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Investigation on chlorine-based sanitization under stabilized conditions in the presence of organic load

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ABSTRACT

Chlorine, the most commonly used sanitizer for fresh produce washing, has constantly shown inferior sanitizing efficacy in the presence of organic load. Conventionally this is attributed indirectly to the rapid chlorine depletion by organics leading to fluctuating free chlorine (FC) contents. However, little is known on whether organic load affects the sanitization process directly at well-maintained FC levels. Hereby, a sustained chlorine decay approach was employed to study the inactivation of *Escherichia coli* O157:H7 under stabilized washing conditions. Chlorine solution was first incubated with organic load for up to 4 h, modeling the chlorination in produce washing lines. The FC level was then stabilized at five targeted values for sanitization study. Our study showed decreased sanitizing efficacy as the organic load increased. At 5 s residence time and pH 6.5, a minimum of 0.5 and 7.5 mg/L FC were needed to achieve a 5 log reduction at 0 and 900 mg/L chemical oxygen demand (COD), respectively. The decrease was more pronounced at lower FC, higher COD, higher pH, and shorter residence time values. The organics-associated interference with FC measurement and disruption of chlorine/bacteria interaction, together with the chlorine demand of concentrated inoculum per se, collectively resulted in inadequate sanitization. Finally, our results were compared with existing studies conducted under dynamic conditions in the context of different experimental settings. This study provided a feasible method for studying the bacteria/sanitizer interaction while ruling out the confounding effect from fluctuating FC levels, and it indicated the direct, negative impact of organic load.

1. Introduction

Fresh produce has emerged as substantial vehicles of foodborne bacterial pathogens, leading to increasing risk of illness and death in the US (Painter et al., 2013). Water washing is an essential step in the fresh and fresh-cut produce processing for removing the debris, soils, and produce latex released from the cut edges, thus maintaining the quality and shelf life of the final products (Simons, 2001). Sanitizers are commonly used during washing to prevent the release and transmission of pathogens (Gómez-López et al., 2014). Chlorine is the most widely utilized sanitizer due to its low cost and rapid bacterial inactivation. However, the efficacy of chlorine is significantly reduced by the presence of organic matters released from the produce (Gómez-López et al., 2014; Shen, 2014; Tomás-Callejas et al., 2012). A number of studies have been conducted under constantly changing conditions (i.e., produce and FC are fed batchwise or continuously) to simulate industrial washing and find out the underlying mechanisms (Luo, 2007; Zhang et al., 2009; Zhou et al., 2015). According to those studies, the inferior efficacy is largely attributed to the rapid depletion of FC by accumulating organic load, followed by periodical replenishment of FC in excess, which collectively lead to considerably fluctuating FC levels (Toivonen and Lu, 2013) (Weng et al., 2016). As a result, the FC level could potentially drop below the effective values, leading to inadequate sanitization efficacy.

However, it remains unclear whether organic load poses a direct impact on chlorine-based sanitization or, in another word, whether FC performs equally effectively at a stabilized level, with or without the organic load. Studies conducted under stable conditions enable the identification of roles for a specific parameter of interest, which is otherwise confounded by the irrelevant parameters that fluctuate under dynamic experimental settings. This provides valuable insights into the interaction among chlorine, organic load, and the bacteria. Ultimately, this would help identifying the key compound or interaction that compromises the sanitizing efficacy significantly, which benefits the

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technological advancement in preventing water-mediated cross-contamination. A recent study by Gómez-López et al. (2014) was a successful attempt with this regard, which demonstrated lower sanitization efficacy of chlorine at higher organic load at a same, stabilized FC level. However, knowledge gap remains since the sanitization time could not be precisely controlled with such an experimental design. In addition, it remains unknown whether the negative effect of organic load exists at different pH levels.

In this study, a feasible method based on sustained chlorine decay was developed to achieve precisely controlled and stabilized working parameters, including FC, chloramine, COD, and pH, all of which were representative for the conditions for fresh produce washing. The effects of organic load and pH on the depletion and stabilization of FC levels were firstly evaluated, using lettuce extract (LE) as a model source of organic load. This allowed us to find optimal strategies for stabilizing the working parameters. Thereafter, a series of sanitization experiments were performed under these well-controlled conditions, using non-pathogenic *Escherichia coli* O157:H7 as model bacteria. Two major questions were to be answered in this study. (1) Within a short time period, does free chlorine perform equally effectively, with or without the presence of organic load when maintained at the same level? (2) Is the impact of organic load on the sanitization pH-dependent? Finally, the data obtained from this study were compared with previous ones measured under dynamic washing conditions, and possible mechanisms underlying our observation were discussed in detail.

2. Materials and methods

2.1. Preparation of lettuce extract

Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) was purchased from a local grocery store in College Park, MD. The lettuce was cut manually into pieces, pressed through a household juicer, and filtered through eight layers of cheesecloth. The filtrate was further centrifuged (9000*g*, 30 min, 4 °C) and then passed through a syringe filter with 1 μm pore size (Pall Corp., Port Washington, NY, USA). These procedures yielded a clear, dark green dispersion defined as lettuce extract (LE), which was frozen at − 20 °C for subsequent assays.

