClO_2 concentration (mg/L) and ORP (mV) within 2 min of contact time (● 10 °C − 22 FAU; ○ 10 °C − 160 FAU; △ 25 °C − 22 FAU; ▼ 25 °C − 160 FAU).

Table 2
E. coli O157:H7 and S. enterica sv. Typhimurium populations expressed as log CFU/g ± standard deviation (SD) on inoculated Red Chard leaves as affected by sanitizer and washing types before and after processing.

Washing type	Sanitizer	Microbial population (log CFU/g)		
		Before processing	After processing	Log reduction
Escherichia coli O157:H7				
A	NaClO	3.51 ± 0.13	2.80 ± 0.12	0.71*
	ClO₂	3.51 ± 0.13	2.79 ± 0.23	0.72*
B	NaClO	3.51 ± 0.13	2.66 ± 0.19	0.85*
	ClO₂	3.51 ± 0.13	2.85 ± 0.08	0.66*
S. enterica sv. Typhimurium				
A	NaClO	4.27 ± 0.10	2.77 ± 0.12	1.50*
	ClO₂	4.27 ± 0.10	3.39 ± 0.23	0.88*
B	NaClO	4.27 ± 0.10	3.15 ± 0.09	1.12*
	ClO₂	4.27 ± 0.10	2.74 ± 0.05	1.53*

*Denotes significant difference ($p < 0.05$) in log reduction within the same row.

washing model type used. Neutralized water samples from pre-washing, washing, rinsing and the de-watering step were processed by using a sterile hydrophobic grid membrane to evaluate the potential risk of E. coli O157:H7 transference from inoculated leaves to the processing water (Table 3). No confirmed colonies in the processing water from prewashing, washing and rinsing steps for any sanitizer and washing type were detected. However, small populations ranging 0.3–0.5 CFU/10 mL of E. coli O157:H7 were recovered from effluent discharge water collected during the centrifugation de-watering step.

In this study PCR-based rapid detection kits were used to evaluate the presence of the pathogens below the quantitative limit of detection in non-inoculated Red Chard leaves (Table 4). For the washing type A, the probe-based GDS system provided molecular evidence of the presence of E. coli O157:H7 after washing with NaClO in 1 out of 3 samples, whereas no positives for ClO₂ treated washes were registered. Positives observed within model system A represented 6% of the total samples for both sanitizers. However, 50% of the analyzed samples were positive for washing system B. Regarding the obtained results, none of the sanitizers in the conditions assayed, could completely prevent the cross-contamination to non-inoculated leaves within the limits of the model system design. Although it is not part of the official protocol, the selective enrichments used for the GDS procedure were direct plated on CHROMagar O157™ (CHROMagar, Paris, France) to confirm the GDS molecular detection results and verified that PCR detection included viable target cells.

As observed for E. coli O157:H7, no colonies of Salmonella were recovered from non-inoculated leaves regardless of the sanitizer

and the washing model type used after processing. However, transference of viable Salmonella from inoculated leaves to the processing water was detected. Water from the centrifugation step was found to harbor significant populations of the target bacteria. Populations of Salmonella in the NaClO treated samples were lower than in comparison to ClO₂, independent of the washing system. The Salmonella recovery in the centrifuge effluent water was 55 and 83 CFU/10 mL for NaClO and 139 and 114 CFU/10 mL for ClO₂ for the washing types A and B, respectively. No significant differences among washing systems for each sanitizer were found.

The evaluation of the presence of Salmonella in non-inoculated leaves below the quantitative limit of detection was carried out by using the BAX® Salmonella system. None of the sanitizers in the conditions assayed were able to prevent cross-contamination to non-inoculated leaves and all samples were determined to be positive by the BAX assay. The selective enrichments used for the procedure were also plated on XLT4 and confirmed the detection of viable target cells by the presumptive diagnostic PCR assay.

4. Discussion

Fresh-cut produce is commonly consumed raw and disinfection in a washing step constitutes a minimum practical means of decontamination for meeting quality and safety goals. Washing with water, particularly with added mechanical action, physically removes some of the attached microorganisms from the surfaces of plant products, and the addition of disinfectants can help to improve the efficacy of this processing step. However, several reports have shown that the level of decontamination is not high enough to ensure safety of fresh-cut commodities (Park, Alexander, Taylor, Costa, & Kang, 2008; Ruíz-Cruz, Acedo-Félix, Diaz-Cinco, Islas-Osuna, & González-Aguilar, 2007; Tomás-Callejas, Martínez-Hernández, Artés, & Artés-Hernández, 2011). Consequently improvements in the application of chemical sanitizers to the washing water are a crucial point to prevent cross-contamination and maximize inactivation of attached human pathogens.

