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Decontamination of fresh parsley leaves by sequential wash treatment in chlorine and lactic acid solutions

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ABSTRACT

Due to concerns about the formation of carcinogenic disinfection by-products (DBPs) during decontamination process of fresh produce by chlorine based sanitisers, there has been interest in finding alternative sanitizers. Despite the intensive research, there is still no widely accepted cost effective sanitizer and most of the sanitizers in the marketplace has limited decontamination efficiency. By using parsley leaves as test vegetable, this study aimed to investigate the possibility of using a two stage sequential wash to increase the disinfection efficiency, while reducing the concentration of chlorine by application of a second stage wash with lactic acid (LA) solution. Washing of parsley leaves in 100 ±10 ppm chlorine solution for 3 min resulted in an average of 1.01±0.64 log reduction, whereas a 1 min wash in 1.0% (w/w) LA returned an average of 1.25±0.39 log reduction for TAC. Increasing washing time in chlorine or LA solutions alone did not increase the decontamination efficiency, significantly. However, 3 min wash in 100 ±10 ppm chlorine, followed by a 1 min wash 1.0% (w/w) LA (sequential wash) increased the decontamination efficiency significantly returning an average of 2.45±0.52 log reduction in total viable count. Sequential wash was also effective in reduction of artificially inoculated non-pathogenic strains of *Escherichia coli*, *Salmonella* and *Listeria* spp. Concentration of DBPs determined by GC-MS in wash water samples were below the limits set by United States Environmental Protection Agency.

I. INTRODUCTION

Fresh produce may contain a high number of microorganisms after harvest. The level of contamination may range between 3 and 7 log units depending on the season and type of the fresh produce [1]. The use of chlorinated water for decontamination of fresh produce is widespread. Therefore, there has been a continuous interest in researching various aspects of decontamination of fresh produce by washing in chlorine solutions. A common finding from these studies is that, depending on the type of produce, chlorine concentration, pH and immersion time, the typical log reduction in natural microbial flora is usually about 1 logarithmic unit [2, 3]. In addition to its limited efficacy, the possible formation of carcinogenic disinfection by-products (DBPs) in wash water such as chloramines, trihalomethanes, chloroform and haloacetic acids has been reported as a matter of concern [4- 8].

Due to growing concerns over the formation of DBPs, there has been increasing interest in identifying alternative sanitizers for fresh produce washing. A wide range of compounds—such as chlorine dioxide, quaternary ammonium compounds, organic acids, hydrogen peroxide, ozone, electrostatic sprays, silver, electrolysed water, and essential oils—have been investigated for their antimicrobial efficacy and potential to replace traditional chlorine-based methods [9]. Despite this intensive research effort, no alternative sanitizer has yet achieved broad acceptance in the industry as a cost-effective, safe, and naturally derived solution. As a result, the food industry in many countries continues to rely on chlorine, typically at concentrations between 50 and 300 ppm, as the primary

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decontamination agent for fresh produce. There are studies suggest that chlorine-based sanitizers could be safely used to wash fresh produce such as, spinach [10] and fresh-cut lettuce [11].

Nevertheless, despite the extensive use of single-agent sanitizers like chlorine, the sequential washing of fresh produce in different sanitizing solutions has received little attention from the scientific community. Singh et al. [12] investigated the efficacy of Chlorine Dioxide (ClO_2), Ozone and thyme essential oil on killing of *Escherichia coli* O157:H7 inoculated onto lettuce and baby carrots by employing a two and three step sequential washing. About 1.48–1.97 log cycle reductions in number of *E. coli* O157:H7 were obtained by aqueous ClO_2, ozonated water or thyme oil suspension. Washing of lettuce in thyme oil followed by aqueous ClO_2, or ozonated water followed by aqueous ClO_2 were significantly ($P<0.05$) more effective in reducing *E. coli* O157:H7 resulting in 3.75 and 3.99 log reductions, respectively. In another study, Singh et al. [13] found that after treatments of alfalfa seeds inoculated with *E. coli* O157:H7 by a sequential wash (thyme oil followed by ozonated water and aqueous chlorine dioxide) resulted in lesser recovery (min of 2.0 ± 0.1 log) of *E. coli* O157:H7 than any other washing treatment alone. Wang et al. [14] investigated efficacy of decontamination treatments in reducing microbial populations on cilantro by washing fresh-cut cilantro samples with tap water, acidic electrolyzed water (AEW), aqueous ozone, chlorinated water, and aqueous ozone followed by AEW (sequential wash). The initial total aerobic plate count (TPC) on the unwashed cilantro leaves was 6.7 log CFU g^{-1}. AEW and sequential wash treatments reduced TPC on cilantro by 0.66 and 0.62 log CFU g^{-1}, respectively. Contrary to expectation of the authors, sequential wash failed to yield a higher TPC reduction than the AEW treatment, which was attributed to internalization of microorganisms on cilantro surfaces or the development of resistance following exposure to the ozone treatment.