2.2. Chlorine decay test

Prior to the test, LE was thawed and determined for its chemical oxygen demand (COD) using the reactor digestion method (Hach method 10236). Sodium hypochlorite solution was diluted in water (deionized, same hereinafter) according to previous literature (Shen et al., 2012; Zhou et al., 2014a) to achieve various initial free chlorine levels (FC_0) ranging from 50 to 320 mg/L (as Cl_2, measured by DPD [N,N-diethyl-*p*-phenyldiamine] free chlorine photometric method [Hach method 10069], same hereinafter). The resultant solution was adjusted to preset pH (3.0, 5.0, and 6.5) using 1 M H_3PO_4. The selection of the three pH values was based on the recommendation in a previous study (Yang et al., 2012). The two higher pHs (6.5 and 5.0) are typically found in lettuce processing facilities based on our recent field study (data to be published), while the lower one (3.0) represents an extremely acidic condition that was occasionally observed, due to poor pH adjustment or insufficient agitation. The purpose for using phosphorous acid was to avoid the interference from oxidant (e.g., HNO_3), unintended chlorine demand (e.g., from citric acid), or chloride (e.g., from hydrochloric acid). In addition, phosphorous acid-based acidulants such as T-128 have already been applied in commercial production of fresh produce (Yang et al., 2012).

After equilibrium at room temperature for 20 min, proper amounts of LE (typically 30 to 70 mL per liter chlorine solution) was introduced to achieve desired COD levels (450, 600, and 900 mg/L) and initiate the depletion process. Our preliminary study showed that the change in free/total chlorine composition followed a very similar pattern at 4 or 25 °C, although the rates of reaction were greater at higher temperatures. The pH of the mixture was monitored and maintained at the desired values (3.0, 5.0, and 6.5) in the first 10 min of experiment using 1 M NaOH, and it remained stable throughout the whole incubation process afterwards (preliminary data not shown). Samples of 2 mL were withdrawn periodically from the mixture and analyzed as follows. The DPD photometric analysis was performed as a conventional method to determine both free (Hach method 10069) and total chlorine (Hach method 8167) levels. Total chloramine was calculated as the difference between total and free chlorine (Zhou et al., 2014b). Meanwhile, the indophenol method (Hach method 10241) for FC was used for comparison, because it was significantly less interfered by chloramines than DPD method according to Hach Company.

Upon completion of the depletion test, the FC level was plotted against incubation time. For each COD/pH combination, various FC_0 levels were tested, and the FC_0 that resulted in stabilized FC levels at 10 (with a tolerance of fluctuation by 5%, same hereinafter), 7.5, 5, 2.5, and 1.25 mg/L for at least 10 min was chosen for the following sanitization test.

2.3. Bacterial culture

A three-strain cocktail of *Escherichia coli* O157:H7 (RM4406, ATCC 43895, and ATCC 700728) with ampicillin resistance and green fluorescence protein marker was used in this study. The isolate RM4406 (lettuce outbreak isolate) was kindly provided by Robert Mandrell (U.S. Department of Agriculture, Agricultural Research Service, Albany, CA, USA). Isolates ATCC 43985 and ATCC 700728 were obtained from the American Tissue Culture Collection. The cells were cultured in 35 mL tryptic soy broth (TSB, Neogen, Lansing, MI, USA) containing 100 mg/L ampicillin at 37 °C in a reciprocal water bath for 20 h. Thereafter, the cell suspension was centrifuged twice at 3000 *g* for 5 min, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in 30 mL of PBS (Zhang et al., 2015). A cocktail of inoculum with approximately 10^8 CFU/mL (determined by turbidity that had been correlated to standard plate counts) *Escherichia coli* O157:H7 was obtained.

2.4. Inactivation of Escherichia coli O157:H7 at different FC and COD levels

A batch of chlorine/LE mixtures prepared with the pH, COD, and FC_0 chosen in the "*Chlorine decay test*" section was incubated under constant stirring. As the FC level declined to 10 ± 0.5 mg/L, samples were drawn and tested by a method from Zhou et al. (2015) with slight modifications. In specific, four samples of 1.9 mL were transferred to four sterile scintillation vials (22 mL capacity). After 30 s of equilibration, 0.1 mL of the bacterial inoculum was introduced to each vial using a mechanical pipette and incubated for 5 or 20 s. Afterwards, 1 mL of neutralizing reagent (sodium thiosulfate, 50 mg/mL in water) was added to each vial, which reduced the free and total chlorine levels to zero instantly (preliminary data not shown). All these procedures were performed under constant stirring (1000 rpm) with a magnetic stirrer. The test was finished within 10 min, after which the FC level of the chlorine/LE mixture was re-checked to ensure successful maintenance. The same experiment was carried out as the FC level of the mixture declined sequentially to 7.5 ± 0.25, 5 ± 0.25, 2.5 ± 0.1, and 1.25 ± 0.08 mg/L. Negative (LE with same COD and pH as the treatment group, no FC) and positive (0.5 mg/L FC in pure water, same pH, no LE) controls were performed in parallel to validate the experimental procedures.

After the abovementioned treatments, bacterial survival was evaluated using a previously described most-probable-number (MPN)

method (Xia et al., 2012) using a 48-well (8 rows × 6 columns) deep microplate. To further confirm the survival of *Escherichia coli*, 1.5 μL from the microplates were pipetted to a tryptic soy agar (TSA) plate containing 100 mg/L ampicillin and incubated at 37 °C for 24 h. Colonies exhibiting green fluorescence under an ultraviolet lamp were counted as positive. Finally, the bacterial population in the samples was compared to that of the negative control, and the sanitizing efficacy was expressed as the reduction in log CFU/mL.

