EFFECTIVENESS OF CHEMICAL WASH INTERVENTIONS FOR KILLING *SALMONELLA ENTERICA* AND *ESCHERICHIA COLI* O157:H7 ON PARSLEY AND GREEN ONIONS

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Abstract
The effectiveness of selected antimicrobial solutions for killing Salmonella enterica and Escherichia coli O157:H7 on artificially inoculated parsley and green onions has been tested. Samples (25 g) of green onions and parsley were artificially inoculated with a 5 strain-cocktail of each pathogen and held at 23 ± 1 °C (16 to 18 hours) before immersing them (2 minutes) in: sterile distilled water (H_2O), chlorine (CHL; 150ppm), 1% (vol/vol) PRO-SAN (PRO1), or 2% (vol/vol) PRO-SAN (PRO2). Subsequently, the vegetables were rinsed in distilled water (3 seconds) to remove residual antimicrobials. Inoculated vegetables, but not immersed in antimicrobial solutions, served as control. Samples were transferred into a stomacher bag containing 50 ml of sterile 0.1% (w/v) peptone water. Aliquots (0.1 ml) of peptone wash solution were surface-plated on xylose lysine tergitol 4 agar or sorbitol MacConkey agar containing nalidixic acid (50 µg/ml) for counting viable S. enterica and E. coli O157:H7, respectively. Following incubation at 35 ^0C for 48 h the bacterial colonies were counted. Immersion of inoculated parsley in water alone reduced initial numbers of E. coli O157:H7 and S. enterica by 1.3 and 1.07 log, respectively. Log reductions on green onions immersed in water were 0.43 (E. coli O157:H7) and 0.58 (S. enterica). Both CHL and PRO1 reduced initial numbers of the pathogens by approximately 2.5 log. The PRO2 solution killed more than 5.0 log of each pathogen on the leafy greens.

Key words: leafy greens, chlorine, PRO-SAN, decontamination, *E. coli* O157:H7 and *S. enterica*

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1. INTRODUCTION

Pathogens can contaminate leafy greens during any point of the *farm-to-table* chain, from production, harvesting, postharvest handling, fresh-cut or value-added operations to distribution and end-user handling. Also, the leafy greens growers recognize that once their produces are contaminated, removal or killing the pathogens is difficult and prevention of microbial contamination at all steps from production to distribution is strongly favored but not always possible. There are many on-farm factors such as the presence of wild animals, birds and insects, soil amendments and irrigation waters can carry human pathogens (Söderqvist, 2017; Pachepsky et al., 2011) which can make almost impossible to avoid on the field contamination of leafy produces. Other sources of contamination include field workers, lack of *on-farm* hand-washing and sanitizing facilities, and the equipment used during harvest and transport to the processing plant (Beuchat, 2006). Because of fewer known effective food safety interventions that can be applied during pre-harvest of leafy greens, Good Agricultural Practices (GAPs) are put in place and focus on water management, testing soil amendments, manure management, sanitation practices, wildlife and pest control, and worker training and hygiene during pre-harvest production (Food and Drug Administration, 1998). The success of GAPs in preventing cross-contamination of and ensuring the safety of the produce is not always achieved and studies have shown that growers do not always follow GAPs on their farms (Ellis et al, 2005; Rodrigues et al., 2014; Kovacs and Davis, 2014) with negative consequences on the safety

of the product before human consumption. Studies on the recent outbreaks associated with leafy greens showed that contamination of at least 20% of the products occurred on the farm, while the rest of the outbreaks was associated with improper handling of produce after leaving the farm (Yaron and Römling, 2014). The current pre-harvest food safety interventions are sometimes limited in achieving their objective and leafy greens could be subjected to subsequent cross-contamination during washing or due to poor hygiene practices during handling and packaging. Furthermore, these vegetables are often eaten raw, the implementation in the produce chain of an efficient washing step would represent a major intervention for controlling the microbial safety of these products.

Among the leafy greens, parsley and green onions are extensively used in foodservice units and in households and can be eaten as garnishes or in mixed salads where they pose a great risk to contaminate the other foods with which they come into contact (Söderqvist, 2017). Herman et al. (2015) reported that 85% of outbreaks were attributed to food prepared in a restaurant or catering facility or an ill food worker was implicated as the source of contamination in 31% of outbreaks.