Due to limited available studies in the scientific literature on the use sequential wash treatments in produce decontamination, the subject is worthy of further investigation. In fact, Food and Drug Administration (FDA) pointed out that research is needed for determination of additive, antagonistic or synergistic effects of sanitation treatments when used in combination [15]. Therefore, the aim of this study was to devise a decontamination process that could effectively inactivate the microorganism while reducing the concentration of the chlorine in wash water. For this purpose, a sequential washing process was designed in which fresh parsley leaves were washed in chlorine and lactic acid (LA) solutions in two consecutive steps. Samples of fresh parsley leaves were either treated (washed) directly to determine the efficiency of the washing on natural microbiota of parsley or, they were inoculated with *Escherichia coli* strain ATCC 25922, *Salmonella enterica* serovar Typhimurium strain ATCC 1428 and *Listeria innocua* strain ATCC 33090 before washing in order to determine the efficiency of sequential wash on commonly encountered pathogenic bacteria. In order to assess the formation of DBPs and the safety of the sequential wash, presence and concentration of chlorinated compounds in water samples taken after the first and second stages of sequential wash were determined by GC-MS.

II. EXPERIMENTAL METHOD

2.1. Growth of Test Microorganisms

E. coli strain ATCC 25922 was revived from a frozen stock and inoculated into 200 ml of sterile tryptic soy broth (Himedia, Bombay, India) in 1 L sterile flasks. The culture was incubated at 37 °C for 24 h to reach approximately

10^9 CFU ml^{-1} in the stationary phase. The cells were then harvested by centrifugation at 7000×g for 10 min and resuspended in sterile phosphate-buffered saline (PBS) pH 7.1 (Oxoid, Basingstoke, UK) to give a concentration of approximately 5×10^9 CFU ml^{-1} stock suspension. Stock suspensions of *S. enterica* serovar Typhimurium strain ATCC 1428 and *L. innocua* strain ATCC 33090 were prepared by the same procedure followed for *E. coli* strain ATCC 25922.

2.2. Sample Preparation

2.2.1. Uninoculated samples

Whole fresh parsley was obtained from a local market at different times during the 3-months period of experimentation. Visibly soiled, spoiled or yellow leaves were separated by gloved hands and stems were trimmed off. Parsley leaves then mixed by gloved hands on a sanitised surface to get an evenly mixed pile of parsley leaves. Prepared samples were kept in the fridge operating at 4.0 ± 2.0 °C until the time of experiment. Total aerobic count (TAC) was determined by homogenizing 25 g of parsley leaves in 225 ml of sterile peptone water, followed by serial dilution and surface plating on Plate Count Agar (PCA, Biolab, Izmir, Turkey), with incubation at 30 °C for 48 hours. In order to determine the optimal duration of the chlorine washing process, parsley samples were washed in 100 ± 10 ppm chlorine solution for 1, 3, and 5 minutes at room temperature, and the effectiveness of each treatment was evaluated by measuring the total aerobic count (TAC). To prevent potential damage to the color and texture of the plant due to exposure to lactic acid (LA), parsley samples were washed in 1.0% LA solution for 1 and 3 minutes. The microbial reduction achieved under these conditions was evaluated by determining the total aerobic count (TAC).

2.2.2. Samples inoculated with test microorganisms

Inoculated parsley leaves were prepared separately for each test microorganisms including *E. coli* strain ATCC 25922, *S. enterica* serovar Typhimurium strain ATCC 1428 and *L. innocua* strain ATCC 33090. For this purpose, sorted parsley leaves (about 120 g) were mixed with 15 ml of stock suspensions of each test organisms on a sanitized benchtop and mixed for 5 min with glowed hands to obtain a homogeneous mix. The samples were then filled in sterile stomacher bags and stored overnight in a refrigerator operating at 4.0 ± 2.0 °C.

2.2.3. Preparation of washing solutions

Mains tap water was used to prepare chlorine and LA solutions. Chlorine solutions (100 ± 10 ppm) were prepared by dissolving calcium hypochlorite tablets (DZF-Tab, Ankara, Turkey) in mains tap water. The pH of the solution was adjusted to 6.5 by adding citric acid (10% w/v) solution. The free chlorine concentration was measured by a portable photometer (Lovibond Maxi Direct, Tintometer, Germany) by diluting the chlorine solution with distilled water and then multiplying the results with dilution factor. After adjusting the free chlorine level, the solution was used within 60 min. LA solutions were prepared by adding calculated amount of L(+) Lactic acid (Merck KGaA, Darmstadt, Germany) to mains water in a 30 L container and mixed well before use on the same day.