2.5. Statistics

All tests were performed in triplicate. The results were expressed as means ± standard error. For statistical analysis, the data were subjected to one-way ANOVA ($P < 0.05$) followed by Tukey's test with an experimentwise confidence level of $\alpha = 0.05$, using SAS 9 software (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Chlorine depletion as a function of initial FC and COD levels

Fig. 1 shows the changes of FC, total chlorine, and chloramine levels at pH 6.5 with different COD (achieved with filtered and diluted LE) and FC_0 levels. A similar trend of FC level was observed under all these conditions (Fig. 1A). Over 60% and 80% of the FC was depleted within the first 5 and 20 min, respectively, which were in good agreement with previous reports (Weng et al., 2016). In addition, a significant decrease in pH (preliminary data not shown) to as low as 3.6 (at 900 mg/L COD, preliminary data not shown) was observed in 15 min. This was probably attributed to the conversion of hypochlorous acid (weak) to hydrochloric acid (strong), as well as the formation of acidic by-products including haloacetic acids. To address the acidification issue, the pH was adjusted by 1 M NaOH. After the rapid depletion stage, a sustained decay of FC was observed with no observable change in pH. The chlorine depletion rate decreased gradually since the reactants were consumed continuously without supplementation. Consequently, the FC level remained stable at the targeted levels for at least 10 min. This provided us a relative wide time window that was sufficient for a series of sanitization tests at various FC and COD levels.

Concomitantly, chloramines as the major disinfection by-product identified so far accumulated rapidly in the first 20 min of the experiment (Fig. 1B), remained stable for a period ranging from 30 min to 2 h (experimental condition dependent), and then decreased by up to 26% to reach another plateau. At the final stage, the chloramine levels remaintained around 30, 14, and 7.5 mg/L at 900, 450, and 225 mg/L COD, respectively. The abundant nitrogenous compounds such as proteins and peptides in lettuce (Deborde and Von Gunten, 2008) are considered good reactants with hypochlorous acid. The three consecutive stages in the chlorine decay experiment could be explained respectively as formation of chloramines, conversion between different forms (mono-, di-, and trichloramines), and conversion of chloramines to other compounds such as chloroform (Jafvert and Valentine, 1992; Qiang and Adams, 2004).

The total chlorine (TC, the sum of FC and chloramines) level underwent a decrease by over 50% in the first 5 min of incubation (Fig. 1C). This indicated substantial conversion of FC to byproducts other than chloramines, which further suggested the reaction between FC and non-nitrogenous substances. For instance, various phenolic compounds such as caffeic acid and chlorogenic acid abound in lettuce, accounting for at most 3.5% of their total dry weight (Huang, 2016; Liu et al., 2007), and they are oxidized rapidly by chlorine. Sulfur-containing compounds such as glucosinolates are found in low abundance, but they react with hypochlorous acid several orders of magnitude faster than phenols or amines (Folkes et al., 1995; Prütz, 1998). Further exploration into the structure and formation mechanism of those chlorination by-products is essential for understanding and enhancing the sanitization process during fresh produce washing.

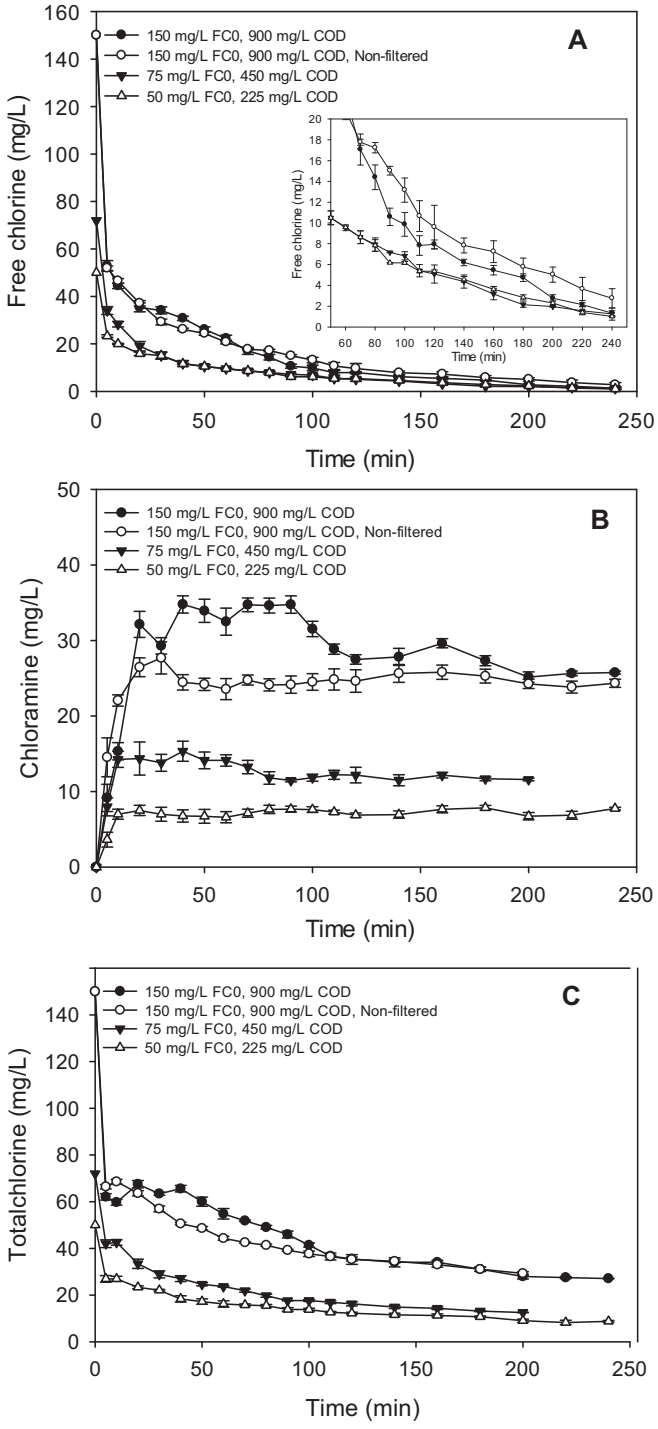

Fig. 1. Progression in free chlorine (A), chloramine (B), and total chlorine (C) levels at pH 6.5 and different initial FC contents (FC_0), with different levels of COD obtained with diluted Romaine lettuce extract.