Moreover, *Salmonella* and *Escherichia coli* O157:H7 have been identified as the primary bacterial pathogens of concern in green onions and parsley. According to the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) parsley is ranked in the *Level 1 Priority* group whereas green onions, which differ in morphology from the other leafy greens, have been ranked in the second highest priority group (*Level 2 Priority*) of concern in terms of microbiological hazards among fresh fruits and vegetables (FAO/WHO, 2008)

Water, chlorine or chlorine derivatives are the traditional washing interventions used to control the foodborne pathogens and they were extensively studied as antimicrobial interventions. Use of potable water for washing leafy vegetables has a very modest antimicrobial effect (WHO & FAO, 1998). The oxidizing chemicals such as chlorine, hypochlorite, chlorine dioxide, peracetic acid, hydrogen peroxide and ozone have been routinely used to reduce microbial contamination on fruits and vegetables (Lopes, 1999). Chlorine is used extensively because it is inexpensive and it is effective in killing pathogens when used on vegetables and fruits. In a quantitative assessment of the impact of cross-contamination during the washing step of ready-to-eat leafy greens, it was found that chlorine concentration should be kept above 10 mg/L to minimize the risk of illness by Salmonella (Maffei, 2017). The pitfall of using chlorine is the potentially long-term health issues that arise from the use of oxidizing sanitizers on produce (Mohamadshafiee and Taghavi, 2012; Singh et al., 2002; Lopes, 1999). Another relevant issue is that many chemical sanitizers tested on fresh produces are hydrophilic solutions and do not have the ability to completely wet the waxy (hydrophobic) surface of certain fresh produce such as peppers and cucumbers to adequately contact pathogens and kill them (Delaquis et al., 1999). This problem may be solved by use of a surfactant that would increase the wettability of the hydrophobic surfaces and exposure pathogens to the antimicrobial treatment.

In addition, several post-harvest interventions were tested for leafy greens decontamination. Huang and Chen (2011) used 2% lactic acid to inactivate *E.coli* O157:H7. Ganesh et al. (2010 and 2012) used electrostatic spraying of leafy greens for inactivating *E.coli* O157:H7 with malic acid, lactic acid, acetic acid alone or in combinations with grape seed extract. Sodium hypochlorite solutions (300 or 600 ppm) were used to reduce biofilm formed by *E.coli* O157:H7 on leafy greens (Niemira and Cooke, 2010).

Other recent studies propose the use of essential oils for their antimicrobial activities against *Salmonella* and *E. coli* O157:H7. It was found that treatments with 0.3% carvacrol or 0.5% cinnamaldehyde reduced *E. coli* O157:H7 and *Salmonella* by 5 log CFU/g on cilantro and

dill (Jitendra et al., 2018). Nevertheless, interventions using essential oils is a very expensive choice when compared with the traditional washes and would not be cost effective for producers or retailers.

Considering all the factors that may affect the efficacy of a washing intervention, some studies have evaluated antimicrobials alone or combined with surfactants to inactivate pathogens on fresh produce items. For example, a vegetable wash (PRO-SAN, 1% w/v) that contains citric acid and an anionic surfactant, reduced viable populations of *Salmonella enterica* on whole tomatoes by more than 3.0 log cycles (Mendonça et al., 2004). Also, log reductions of *S. enterica* on Serrano peppers and whole tomatoes immersed for 2.0 minutes in 1% (w/v) PRO-SAN solutions ranged from 2.31 to 3.0 (for tomatoes) and 3.39 to 4.1 (for serrano peppers) (Mendonça et al., 2011). Based on these reported results, combinations of organic acid and anionic surfactant seem to be a promising alternative to chlorine-based chemicals for decontaminating fresh produce. In addition, PRO-SAN has the Food and Drug Agency (FDA) approval for use in washing fruits and vegetables at concentrations from 1 to 5% (w/v) and is also approved by Environmental Protection Agency (EPA) being considered environmentally friendly because of its biodegradable character. Also, PRO-SAN may be used at 2% for sanitizing food contact surfaces without a subsequent rinse step.

Accordingly, the objective of this study was to evaluate the effectiveness of the commercially available fruit and vegetable wash, PRO-SAN LC (Microcide, Inc. Detroit, Michigan) which contains an organic acid and surfactant, for inactivating *Salmonella enterica* and *Escherichia coli* O157:H7 on parsley and green onions.

2. MATERIALS AND METHODS

Preparation of inocula. A 0.1 ml of working nalidixic-acid-resistant culture of *Salmonella enterica* or *Escherichia coli O157:H7* was transferred to 10 ml of tryptic soy broth supplemented with 50µg/ml nalidixic acid (TSBNA) and incubated at 35 °C for 24 h. Two consecutive 24-hour transfers of each culture in TSBNA were performed. To obtain 40 ml of a 5-strain mixture of each pathogen, 8.0 ml of each strain or serotype were combined in an appropriately labeled sterile centrifuge tube. The cells were harvested by centrifugation (10,000 x g; 8.0 min; 4 °C), the supernatant was discarded and the cells were suspended in 10 ml of sterile saline (0.85% NaCl).