2.3. Washing of Parsley

2.3.1. First stage wash

Parsley leaves (~120 g) were washed using household fruit & vegetable washer (Model: ZA-AF, Zaet Co. Ltd. China) which gently rotated the parsley leaves in washing solutions. The ozone generator of the washer was switched off by a switch installed by the manufacturer for this study. The machine was filled up with 8 liters of wash solution to wash parsley leaves. In the first-stage wash, two separate washing procedures were applied: one involved a 3-minute chlorine wash (100 ± 10 ppm), and the other involved a 1-minute LA (1.0% w/w) wash. After a pre-set washing time, parsley leaves were removed from solution and placed into a spinner (model: SWM 6306, 10 L, Sinbo, Istanbul, Turkey) and spun for 1 min at about 250 rpm to get rid of excess moisture. Between each wash and spun, the washing barrel of fruit and vegetable washer and the centrifuge basket were sanitised by spraying 70% (v/v) alcohol and then rinsed with previously prepared chlorinated (<1 ppm) water.

2.3.2. Second stage wash

As part of the sequential washing procedure, the samples were first subjected to a 3-minute chlorine wash (100 ± 10 ppm). Following this, the samples were spun for 1 minute to remove excess water. Subsequently, a 1-minute LA (1.0% w/w) wash was conducted using a fruit and vegetable washer. The parsley leaves were then removed from the washer and spun for 1 min to get rid of the access water. The dewatered samples were then placed into sterile stomacher bags and stored in the fridge (4.0 ± 2.0 °C) until the time of microbial analysis on the same day.

2.4. Microbiological Analysis

Samples of parsley leaves (10 g) were placed into stomacher bags and 90 ml of PBS were added. The bags were homogenised for 1 min at 230 rpm in a stomacher (Seward 400, London, UK). Serial dilutions were prepared by adding 0.1 ml of the solution taken from stomacher bag into 0.9 ml of PBS in 1.5 ml Eppendorf tubes and mixing by a vortex mixer (Heidolph, Schwabach, Germany). Serial dilutions were plated in duplicate on appropriate media by using the Miles–Misra–Irwin counting method [16] and the average values from two counts were used for calculations. The total aerobic plate count (TAC) was determined by plating the dilutions on plate count agar (PCA, Biolab, Izmir, Turkey). Total counts of *E. coli*, *Salmonella* spp. and *Listeria* spp. were determined by plating the serial dilutions on Eosin Methylene Blue (EMB) agar (Himedia, Bombay, India), Xylose-Lysine-Desoxycholate agar (Oxoid, Basingstoke, UK) and Palcam agar (Oxoid, Basingstoke, UK), respectively. All plates were incubated at 37 °C for 24-48 h before counting the colonies. Microbial reduction was expressed in terms of logarithmic reduction corresponding to the logarithmic difference between the initial number of microorganisms in control samples and the number of microorganisms surviving after wash treatments.

2.5. Determination of Carcinogenic Disinfection By-Products (DBPS) in Wash Water

As an indication for the amount of chlorinated compounds in washed parsley leaves, the concentration of chlorinated compounds (bromodichloromethane, dibromochloromethane, 1,2 dichloromethane, tetrachloroethane, tribromomethane, trichloroethane, trichloromethane and benzene) in water samples taken after first stage wash in

100±10 ppm chlorine, and after the sequential wash were determined. The water samples were filtered through a filter paper (type751/75/20, Macherey-Nagel GmbH & Co. KG., Germany) into a sterile screw capped plastic container up to the brim without leaving a head space and the container was tightly closed. The samples were then quickly cooled in a refrigerator and kept cool until the time of analysis on the same day.

2.5.1. GC-MS Analysis

A gas chromatograph (GC) coupled with mass spectrometer (MS) (Bruker, Model: SCION TQ 456GC, Massachusetts, USA) was used to determine the chlorinated compounds. A 10 µl syringe was used to obtain samples by puncturing the plastic lid of the sample bottle and 1 µl sample was injected to the instrument for analysis. Before the start of the analysis the oven temperature was raised to 230 °C for cleaning the residues from previous runs. Then, following temperature profile was used for the oven, after an 8 min holding time at 30 °C, temperature was first increased to 150 °C at a rate of 5°C min^{-1} and then to 230 °C at a rate of 25°C min^{-1} and maintained at 230 °C for 2 min. Manifold, ion source and transfer line temperatures for MS were 40 °C, 260 °C and 300 °C, respectively. Electron impact mass spectra was recorded with an ionization voltage of 70 eV. The flow rate for Helium was 1.00 ml min^{-1} and total run time was 17.8 min. Data were collected and analysed by MSWS v.8 software of the GC-MS. Estimation of concentration of the chlorinated compounds were based on the areas of the peaks detected by MS, which was calibrated by standard solutions previously.