Lastly, a separate set of experiments was conducted to compare the chlorine depletion by filtered and non-filtered LE. When both diluted to 900 mg/L COD, the non-filtered LE it depleted FC and produced chloramine at a slower rate compared to the filtered one (Fig. 1A and B). This could be explained by the existence of water-insoluble components which contributed significantly to COD but negligibly to the chlorine demand (LeChevallier et al., 1981).

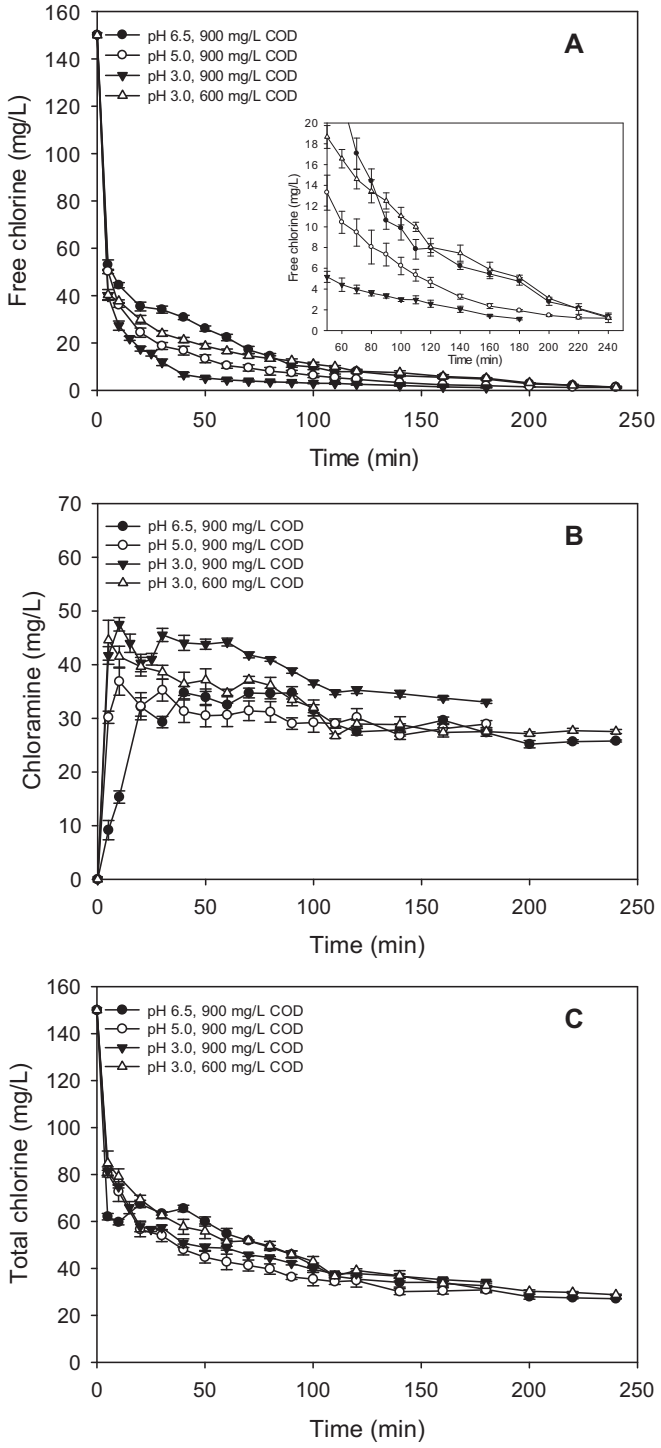

Fig. 2. Progression in free chlorine (A), chloramine (B), and total chlorine (C) levels at various pH with 150 mg/L of initial FC contents (FC_0), and different levels of COD obtained with diluted Romaine lettuce extract.

3.2. Chlorine depletion as a function of pH

We further studied the chlorine depletion profile at three pHs (6.5, 5.0, and 3.0), one FC_0 (150 mg/L), and two COD levels (900 mg/L for pH 6.5 and 5.0; 600 mg/L for pH 3.0 to achieve slower FC decay). As shown in Fig. 2A, a similar biphasic pattern of FC depletion was observed at all three pHs, but the depletion rate increased significantly at lower pH. This was evidenced in more rapid FC decay (Fig. 2A), chloramine formation/degradation (Fig. 2B), and conversion of FC to other byproducts (Fig. 2C). All these results suggested the higher reactivity between hypochlorous acid and the organic load, which was consistent with previous studies conducted in drinking water systems (Jafvert and Valentine, 1992; Qiang and Adams, 2004; Weil and Morris, 1949). From a practical point of view, the greater consumption of chlorine at lower pH suggested the faster accumulation of unwanted byproducts. This should be avoided given their known adverse impact on human health and food quality.

Table 1
Optimal experimental conditions to achieve stable FC and chloramine levels at different pH and COD levels.

