Chlorine is widely used by fresh-cut produce processors to reduce pathogens and prevent cross-contamination. However, limited information is available on the efficacy of chlorine preventing pathogen survival and the cross-contamination during washing of produce in consideration of wash-water quality change. A study of chlorine against *Escherichia coli* O157:H7 survival and cross-contamination was conducted simulating produce processors’ washing process. Spinach was inoculated with *E. coli* O157:H7 and manually washed with uninoculated iceberg lettuce in water with initial free chlorine (FC) of 40mg/L. A total of seven cycles of consecutive washing were conducted until FC ≤0.5 mg/L. After every 1 min wash, water and produce were collected to enumerate the surviving *E. coli* O157:H7 and test water qualities. Water qualities were impacted by consecutive washing and significantly affected the survival and transference of *E. coli* O157:H7. A reduction of 0.7-0.9 log-CFU/g was observed on spinach. The pathogen survived in wash-water (0.15-13 MPN/ml) and cross-contaminated onto lettuce (11-33 MPN/g), when wash-water’s residual FC was <4.0-mg/L. Results suggested that wash-water quality change severely limits chlorine inactivation of *E. coli* O157:H7 on produce and in wash-water, which provides useful information for produce processors to develop wash-water sanitizing control systems.

**Key Words:** Chlorine Water, *Escherichia Coli* O157:H7, Water Quality, Produce.
experiment [13]. Therefore, this study aimed to evaluate the efficacy of chlorine against *E. coli O157:H7* survival and cross-contamination during continuous produce-washing process taken in consideration of wash water quality change.

### Materials and Methods

#### Bacterial strains and inoculum preparation

The two-strain mixture of *E. coli O157:H7* used in this study included nalidixic acid-resistant derivatives of strains ATCC 43888 and 43889 obtained from Bacteria Strain Collection Center at Western Kentucky University. Nalidixic acid resistant strains were used to allow the selective isolation of the inoculum from natural contaminating flora. Each strain was activated (35°C, 24 h) separately in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) plus 0.1 ml nalidixic acid (100 µg/ml, Sigma-Aldrich, Inc., St. Louis, MO), and subcultured (0.1 ml) into the same broth at 35°C for another 24 hours. The two strains were then combined and centrifuged (Eppendorf model 5810R, Brinkmann Instruments Inc., Westbury, NY) at 4,629×g for 15 min at 4°C. The harvested cells were washed with 30 ml of phosphate-buffered saline (PBS, pH 7.4; 0.2 g of KH$_2$PO$_4$, 1.5 g of Na$_2$HPO$_4$·7H$_2$O, 8.0 g of NaCl, and 0.2 g of KCl in 1 liter of distilled water), centrifuged as previously described, and resuspended in 30 ml of fresh PBS. The washed inoculum was serially diluted in PBS to obtain a target inoculation level of 6.00 log CFU/g when 5 ml of washed cultures was added to 5 L fresh sterile PBS.

#### Produce and spinach inoculation

Fresh iceberg lettuce (*Lactuca sativa L.*) and baby spinach (*Spinacia oleracea L.*) were obtained from a local fresh produce wholesale establishment and used within 2 hours before the experiment. Iceberg lettuce was manually shredded to 1.5-2.0 cm strips using a sterile knife about 2 hours prior to the washing. Baby spinach leaves (300 g) were completely immersed in the prepared inoculum solution and kept under manually constant agitation for 15 minutes. Inoculated spinach leaves were then drained in biosafety hood for 1 hour, and stored at 4°C in 20 g portions for 24 hours before treatment.

#### Preparation of chlorine solution and measurement of wash water quality

A chlorine wash solution with ~40.0 mg/L free chlorine was prepared by diluting 6% NaOCl (Clorox, Oakland, CA) into cold distilled water (5°C) and adjusted to pH of 6.8 with citric acid. The FC and total chlorine (TC) concentration of solutions after each washing cycle were measured with a commercial test system including a DPD powder dispenser and chloride photometer (CP-15, HF Scientific Inc., Ft. Myers, FL). Water quality characteristics of wash water, including pH, turbidity, oxidation-reduction potential (ORP), and COD, were determined using a digital pH meter (Fisher Scientific, Fair Lawn, NY), a portable turbidity meter (2020 WE, Fisher Scientific, Fair Lawn, NY), a digital ORP meter (HI 98120, Hanna Instruments, Smithfield, RI), and a reactor digestion method (COD2 Mercury-Free COD Reagent, HACH company, Loveland, CO), respectively.