2.6. Statistical Analysis

Sequential washing experiments on uninoculated parsley leaves, which comprise the main body of this study, were carried out on 12 different days. On each day of experiment, five samples from each treatment condition were used for microbiological analysis. For the experiments that were conducted to investigate the effect of washing time, washing temperature, concentration of washing solutions and to determine the efficiency of sequential wash on *E. coli*, *Salmonella* spp. and *Listeria* spp. in inoculated samples, 3 replicates were used for each experimental condition. Standard deviations (StDev) were calculated by using MS® Excel® 2016. Data were analysed using the Statistical software (SPSS v.16, IBM, Chicago, IL, USA) by one-way analysis of variance. Tukey's Post Hoc test was used to determine differences at a=0.05.

III. RESULTS

3.1. Effect of Chlorination Wash on Microbiota of Parsley Leaves

Microbial load of fresh parsley leaves determined by TAC carried out on 12 different days was on average 6.15 ± 0.64 log unit. Studies show that 100 ppm is generally used as the free chlorine concentration [17]. As the aim of the study was to minimize the chlorine concentration while optimising the disinfection power of the washing process, 100 ppm chlorine concentration was chosen for wash treatments. Reduction in amount of TAC in parsley samples as a result of washing in 100 ±10 ppm chlorine solution for 1, 3 and 5 min were on average 0.91 ± 0.34, 1.01 ± 0.64 and 0.98 ± 0.41 log, respectively. It is known that extending contact time does not contribute

significantly to the disinfection power of chlorine and similar results were reported for fresh produce previously [3, 18].

3.2. Effect of LA Concentration and Washing Time on Microbiota of Parsley Leaves

In order to minimise any damage to the colour and tissue of the plant due to exposure to LA solution, an approach to minimise the concentration of LA and contact time was adopted. Results showed that increasing washing time in 1.0% LA solution from 1 min to 3 min resulted in 1.25±0.39 and 1.10±0.44 log reductions, respectively. The difference was not statistically significant ($P>0.05$), the lesser inactivation after prolonged exposure to LA could be explained by activation of proton pumping mechanisms in microorganisms. Lambert et al. [19] studied the modelling of microbial inhibition by weak acid preservatives, demonstrated that it was feasible to pump protons out of the cell by a mechanism known as H+-ATPase, and concluded that the recovery of the cell upon exposure to weak acids is time and concentration dependent. There might be an optimum exposure time to weak acids for the maximum killing of microorganisms. Extending the contact time with organic acids may activate the proton pumping mechanisms which could raise the internal pH of the cytoplasm and give better survival chance to the microorganisms. Increasing LA concentration from 0.8% to 1.2% (w/w) did not increase the microbial load significantly after 1 min wash. The average reductions in TAC were 0.85 ± 0.31, 1.25 ± 0.39 and 1.15 ± 0.42 log unit for 0.8, 1.0 and 1.2% (w/w) LA solutions. Gurtler et al. [20] reported a 1.39 log reduction as a result of 2 min washing of strawberries in 1.0% LA. Based on our results 1 min wash in 1.0 (w/w) LA was used for wash treatments.

3.3. Effect of Washing Solution Temperature on Reduction of Microbial Load

A separate set of experiment were conducted to understand whether using a mild washing solution temperature (40 ± 2 °C) could increase the disinfection efficiency as compared to washing treatments carried out at room temperature (20 ± 2 °C). As seen in Figure 1, first chlorine wash at 40 °C resulted in about 0.32 log higher log reduction on average, compared to washing at 20 °C. Increased microbial reduction as a result of increased chlorine washing temperature has been shown previously [21]. Increasing the washing temperature for first LA wash increased microbial reduction only slightly whereas, no difference in microbial reduction as a result of increased washing temperature was observed for sequential wash (Figure 1). Therefore, subsequent sequential washing experiments were carried out at room temperature.

3.4. Effect of Wash treatment on Reduction of TAC on Parsley

In line with literature data, our results show that chlorination wash (100±10 ppm, 3 min) could reduce the natural microbiota of parsley by 1 order of magnitude. Similar or slightly better results (1.25 log) are achieved when 1.0% LA (w/w) solution is used for washing of parsley. In contrast, the sequential wash procedure—combining both treatments, washing of parsley first in 100 ± 10 ppm chlorine for 3 minutes followed by a wash in 1.0% (w/w) LA for 1 min, —achieved a significantly higher microbial reduction, reaching between 1.45 to 3.27 log reduction, average reductions being 2.45 ± 0.52 log unit (Figure 2). As the experiments were carried out on natural microbiota of 60 samples on 12 different days, the variation in initial microbial load was high (8.5×10^4 to 6.3×10^6 CFU g^-

[1]) and therefore, the observed standard deviations in log reductions were also high. Although the individual treatments provided moderate decontamination, the sequential application proved more effective, suggesting a synergistic effect between chlorine and LA.