FC_0* (mg/L)	COD (mg/L)	Time for reaching FC** level of (min)					Chloramine level (mg/L)
		10	7.5	5.0	2.5	1.25	
pH 6.5							
150	900	100	130	160	210	250	28.5 ± 0.7c***
75	450	45	85	95	150	190	13.2 ± 1.3d
pH 5.0							
150	900	55	75	100	150	200	29.5 ± 0.6c
pH 3.0							
200	900	45	65	90	115	145	44.3 ± 1.1a
150	600	90	130	170	210	230	34.5 ± 0.6b

* FC_0: initial free chlorine content.
** FC: free chlorine content in mg/L.
*** Different letters denote statistically significant difference ($P < 0.05$).

Based on the abovementioned results, we performed the sanitization test under the conditions specified in Table 1. These conditions allowed the stabilization of FC and chloramine contents at each targeted level (10, 7.5, 5, 2.5, and 1.25 mg/L) for at least 10 min, which was sufficient for the subsequent experiment. It should be noted that those conditions are only optimal under the current experimental settings. Switching to other types of produce or different chlorination levels requires further optimization process following the same methodology.

3.3. Inactivation of Escherichia coli O157:H7 by chlorine as a function of FC and COD levels

As discussed in the Introduction, the direct impact of organic load on the sanitization efficacy was the major focus of our study. Fig. 3 presents the inactivation of *Escherichia coli* O157:H7 by chlorine at pH 6.5 with different COD and FC levels, which were stabilized under the conditions defined in Table 1. Without any organic load, a solution containing 1.25 mg/L FC exhibited 5.76 log reduction in the bacterial dispersion in 5 s (Fig. 3A), and a log reduction over 7 was observed at FC level above 5 mg/L. Introduction of 225 mg/mL COD (or approximately 7 mg/L chloramine) did not produce any significant ($P < 0.05$) effect (preliminary data not shown), but greater levels (450 or 900 mg/L) of COD resulted in a substantial decrease in the sanitizing efficacy. Overall, the adverse effect of organic load was more significant at high COD levels, low FC contents, or shorter residence time. As an extreme example, at 1.25 mg/L FC and 5 s of residence time, a log reduction over 5 was achieved without organic load, but virtually no log reduction was achieved at 900 mg/L COD (or approximately 28.6 mg/L chloramine). A moderate sanitizing efficacy of 3.42 log reduction was observed at the same residence time and FC level, with a COD level at 450 mg/L. For verification purpose, the results in this section were measured in at least eight replicates, and they were in good consistence. However, the applicability of this result to other washing conditions or other types of produce is yet to be confirmed.

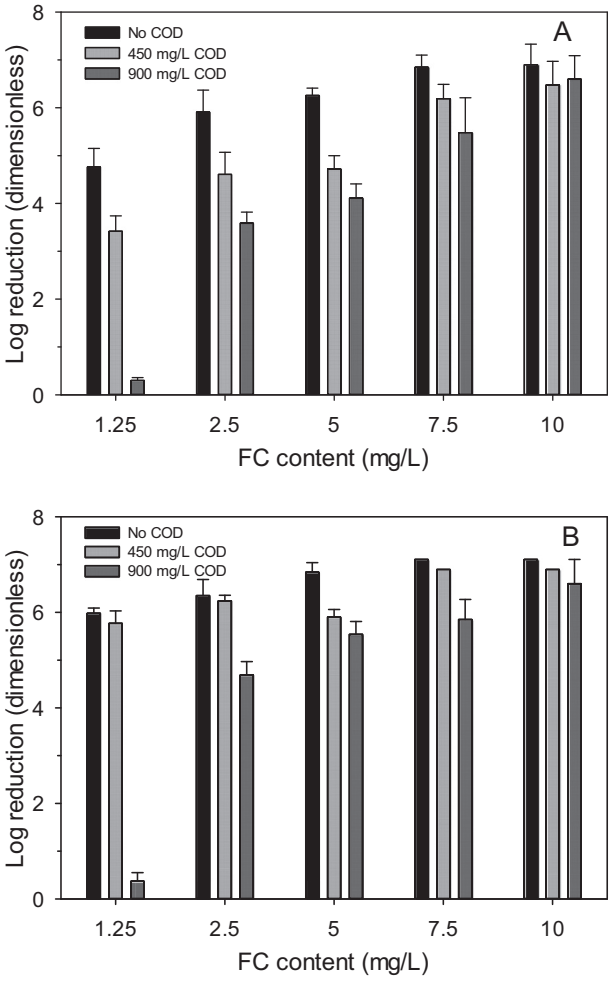

Fig. 3. Inactivation of *E. coli* O157:H7 by chlorine at pH 6.5 with different levels of organic load in 5 (A) and 20 (B) seconds.