#### Produce washing process

One portion of inoculated spinach leaves (20 g) was immersed in 20 L of chlorinated washing solution, immediately followed by one portion of shredded lettuce (1,000 g). Moderate manual stirring (~90 rpm/min) was applied to keep lettuce and spinach leaves completely submerged for 1 min. Spinach leaves and washed lettuces (10 g) were immediately sorted out from the wash container into a filtered stomacher bag. Wash water was immediately sampled into 50-ml centrifuge tubes, neutralized with sodium thiosulfate, and filtered (Whirl-Pak filter bag, Nasco, Modesto, CA) for water quality and microbial analyses. After all samples were taken, the remaining washed lettuce was removed and discarded. A second portion of inoculated spinach and non-inoculated lettuce were washed the same way in the same solution. A total of seven cycles of consecutive washing were conducted until free chlorine depleted (<0.5 ppm). The weight equivalent for lettuce for each washing (1,000 g) was 5% of that of the wash solution (20 L). At the end of 7 washes, the accumulative produce to water ratio was 35%.

#### Microbiological analysis of wash water and produces

After every washing cycle, spinach, lettuce, and water samples were collected to conduct microbial analysis. Spinach leaves (20 g) were placed in a Whirl-Pak filter bag (1627 ml, 19 × 30 cm, Nasco, Modesto, CA) with 100 ml of TSB and homogenized (Masticator, IUL Instruments, Barcelona, Spain) for 2 minutes. Serial tenfold dilutions of each sample, in PBS, were surface-plated onto tryptic soy agar (Acumedia, Lansing, MI) supplemented with 0.1% sodium pyruvate (Fisher Scientific, Fair Lawn, NY; TSAP) and sorbitol MacConkey agar (BD, Franklin Lakes, NJ) with NaL (100-ppm) for enumeration of total bacterial populations and inoculated *E. coli O157:H7*, respectively. Colonies were counted manually after incubation at 35°C for 24 h. Triplicate 10-g lettuce after each washing cycle was also placed into a Whirl-Pak filter bag with 90 ml of TSB and homogenized for 2 min. The filtered 20 ml-water samples were immediately poured into 50-ml centrifuge tubes with 5 ml of 5×TSB plus 0.1% sodium pyruvate and sodium thiosulfate to dechlorinate residual chlorine. The pathogen survival in lettuce and wash water were enumerated using a modified MPN method previously described. Briefly, eight aliquots (5 ml) from lettuce or water samples were added into an 8x6 deep-well microplate, 10-fold serial diluted in TSB, covered by a sterile foil paper, and incubated overnight (18-24 h) at 35oC. Turbidity of each well was recorded after incubation, and 3-µL of droplets from each well were arrayed onto MacConkey agar (BD, Franklin Lakes, NJ) with NaL (100-ppm) and incubated at 37°C for 24 h. The characteristic *E. coli O157:H7* colonies were also confirmed by a rapid RIMTM latex agglutination assay (Remel Inc., Lenexa, KS). The growth pattern was recorded and calculated using an MPN calculator (VB-6 version).

#### Statistical analysis

The experiments were conducted with 4 replications. Water quality data including free chlorine, total chlorine, ORP, pH, turbidity, and COD, and microbiological data (survivors or reductions; converted to log CFU/g) were analyzed using the One Way ANOVA in the GLM procedures of SAS (SAS Institute, 2002). Means and
standard deviations for all data were calculated, and the mean differences were separated with the least significant difference procedure at a significance level of $\alpha=0.05$.

**Results and Discussion**

**Water quality variance during produce washing**

The initial pH, turbidity and COD of chlorinated water solution was 6.81, 0.4 NTU, and 265 mg/l, respectively, before introducing shredded iceberg lettuce and spinach (Fig 1). Wash water pH value decreased to 4.99 and returns to 5.45 by the end of 7 washing cycles (Fig 1). As expected, turbidity and COD value of wash water increased proportionally with increasing amount of fresh produce continuously introduced into wash water. By the end of the washing, the water turbidity reached 48 NTU was accompanied by COD increased to 1356 mg/l (Fig 1), which agreed with previous studies of Luo (2007) [14] and Luo et al. (2012) [15]. Turbidity indicates the wash water quality is associated with soil, debris, and organic matter. COD is used to indirectly determine organics amounts in wash waters. Both are important indexes for monitoring wash water quality during commercial fresh produce washing process. The increasing of turbidity and COD can be explained by the soil and debris from lettuce and spinach and their exudates entering into wash water.

The initial FC and TC of wash water were 40.6 and 40.4 mg/l, respectively (Fig 2). As shredded lettuce and spinach leaves were continuously washed in wash waters, the FC and TC concentration decreased significantly, and the final residual FC and TC concentration were 0.5 and 8.2 mg/L, respectively (Fig. 2). Thus, the ORP value is not a reliable indicator to monitor the FC concentration changes in wash water.