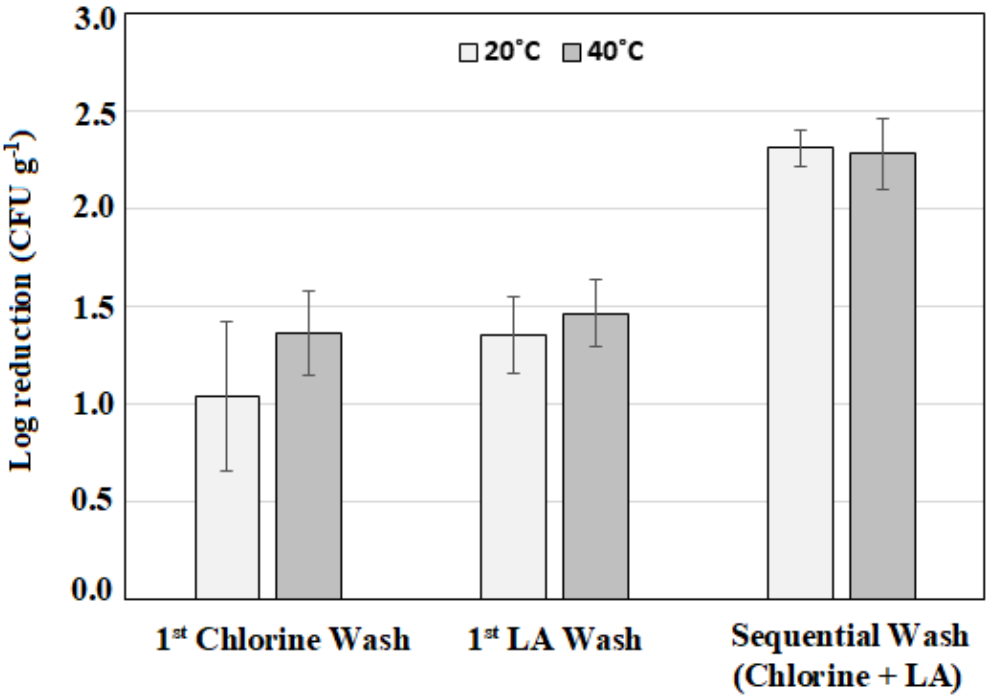

Figure 1. Reduction in the number of TAC as a function of washing solution temperature after a 3 min wash in 100 ± 10 ppm chlorine, 1 min wash in 1.0% (w/w) LA solution and sequential wash. Error bars shows ± 1 standard deviation, n=3.

3.5. Reduction in Number of E. coli, Salmonella and Listeria spp. in Inoculated Samples as a Result of Wash Treatments

Further experiments were carried out to validate the effect of sequential wash on samples inoculated with *E. coli* strain ATCC 25922, *S. enterica* serovar Typhimurium strain ATCC 1428 and *L. innocua* strain ATCC 33090. Before the inoculation, the number of *E. coli* spp. and *Listeria* spp. determined by plating on selective agar plates were 4.2 x 10^6 CFU g^{-1} and 6.7 x 10^2 CFU g^{-1}, respectively. *Salmonalla* spp. was not detected (<100 CFU g^{-1}) on uninoculated parsley leaves. After the inoculation the number of *E. coli* spp., *Salmonella* spp. and *Listeria* spp. in inoculated samples determined by plating on appropriate selective media were, 9.00 ± 0.01, 8.77 ± 0.02 and 8.89 ± 0.09 log CFU g^{-1}, respectively. As seen in Figure 3, the individual washing treatments with chlorine and lactic acid (LA) demonstrated moderate reductions in microbial load across all tested pathogens. For *E. coli*, chlorine and LA treatments achieved log reductions of 1.44 ± 0.08 CFU g^{-1} and 1.28 ± 0.16 CFU g^{-1}, respectively. In the case of *Salmonella* spp., chlorine resulted in a reduction of about 1.09 ± 0.35 CFU g^{-1}, while LA yielded a decrease of 1.46 ± 0.25 CFU g^{-1}. For *Listeria* spp., the log reduction was slightly higher, reaching 1.41 ± 0.08 CFU g^{-1} with chlorine and 1.58± 0.12 CFU g^{-1} with LA. Although both agents were effective to some extent, neither treatment alone was sufficient to achieve substantial microbial inactivation (less than 1.5 log reduction). However, reduction in number of inoculated *E. coli*, *Listeria* spp. and *Salmonella* spp. as a result of sequential wash were significantly

better (2.36 ± 0.22, 2.27 ± 0.03 and 2.46 ± 0.29 log, respectively), similar to the decrease in TAC in the natural microbiota of parsley leaves. These findings indicate that the combined application of chlorine and LA can effectively reduce the pathogenic bacterial load in fresh produce by increasing washing efficiency.