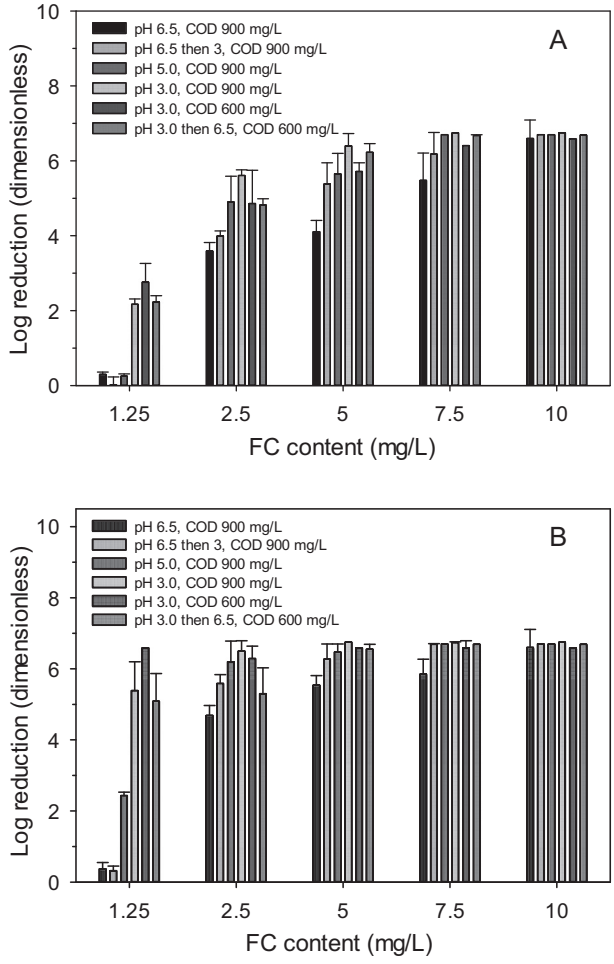

Fig. 4. Inactivation of *E. coli* O157:H7 by chlorine at different pH with different levels of organic load in 5 (A) and 20 (B) seconds.

3.4. Inactivation of Escherichia coli O157:H7 by chlorine as a function of pH

As shown in Fig. 4, the sanitizing efficacy of chlorine was improved significantly under more acidic conditions. Our preliminary trial showed no significant decrease in bacterial population under those conditions without added FC (data not shown). At pH 3.0, 5 s of residence time, and 900 mg/L COD (44.3 ± 1.1 chloramine), the log reduction achieved by 10, 7.5, 5, 2.5, and 1.25 mg/L FC was 0.15, 1.27, 2.29, 2.02, and 1.92 units higher than those obtained at pH 6.5. Nevertheless, those numbers remained considerably lower than those achieved without any organic load. Lowering the COD level to 600 mg/L at pH 3.0 resulted in virtually the same sanitization efficacy as at 900 mg/L of COD. Improved sanitizing efficacy was also observed at pH 5.0 and 900 mg/L COD (29.5 ± 0.6 mg/L chloramine), except for the very low sanitization efficacy at 1.25 mg/L FC. Regardless of the pH or FC level, the sanitizing efficacy was improved as the residence time was extended to 20 s (Fig. 4B). However, only at pH 3.0 could 1.25 mg/L of FC achieve over 5 log reduction.

3.5. Minimal FC level recommended to achieve sufficient bacterial inactivation

Table 2 summarizes the minimal FC level required to achieve at least 5 log reduction in *Escherichia coli* O157:H7 population under different experimental conditions. Without any organic load, only 0.5 mg/L was necessary to achieve sufficient sanitization within 5 s. The required FC content increased with the COD gradually, reaching a maximum of 7.5 mg/L (5 s) or 5 mg/L (20 s) at pH 6.5 and 900 mg/L COD. Lowering the pH to 5.0 or 3.0 resulted in a significant decrease in the required FC level. However, the accelerated formation of toxic and off-gassing by-products and potential quality deterioration of fresh produce bound to the low pH should be considered when this strategy is applied in practice.

Table 2
Minimal FC levels required to achieve 5 log reduction in *E. coli* O157:H7 under different experimental conditions.

pH (sanitization)	COD	Chloramine*	FC (mg/L)**	
	mg/L	mg/L	5 s***	20 s***
6.5	0	0.0 ± 0.0e****	0.5	0.5
6.5	450	13.2 ± 1.3d	7.5	2.5
6.5	900	28.5 ± 0.7c	7.5	5
5.0	900	29.5 ± 0.6c	2.5	2.5
3.0	900	34.5 ± 0.6b	2.5	1.25
3.0	600	44.3 ± 1.1a	2.5	1.25

* Chloramine levels were calculated as the difference between free and total chlorine levels, both measured by DPD photometric method.

** FC: free chlorine content.

*** FC level required to achieve 5 log reduction in two exposure times.

**** Different letters denote statistically significant difference ($P < 0.05$).

Table 3
Comparison between DPD and indophenol free chlorine methods at different FC and COD levels at pH 6.5.

FC (DPD)* mg/L	Chloramine (DPD)** mg/L	FC (indophenol) mg/L	Difference (indo-DPD)/DPD* 100%
1600 mg/L COD			
9.55	58.25 ± 1.24a***	6.63 ± 0.15c	− 31%
6.84	58.56 ± 1.09a	4.07 ± 0.14d	− 41%
5.32	58.88 ± 0.97a	2.16 ± 0.12f	− 59%
2.16	56.54 ± 0.88a	0.75 ± 0.13 h	− 65%
1.08	56.82 ± 2.15a	0.36 ± 0.09i	− 66%
900 mg/L COD			
10.75	29.50 ± 1.14b	8.30 ± 0.25b	− 23%
7.71	28.79 ± 0.35b	5.76 ± 0.28c	− 25%
5.85	29.30 ± 0.77b	4.17 ± 0.12d	− 29%
2.34	29.76 ± 1.01b	1.62 ± 0.14 g	− 31%
1.04	28.01 ± 0.82b	0.73 ± 0.09 h	− 30%
600 mg/L COD			
12.60	22.60 ± 1.12c	11.79 ± 0.45a	− 6%
10.40	23.30 ± 0.65c	8.61 ± 0.43b	− 17%
7.00	22.10 ± 0.22c	5.78 ± 0.33c	− 17%
4.92	20.68 ± 0.47c	3.22 ± 0.17e	− 35%
2.66	20.44 ± 0.52c	1.97 ± 0.15f	− 26%
0.96	19.64 ± 0.51c	0.68 ± 0.21 h	− 30%
450 mg/L COD			
12.30	16.35 ± 0.12d	12.11 ± 0.87a	− 2%
6.53	16.57 ± 0.14d	6.01 ± 0.25c	− 8%
5.55	15.10 ± 0.37e	5.82 ± 0.42c	5%
2.38	15.92 ± 0.51d	2.05 ± 0.15f	− 14%
1.15	15.70 ± 0.25d	0.88 ± 0.11 h	− 23%

* FC: free chlorine content. DPD: N,N-diethyl-*p*-phenyldiamine photometric method.