**Pathogen survival in wash waters during continues washing**

Survival of *E. coli* O157:H7 in wash waters directly related to the amount of residual free chlorine during 7 washing cycles. *E. coli* O157:H7 cells were not recovered from the wash water when the residual free chlorine was more than 4.56 mg/L (Table 1), indicating that free chlorine is a strong sanitizing reagent for controlling *E. coli* O157:H7 in water. In a most recent study, the author reported that *E. coli* O157:H7, non-O157 STEC, or *Salmonella* spp. cells did not survive in chlorinated water with residual free chlorine concentrations more than 1.0 mg/L, with animmediate contact time of 5-10 s [9]. Similar studies of Yang et al., (2013) [18], Luo et al. (2011) [19], and others [20, 21]also confirmed a rapid bactericidal effect of free chlorine in washing water. In this study, when the residual free chlorine concentration decreased to below 2.0 mg/L, even the ORP was above 720 mg/L, the pathogen started to survive in wash water, resulting a recovered population ranging from 11.3 to 32.3 MPN/ml (Table 1). In tomato and some produce processors, the option of maintaining an ORP value of 650 mV instead of measuring free chlorine was widely accepted.
as a reliable index to monitor wash water sanitizing effects [9]. However, the results of this study, in agreement with other studies [17,22], questioned the reliability of applying ORP index as a proxy for the sanitizing effects of wash water.

**E. coli O157:H7 cross-contamination**

The transference of *E. coli* O157:H7 from contaminated lettuces or baby spinachs to un-contaminated clean produce when free chlorine was depleted in wash waters has been well documented in the studies of Allende et al. (2008) [12], and Luo et al. (2011) [19]; (2012) [15]. In the present study, it is noticeable that when the residual FC concentration was more than 9.4 mg/L, no *E. coli* O157:H7 cells were detected on fresh clean lettuces (Table 1). As shredded lettuce mixed with inoculated spinach was continuously introduced into chlorinated wash waters, the rapid increase of turbidity and COD caused a significant decline of FC concentration. When the FC concentration decreased to 0.54 to 4.56 mg/L, even though the ORP of water was more than 720 mv, the pathogen transferred from contaminated spinachs to fresh uninoculated lettuces, ranging from 0.15 to 13.4 MPN/g (Table 1). Previous study of Luo et al. 2011 [19] found that 10 mg/L of residual free chlorine was sufficient to prevent cross-contamination of *E. coli* O157:H7 between contaminated and fresh clean lettuces. The required residual free chlorine concentration to prevent cross-contamination during lettuce washing is higher than that of wash water sanitization. It might be explained by pathogen cells are likely to be embedded in organic matters, and the accumulated organic materials in wash waters may protect them from exposure to sanitizers, allowing them to survive for a long time, and transfer to clean produce [12]. Once clean lettuces were attached with pathogens, a following-up washing will not inactivate the pathogen cells [23]. The findings from this study are important, because the current HACCP plan of chlorinated water sanitization applied by industry produce processors sets the critical control limit of available free chlorine level as ≥ 1 mg/L. Recently, a high amount of free chlorine (2-5 mg/L) in wash waters being determined as a critical control limit and is ready to be applied.
E. coli O157:H7 reduction on spinach

The overall total average E. coli O157:H7 inoculum level in control (unwashed) spinach leaves was 5.3 ± 0.1 log CFU/g. Total bacterial counts on TSA were similar (P>0.05) to those observed on TSA+NaL for all washing cycles, indicating that the majority of colonies found on TSA were inoculated E. coli O157:H7. Washing of inoculated spinach and uninoculated lettuce caused overall pathogen reductions on spinach by 0.7 to 0.9 log CFU/g among 7 washing cycles with FC concentration ranging from 0.5 to 40.8 mg/L. This result agrees with most other authors [24,25], who reported that various chlorine concentrations have no effect on pathogen reductions. For most wash water chemical sanitizers, especially chlorinated water solution, pathogen reductions of 1-2 log CFU/unit were widely reported by both laboratory scale and pilot plant scale studies [11,15]. This finding was slightly lower but close to the other researchers’ results, suggesting that pre-harvest decontamination approach is critical in prevention of pathogen contamination during produce processing, and simply relying on post-harvest chlorinated water washing may fail to have any sanitizing effects.

In summary, water quality changed dramatically during continuous produce washing process; the accumulated high organic loads severely limit chlorine inactivation of E. coli O157:H7 on produce and in wash-water. The results from this study verified that the pathogen reduction and cross-contamination prevention ability of chlorinated water depends on the available residual free chlorine concentration in wash solution rather than the amount of chlorine initially dumped into wash water. Therefore, monitoring and maintaining an adequate level of FC during fresh-cut produce washing is extremely important for the control of pathogens in wash water and the prevention of cross-contamination.

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