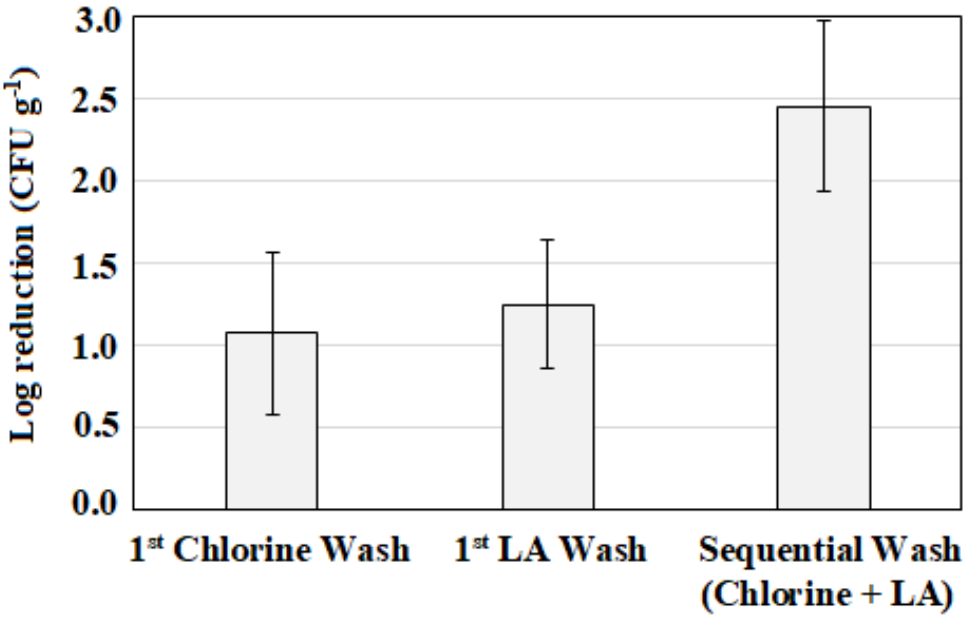

Figure 2. Reduction in the number of TAC after a 3 min wash in 100 ± 10 ppm chlorine solution, 1 min wash in 1.0% (w/w) LA solution and sequential wash at room temperature (20 ± 2 °C). Error bars shows ±1 standard deviation, n=12.

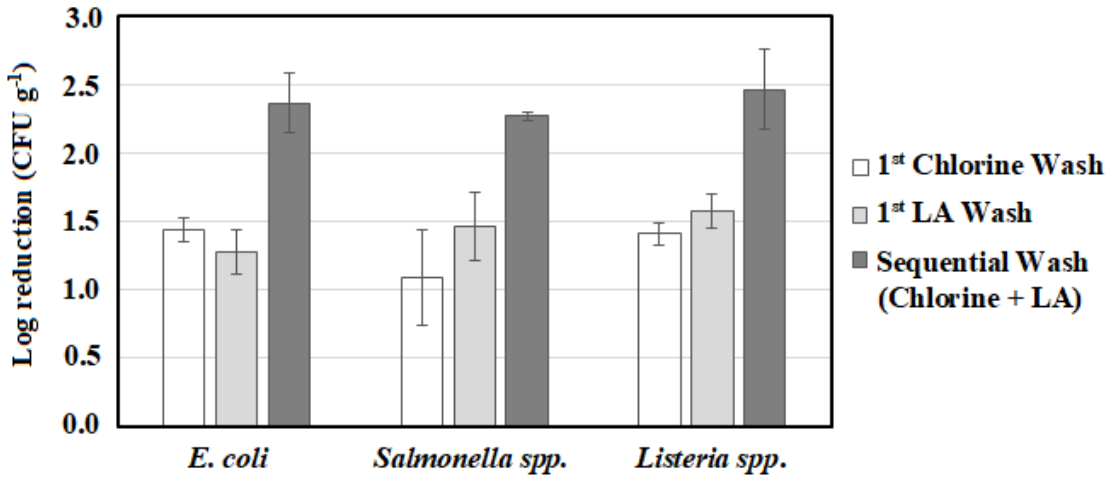

Figure 3. Reduction in number of *E. coli*, *Salmonella* spp. and *Listeria* spp. in inoculated samples as a result of sequential wash at room temperature (20 ± 2 °C). Error bars shows ± 1 standard deviation, n=3.