** Chloramine levels were calculated as the difference between free and total chlorine levels, both measured by DPD method.

*** Different letters denote statistically significant difference ($P < 0.05$).

3.6. Possible factors underlying the observed negative impact of organic load and pH

3.6.1. Effect of organic load

In this study, we observed a direct, negative impact of organic load on the sanitization, under precisely controlled and well-maintained conditions. The results from this study could be attributed to multiple factors. First, it has been established that both inorganic (Hach.Company, 2014) and organic (Jensen and Johnson, 1989) chloramines cause a false increase in the FC readings by the conventional DPD method. Therefore, it was hypothesized that a same reading in DPD measurement translated to lower actual FC content at higher COD, which might have resulted in the observation of lower sanitization efficacy under these conditions. To test this hypothesis, we calibrated the DPD FC method with the indophenol assay in the presence of organic load. The latter assay introduced by Hach® Company converts FC exclusively to inorganic monochloramine which then reacts with a proprietary reagent. The company claims minimal interference from chloramines for this method.

As shown in Table 3, the FC readings measured by DPD method were significantly higher than those obtained by the indophenol assay. The effect of pH on the measurement was minimal because of the buffering agents in both methods. The discrepancy between the readings from the two assays was more noticeable at low FC contents and high COD levels, ranging from three-fold (1600 mg/L or 58 mg/L chloramine) to < 23% (450 mg/L COD or 16 mg/L chloramine). These results were comparable to previous reports on monochloramine and simple amino acid-derived chloramines (Jensen and Johnson, 1989).

Another possible factor to consider is the depletion of FC by bacterial cells per se. According to Helbling et al., a suspension containing 10^7 CFU/mL *Escherichia coli* may induce a chlorine demand of approximately 0.3 mg/L in 30 s, and the bacterial cells already inactivated could continue to consume FC due to the leakage of biomacromolecules. Therefore, compared to the bacterial cells inactivated at earlier stages of sanitization, those inactivated later might have received significantly lower dose of FC. The actual FC level could be even lower in the presence of organic load, since it generates a false signal recognized as FC by the DPD method.

Furthermore, the organic compounds and chlorination byproducts in the wash water might have interfered with the bacteria-chlorine interaction, thus also altering the sanitizing efficacy. As reported by Virto et al. (2005), certain organic matter present in the TSB form a coating layer on the cell membrane of Gram negative bacteria, protecting them from being killed by chlorine. However, there is a considerable knowledge gap on the composition of organic compounds and their byproducts in fresh produce wash water. Further investigation is recommended to elucidate if the same protective effect is found in this system.

The last possible mechanism involves the organic chloramines, the predominant byproducts in the wash system. Our preliminary study using indophenol and DPD methods showed negligible level of inorganic monochloramine (NH_2Cl) and high levels of organic chloramines. Unlike monochloramine that functions synergistically with FC (Kouame and Haas, 1991), organic chloramines exhibit little (1000 mg/L expressed in FC was needed to induce 4 log reduction for *Salmonella* Enteritidis) (Bastos et al., 2005) to no (Donnermair and Blatchley III, 2003) bacterial inactivation effect. Moreover, they were found to reduce the sanitizing efficacy of monochloramine (Wolfe et al., 1985), although no literature is available so far on FC. It is speculated that organic chloramines as large biomolecules may diffuse less effectively due to their bulky structure and viscosity. However, follow-up study is needed to validate these hypotheses.

3.6.2. Effect of pH

pH might have affected the sanitization by three mechanisms. First, it determines the proportion of hypochlorous acid molecules, the active form of FC, which has been well documented (El-Kest and Marth, 1988; Stopforth et al., 2008). However, since hypochlorous acid is the predominant form at pH lower than 7, the effect of this factor is expected to be minimum. Second, it could alter the chemical equilibrium of chlorination reaction, generating various byproducts that affected the sanitization in different ways. Third, it may directly alter the activity of FC and/or the chlorination by-products at the sanitization stage. To find out which of the latter two was the predominant mechanism in our study, we adjusted the pH at two stages: chlorine depletion (i.e., reaction between FC and LE) and sanitization (i.e., interaction between FC and bacteria). As shown in Fig. 4A, when chlorination took place at pH 3.0 (and pH 5.0 as well; data not shown), higher sanitization efficacy was observed than was at pH 6.5, regardless of the pH at the following sanitization stage. These results indicated the major role of pH in the chlorination process, which produced different quantities (and possibly categories) of by-products that affected the subsequent sanitization process. In comparison, within the tested range, pH might have had a less significant impact on the FC/bacteria interaction. This was suggested by the fact that such a hypothesis was consistent with a previous study conducted without organic load (Yang et al., 2012), where FC (0.4 mg/L or above) performed equally well over the pH range of 3.0 to 7.0. However, as suggested in the previous study, the mechanism of chlorine depletion and by-product formation under the conditions relevant to fresh produce washing need to be investigated extensively to elucidate the complex role of pH.