The population of E. coli O157:H7 and Salmonella inoculated onto Red Chard leaves was determined after the chemical treatment. In this study, 0.7 and 0.8–1.5 log reductions of E. coli O157:H7 and Salmonella, respectively, after processing with 3 mg/L ClO₂ were observed. This result is consistent with that previously reported by López-Gálvez, Gil, Truchado, Selma, and Allende (2010) where generic E. coli reductions of 0.8 log CFU/g in fresh-cut lettuce treated with 3 mg/L ClO₂ were detected. However, inconsistent results have been reported depending on the commodity, initial

Table 3
Transference of E. coli O157:H7 and S. enterica sv. Typhimurium from inoculated Red Chard leaves to processing water as affected by sanitizer and washing types.

Escherichia coli O157:H7				S. enterica sv. Typhimurium			
Washing type	Sanitizer	Washing step	CFU/10 mL	Washing type	Sanitizer	Washing step	CFU/10 mL
A	NaClO	Prewashing	ND	A	NaClO	Prewashing	ND
		Washing	ND			Washing	ND
		Rinsed	ND			Rinsed	ND
		Centrifuged	0.3			Centrifuged	55
	ClO₂	Prewashing	ND		ClO₂	Prewashing	ND
		Washing	ND			Washing	ND
		Rinsed	ND			Rinsed	5
		Centrifuged	0.8			Centrifuged	139
B	NaClO	Prewashing	ND	B	NaClO	Prewashing	ND
		Washing	ND			Washing	ND
		Rinsed	ND			Rinsed	2
		Centrifuged	ND			Centrifuged	83
	ClO₂	Prewashing	0.5		ClO₂	Prewashing	2
		Washing	ND			Washing	ND
		Rinsed	ND			Rinsed	ND
		Centrifuged	0.5			Centrifuged	114

ND: Not detected.

Table 4
Cross-contamination of *E. coli* O157:H7 and *S. enterica* sv. Typhimurium from inoculated material to non-inoculated Red Chard leaves as affected by sanitizer and washing types above and below the limit of quantitative detection.

Washing type	Sanitizer	Microbial counts (log CFU/g)[a]	GDS O157[b]	BAX Salmonella[c]
Escherichia coli O157:H7				
A	NaClO	ND	1/3	–
	ClO_2	ND	0/3	–
B	NaClO	ND	1/3	–
	ClO_2	ND	2/3	–
S. enterica sv. Typhimurium				
A	NaClO	ND	–	3/3
	ClO_2	ND	–	3/3
B	NaClO	ND	–	3/3
	ClO_2	ND	–	3/3

ND: Not detected.
[a] Limit of quantitative detection LOD = 1.52 log CFU/g.
[b] BioControl Assurance GDS *E. coli* O157 PCR system.
[c] Qualicon BAX *Salmonella* PCR system.

inoculum level, ClO_2 concentration, contact times, washing conditions and recovery procedures (Lee & Baek, 2008; Pao, Kelsey, Khalid, & Ettinger, 2007, Pao, Kelsey, & Long, 2009; Singh, Singh, & Bhunia, 2003; Singh, Singh, Bhunia, & Stroshine, 2002). Washing iceberg lettuce for 2 min with TriNova (20, 100 and 200 mg/L of ionic ClO_2^-) resulted in reductions of *E. coli* O157:H7 of 0.96, 1.05 and 1.13 log CFU/g respectively (Keskinen et al., 2009). Increasing the ClO_2 concentration does not seem to result in greater reductions of the initial pathogen population. In the same manner, Kim, Kim, and Song (2009) reported log reductions of 1.66 and 1.54 log CFU/g of *E. coli* O157:H7 and *S. enterica* ser. Typhimurium in inoculated broccoli sprouts after washing with 50 mg/L ClO_2 for 5 min. However, the current residual ClO_2 concentration in water approved by the U.S. Food and Drug Administration must not exceed 3 mg/L for fresh-cut produce (21 CFR 173.300; 2007). Despite a concerted effort by the authors, no confirmation could be found to support the widely held industry belief that 5 ppm ClO_2 (used in several of the research journal citations referenced above and subsequently) may currently be used on raw and fresh-cut produce according to an FDA Opinion Letter from the late 1990's.