3.6. Carcinogenic Disinfection By-Products in Wash Water

The concentrations of DBPs in the tap water used during the first chlorine wash and the sequential wash, as determined by GC-MS, were given in Table 1. As seen from the table there was no statistically significant

difference in concentration of chlorinated compounds between tap water and any of the washing treatments, except for tribromomethane (P=0.031), where the concentration was very low (0.000 to 0.003 ppb). According to regulations by United States Environmental Protection Agency, maximum contaminant level (MCL) for Benzene, 1,2-dichloroethane and dichloromethane is 5 µg L^{-1} [22]. Our results show that none of the water samples contained DBPS above the MCL set by US EPA. The amount of DBP_S in wash water can be taken as an indication for the amount of DBP_S in washed parsley leaves. Gómez-López et al. [6] reported that the amount of chlorinated compounds in baby spinach after washing in chlorine solution was 3 to 4 orders of magnitude less than the amount of chlorinated compounds in wash water. López-Gálvez et al. [11] reported that carcinogenic halomethanes formation in fresh-cut lettuce was negligible.

Table 1. Concentration of DBPs in mains tap water used for washing experiments and water samples taken immediately after first chlorine wash and sequential wash (n=3, α=0.05).

Chlorinated compounds	Tap Water		Water from 1st chlorine wash		Water from sequential wash (Chlorine + LA)		P value
	Mean (µg L^{-1})	StDev	Mean (µg L^{-1})	StDev	Mean (µg L^{-1})	StDev	
Bromodichloromethane	0.097	0.010	0.960	0.019	0.830	0.083	0.565
Dibromochloromethane	0.157	0.012	1.270	0.032	1.280	0.030	0.326
1,2 dichloromethane	0.194	0.082	0.178	0.055	0.172	0.044	0.799
Tetrachloroethane	0.328	0.050	0.256	0.067	0.266	0.066	0.286
Tribromomethane	0.001[a]	0.000	0.004[b]	0.002	0.002[a]	0.001	0.031
Trichloroethane	0.535	0.100	0.521	0.107	0.459	0.107	0.554
Trichloromethane	0.033	0.003	0.032	0.005	0.030	0.006	0.697
Benzene	0.212	0.067	0.298	0.088	0.214	0.014	0.147

Values with different superscripts within rows are significantly different (P≤0.05, n = 3).

IV. DISCUSSION

It has been stated that the majority of the experiments on decontamination of fresh produce are carried out in unrealistic conditions that renders the results because of the extreme doses, excessive washing times and the use of unauthorized substances [23]. In addition, artificially inoculated samples with high number of microorganism may not give a true picture of the efficiency of a decontamination process, as attachment of the microorganism to the surface of a test vegetable and biofilm formation may not take place to the extent of a vegetable contaminated naturally while growing. In order to evaluate the efficiency of the wash treatments under more realistic conditions, the main experiments were carried out on uninoculated parsley samples. Artificial inoculation was considered to be potentially misleading in assessing the actual efficacy of the treatments, as microorganisms may not adhere to the leaves to the same extent as they would under natural contamination, due to limited biofilm formation. In our experiments, we tested the effectiveness of sequential wash on natural microbiota of parsley substantially, by conducting experiments on 12 different lots of parsley. The results were also confirmed on inoculated samples with *E. coli*, *Listeria* spp. *and Salmonella* spp. slightly higher inactivation obtained as a result of washing in chlorine and LA alone could be explained by the inability of bacterial attachment to the surface of parsley leaves due to artificial inoculation.

Although organic acids have certain disadvantages—such as high cost, strong odour, and corrosiveness—and their use at high concentrations or with prolonged contact times may lead to tissue damage in leafy vegetables [24], they remain widely studied for their antimicrobial potential. Among these, LA which has a GRAS (Generally Recognized as Safe) status, was used for decontamination of fresh produce by several researchers [25-29]. Organic acids share a common mode of action. In aqueous solution, weak-acids exist in pH-dependent equilibria between

uncharged acid molecules and their respective charged anions. The proportion of undissociated acid increases as the pH declines. It is generally agreed that only undissociated acids have antimicrobial activity, although some activity by anions has been suggested [19]. Less direct antibacterial activities include interference with nutrient transport, cytoplasm membrane damage resulting in leakage, disruption of outer membrane permeability, influence on macromolecular synthesis [30] and disturbing of transmembrane proton motive force and causing an inhibition of acid sensitive enzymes [31].