3.7. Comparison with previous studies under simulated produce washing conditions

Since sanitization efficacy is highly dependent on experimental

design, it is important to compare our results with others obtained during simulated washing process. To begin with, our results indicated over 5 log reduction in *Escherichia coli* O157:H7 by 0.5 mg/L FC without organic load, which was similar to the reports by Shen et al. (2013)) and Luo et al. (2011). Recently, Zhang et al. (2015) found over 5 log reduction in transient periods (e.g., 0.1 ms) at a C × t value of 2.5 mg·s L^{-1}, which was also in line with our study (0.5 mg/L × 5 s equals 2.5 mg·s L^{-1} as well). These results suggested comparable mixing efficiency among different testing systems.

With organic load introduced, the results from our study were comparable with some of previous findings but different from some other reports. Gómez-López et al. (2014) investigated the sanitizing efficacy of chlorine during simulated dynamic washing. The FC level was maintained by simultaneous and continuous introduction of contaminated wash water and sanitizer. Despite the significant difference in experimental design between that and our studies, similar results were obtained in that higher FC levels are needed for complete bacterial removal at higher organic load. Recently, Gereffi et al. (2015) investigated the cross-contamination of tomatoes and recommended higher FC levels (25 mg/L compared to 10 mg/L without organic load) to prevent cross-contamination with organic load. The authors also reported the migration of bacteria from inoculated to uninoculated tomatoes in 2 s, which underscores the need for investigating the sanitizing efficacy in short periods. Another relevant study on chlorine generated in electrolyzed water reported no bacterial survival when the FC level was maintained above 3 mg/L at up to 600 mg/L COD (Gomez-Lopez et al., 2017). This value was higher than the requirement (0.5 mg/L) without organic load and comparable with the results from our study. However, the authors did not report whether different concentrations of FC were required at different COD levels.

Van Haute et al. (2013) compared the disinfection efficacy of free chlorine under dynamic washing conditions. In that study, lettuce was fed batchwise, and FC content was maintained at approximately 1 mg/L by continuously pumping sodium hypochlorite solution. Contrary to our results, the authors found no significant effect of COD on the disinfection efficacy, which could be attributed to different experimental designs between the two studies. First, the exposure time was up to 20 s in our study compared to a minimum of 1 min in Van Haute's report. Second, sanitization was performed after a relatively long chlorination process in our study to produce a stable FC, as well as a stable and relatively high level of chlorination byproducts (worst scenario). On the other hand, Van Haute's investigation aimed at replicating real-world washing process, where sanitization took place during chlorination. Finally, the ratio between bacteria and sanitizer differed between the two studies. In Van Haute's study, the bacteria migrated from lettuce (4 log CFU per gram lettuce) into water continuously at an unknown yet possibly low rate. The final bacterial count in Van Haute's study was 5.4 log CFU/mL after 1 h of accumulation, compared to 7 log CFU at the beginning of sanitization in our study. All those factors could have contributed to the lesser effect from organic load in their study.

Shen et al. (2013) reported over 5 log reduction of *Escherichia coli* at 0.5 mg/L FC and up to 460 mg/L COD during dynamic lettuce washing. However, this FC level was the final value observed after 120 s of incubation using hypochlorous acid (FC_0 = 2 mg/L) and the lettuce extract, while the sample was withdrawn for microbial analysis after 5 s of incubation. Although no information was given on the exact FC level at the time of sample withdrawal, it was likely that this value was well above 0.5 mg/L. In addition, the highest COD level used in that study (460 mg/L) was comparable to our medium level (450 mg/L), which only exhibited a moderate impact on the sanitization in our study as well.

In summary, the different results reported by previous and our studies have suggested the diversity of chlorine efficacy under various experimental designs, each of which represents a specific washing procedure, produce type, and relevant working parameters. Therefore, it is advisable that the actual sanitizing efficacy of chlorine be validated for each washing procedure in the actual produce processing facilities.

4. Conclusions

In this study, the sanitizing efficacy of chlorine on *Escherichia coli* O157:H7 was evaluated under well-maintained conditions covering a range of FC, COD, and pH levels. The chlorine depletion and chlorination by-product formation proceeded more rapidly at higher COD levels or lower pH. A significant decrease in the sanitizing efficacy was observed at 450 and 900 mg/L COD, compared to that achieved without organic load. This effect was more noticeable at shorter residence time, higher COD levels, or elevated pH. When DPD method was used for FC measurement, 0.5 mg/L FC was sufficient to reduce the bacterial population by 5 log units at pH 6.5 in pure water, whereas a minimum of 7.5 mg/L FC was required at 900 mg/L COD. Multiple factors could have accounted for these observations, including biased FC measurement in the presence of organic chloramines, rapid depletion of FC by concentrated bacterial inoculum, and possible interference with the bacteria-sanitizer interaction by organic matters or chlorination by-products. This study provided a simple and feasible method for investigating the bacteria/sanitizer interaction under stable conditions in the presence of organic load, and it underscored the potential risk of insufficient sanitization in the presence of organic load at FC levels previously demonstrated effective for drinking water disinfection procedures. However, since this is the first report to our knowledge using the chlorine decay technique, further efforts are needed to assess or improve the relevance of the experimental conditions to the chemical interactions found in actual industry-scale fresh produce washing processes.

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