The inoculated plant material represented only 3–5% of total weight when mixing with non-inoculated Red Chard leaves. Low levels of inoculum and the inoculated/non-inoculated plant material ratio was selected to mimic real conditions of contamination in the industrial processing plants. Plant material might arrive from the production fields with sporadic levels of contamination within and among lots received by the processor. Lots may include product harvested from different production fields and even different growers many kilometers apart. Non-quantitative evidence of cross-contamination of non-inoculated leaves by *E. coli* O157:H7 was detected after plating on selective culture media (LOD 1.52 log CFU/g). Based on the Assurance GDS results, 33% of the total samples were positive for *E. coli* O157:H7, and only ClO_2 for the washing type A was able to completely prevent cross-contamination. Prior reports demonstrated that once cross-contamination occurs during produce washing, further washing with disinfectant solutions, including ClO_2, is unable to inactivate the contamination (López-Galvez et al., 2010). While no *Salmonella* colonies were recovered on selective media from non-inoculated Red Chard, detection by PCR-BAX confirmed that all samples were positive regardless of the washing and sanitizer type used. Pao et al. (2007) studied the effect of ClO_2 for preventing *Salmonella* cross-contamination through contaminated water to non-inoculated tomatoes and determined that 5 mg ClO_2/L effectively prevented cross-contamination and survival on the smooth surface commodity.

It is known that water quality can affect the efficiency of chemical sanitizers. Although the interaction between ClO_2 and organic matter does not produce toxic by-products, as occurs with NaClO, ClO_2 is able to oxidize a large fraction of natural organic matter (Swietlik & Sikorska, 2004), reducing its availability in inactivating target microbes. Cross-contamination with *E. coli* between inoculated and non-inoculated fresh-cut escarole washed with different water quality was influenced by microbial and organic load present in re-circulating water (Allende, Selma, López-Gálvez, Villaescusa, & Gil, 2008). In this study, ClO_2 stability test results showed that ORP values were affected by water turbidity. It has been established that ORP values >650 mV are required to effectively kill human pathogens in the limited contact times typically present during produce washing processes (Suslow, 2004). The ORP often correlates well with the antimicrobial potential of the water and it is associated with the concentration of the oxidant, though not in a direct linear fashion under all conditions (Robbs, Bartz, & Sargent, 1995). Previous studies showed that residual ClO_2 in hot water was lower than in cold water, presumptively related with faster solubilization of ClO_2 (Zhang, Stout, Yu, & Vidic, 2008).

Though further study is warranted, our results suggest that special attention to water from a centrifugation-de-watering step should be taken into account as a control point in minimal processing of fruits and vegetables. Large populations of *Salmonella* were recovered from centrifugation discharge effluent water whereas no colonies were detected from water in contact with inoculated leaves in earlier washing unit operations. This result confirms the ability of the centrifugal force applied during the de-watering step to remove at least a proportion of more firmly attached cells with the unbound water from the leaf surface. This represents a potential risk of cross-contamination transference to product and equipment at the step immediately prior to packaging. These results further suggest that the centrifugation effluent water could be used as a potential sample point to evaluate lot contamination and cross-contamination in the processing chain by low levels of pathogens, as were used in this study, undetectable by conventional sampling methods. In this way, a rapid molecular protocol for evaluating aerobic bacterial load on fresh-cut lettuce by using centrifugation water as an alternative to the food product itself has been recently developed (Gómez et al., 2010). In the same way, Bhagwat (2004) proposed to use vegetable rinse-water as a broader lot screening strategy for detecting *Salmonella* by real time PCR.

In summary, selected levels of ClO_2 and NaClO used in this experiment were unable to fully disinfect the applied pathogen surrogates from inoculated leaves regardless of the washing type. While ClO_2 substantially prevented *E. coli* O157:H7 cross-contamination, of the isolate used in this study, it was not effective for the *Salmonella* isolate. Due to the certainty of generating aerosols and other means of handling contamination, only individual isolates of the available attenuated pathogen surrogates could be used to represent possible outcomes with pathogenic forms, as would be typical in more contained lab studies of wash processing.

The outcomes of this research provide further evidence that the application of chemical sanitizers during the washing-disinfection step does not guarantee the inactivation of pathogens and elimination of cross-contamination concerns. Water quality management parameters should be designed thoughtfully and implemented with great attention to detail to optimize safety and overall quality for consumers.

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