Alakomi et al. [32] reported that a 5 mM (~0.05%, w/v) LA solution (pH 4.0) caused prominent permeabilization in outer membrane of *E. coli* O157:H7, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium, the effect in the fluorescence assay being stronger than that of Ethylenediaminetetraacetic acid (EDTA, a classical cell membrane permeabilizing agent). Considerable proportions of lipopolysaccharide were liberated from S. *enterica* serovar Typhimurium by lactic acid. Analysis of liberated material by electrophoresis and by fatty acid analysis showed that LA was more active than EDTA or Hydrochloric acid in liberating lipopolysaccharide from the outer membrane. Thus, authors concluded that lactic acid, in addition to its antimicrobial property due to the lowering of the pH, also functions as a permeabilizer of the gram-negative bacterial outer membrane and may act as a potentiator of the effects of other antimicrobial substances.

It has been stated that chlorine could facilitate the diffusion of LA through cell membrane. Venkobachar et al. [33] reported that treatment with chlorine induced the leakage of macromolecules from *E. coli* cells indicating the permeability changes of the membrane. Proteins and RNA were detected in the supernatant when the cells were treated with chlorine dose of 1.5 mg L^{-1}. The presence of DNA was observed only at high chlorine doses. Boulos et al. [34] studied viable and total counts of bacteria in drinking water by a rapid epifluorescence staining method using the LIVE/DEAD® Bacterial Viability Kit (BacLight™). Increased chlorine concentration up to 3 ppm induced a decrease in viability, indicating bacterial cell membrane damage by chlorine.

Due to membrane permeabilization power of both chlorine and LA, it is likely that the combination chosen in our study has an additive (if not synergistic) effect on microbial inactivation. The particular order of sequential wash conducted in our study was chosen based on the assumption that residual chlorine on parsley leaves from 1st stage wash could be washed off by LA solution. In addition, after the final stage of washing the surface of the produce could be left slightly acidic which could suppress the bacterial growth. Nevertheless, a rinsing step could be added if the residual LA is not wanted on the produce surface.

Literature search on the subject returned no sequential wash studies employing chlorine with LA for sanitising of food products. The only comparable study was done by Lang et al. [35] who studied efficacy of organic acids and hypochlorite treatments for eliminating *E. coli* O157:H7 (inoculated at a level of 10^6 CFU g^{-1}) from alfalfa seeds prior to sprouting. They used various combinations of high concentrations (2.5-5.0%, v/v) of LA and acetic acid with chlorine (up to 20 000 ppm). Soaking treatments with 5.0% LA (10 min at 42°C) returned 3.0 log reduction whereas, successive treatments with 5.0% LA for 10 min at 42°C followed by 2000 ppm active chlorine for 15 min at 25°C resulted in 4.1 log reduction.

Our results suggest that after sequential washing of parsley does not generate significant amount of DBPs. Similar results were reported by Klaiber et al. [21] where they determined that the by-product formation due to chlorination of minimally processed carrots with tap water containing 200 mg L^{-1} free chlorine was negligible (<0.2 mg L^{-1}).

Although this study was not specifically designed to eliminate viruses, the use of chlorine in fresh produce decontamination may offer additional safety benefits, particularly during viral pandemics. Virus contamination on produce can occur through polluted water during cultivation or via infected food handlers—symptomatic or asymptomatic—during harvest and postharvest stages [36, 37]. Emerging zoonotic viruses, such as respiratory coronaviruses and influenza, may also pose a transmission risk via contaminated food, especially since some, like SARS-CoV, can replicate in the gut, suggesting possible oral transmission through produce consumption [38]. SARS-CoV-2 has been detected in faeces [39–42] and wastewater [43, 44], and indirect transmission via contaminated hands and mucous membrane contact has been reported [45]. Coronaviruses such as HCoV 229E, MHV, and TGEV are more stable at acidic pH [46], making acid-based disinfectants less effective. In contrast, chlorine is highly effective against coronaviruses even at 10 ppm [47], suggesting that its controlled use in produce washing may offer an added layer of protection during pandemics.

V. CONCLUSIONS

Under the experimental conditions tested in our study, results were obtained indicating that washing parsley leaves in 100±10 ppm chlorine solution followed by 1.0% (w/w) LA solution did not result in the formation of DBPS above the limits set by the US Environmental Protection Agency. Although the results need to be tested under industrial conditions, our results indicate that a sequential washing process using moderate chlorine and LA concentrations can be safely used for decontamination of parsley leaves under controlled conditions. The extra safety margin obtained by the sequential washing proposed in this study may be valuable for the fresh and fresh-cut vegetable industry. It may also help to provide a significant reduction in the chlorine concentration used in washing sprouting seeds, while also increasing the efficiency of the disinfection process. Further studies shall focus on testing and optimizing the sequential washing process on other vegetables and sprouting seeds where very high chlorine concentrations are used. Larger scale experiments are also needed to prove the effectiveness of sequential washing with reduced chlorine and/or organic acids and to investigate the formation of DBPs in industrial applications.

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