Inoculation of green onions and parsley. Green onions or parsley were placed in separate sanitized plastic containers and were manually mixed, using new surgical gloves, to distribute evenly the natural microflora over the surfaces of these fresh produce items. A fresh pair of gloves was used for each type of produce. Portions of 25-gram green onions or parsley were weighted out aseptically using sanitized scissors (90% ethanol and flame) to adjust the weight of the portions. Green onions or parsley were each inoculated with 0.05 ml (50 µl) of the *Salmonella enterica* or *Escherichia coli O157:H7* inocula to give approximately 10^7 CFU/sample. The 50 µl of inoculum was distributed in drops placed in a linear pattern along the length of the green onions and randomly distributed onto parsley leaves. The inoculated samples were placed on sterile aluminum foil in the laminar flow hood (at 23 ± 2 °C) with the fan operating for 30 minutes to air dry. Subsequently, the fan was turned off and inoculated samples remained at 23 ± 2 °C (16 to 18 hours) under the laminar flow hood to allow the attachment of pathogen cells to the samples' surface before immersion in the antimicrobial solutions.

Preparation of antimicrobial solutions. Sterile distilled water was used for control solution and for preparing all antimicrobial solutions. The 150 ppm sodium hypochlorite solution (CHL) was prepared by adding 3.75 ml of bleach to 1,496.25 ml distilled water. The pH of CHL solution was measured and adjusted to 6.5 with citric acid as needed for ensuring maximum efficacy of the chlorine solution. The

level of free chlorine was tested using Hach Company (Ames, Iowa) chlorine test kit. The PRO-SAN solutions were prepared by adding 15 ml or 30 ml of PRO-SAN LC (liquid concentrate) to 1,485 ml of distilled water for obtaining 1.0% (v/v) PRO-SAN (PRO1) or 2.0% (v/v) PRO-SAN (PRO2), respectively. All antimicrobial treatment solutions were labeled with name, concentration, and date of preparation.

Immersion of the green onions and parsley in washing solutions. Sterile thongs were used for the immersion of each inoculated 25-g portion of green onions or parsley in distilled water (control) or antimicrobial solutions. The sample was fully covered by the solution and immersed for 2.0 minutes. Subsequently, each sample was rinsed for 3.0 seconds in sterile dilled water to remove residual antimicrobial solution. Individual sterile thongs were used for transferring each sample to a separate appropriately labeled stomacher bag containing 50 ml of sterile 0.1% (w/v) peptone.

Microbiological analysis. Each bagged sample was pummeled for 30 seconds in a laboratory Stomacher blender, at medium speed, to dislodge the pathogens from the surface of the fresh produce and suspend them in the peptone solution. From the sample homogenate appropriate 10-fold serial dilutions were prepared in 0.1% (w/v) peptone and aquilots of 0.1-ml were surface-plated on xylose lysine tergitol 4 agar with added nalidixic acid (50 µg/ml; XLT-NA) for counting viable *S. enterica* or sorbitol MacConkey agar with added nalidixic acid (50 µg/ml; SMA-NA) for counting viable *E. coli* O157:H7. All inoculated plates of selective agar were incubated at 35 °C for 48 h before counting the bacterial colonies.

3. RESULTS AND DISCUSSION

The initial contamination levels, via artificial inoculation, of parsley and green onions were 10^7 CFU/sample. After drying of the cell suspension on the leafy greens and before their immersion in water or sanitizers, a slight decrease in viable numbers of *Salmonella enterica* and *E. coli* O157:H7 has been noted. However, this is not uncommon since during the drying of the inocula it is expected that some cells will die or may be sub-lethally injured and they will fail to grow on the selective media which has been used for this study. Before immersion in water or chemical solutions, on parsley, the initial numbers of viable *E. coli* O157:H7 were 6.8 $\log_{10}$ CFU/sample and 6.72 $\log_{10}$ CFU/sample of viable *S. enterica*, respectively. Initial numbers of viable pathogen on green onions were 7.09 $\log_{10}$ CFU/sample for *E. coli* O157:H7 and 6.6 $\log_{10}$ CFU/sample for *S. enterica*.

The survivors of *E. coli* O157:H7 and *S. enterica* on parsley following the immersion in DW and in antimicrobial solutions are shown in Figures 1 and 2. Amongst all the washing solutions, the immersion of parsley in DW produced the most modest log reduction of pathogens, namely of l.3 log of the initial viable *E. coli* O157:H7 numbers and of 1.07 log of the initial viable counts of *S. enterica*. Similarly, the immersion of green onions in DW resulted in small reduction of viable *E. coli* O157:H7 (0 .43 log per sample) and 0.58 log per sample in the case of *S. enterica* (Figures 2 and 3).

The noted reduction in initial viable count of *E. coli* O157:H7 and *S. enterica* on the two types of fresh produce following the immersion in DW may the attributed solely to the physical removal of the cells which are loosely attached to the product. Water it is known to not present any antimicrobial action on bacterial cells. Use of potable water for washing leafy vegetables reduces the number of bacteria by 0.1–1 log units, i.e. by 90% at best (WHO&FAO, 1998). Some microorganisms may also adhere to cut surfaces or in stomata and are thus inaccessible to wash water (Seo and Frank, 1999).

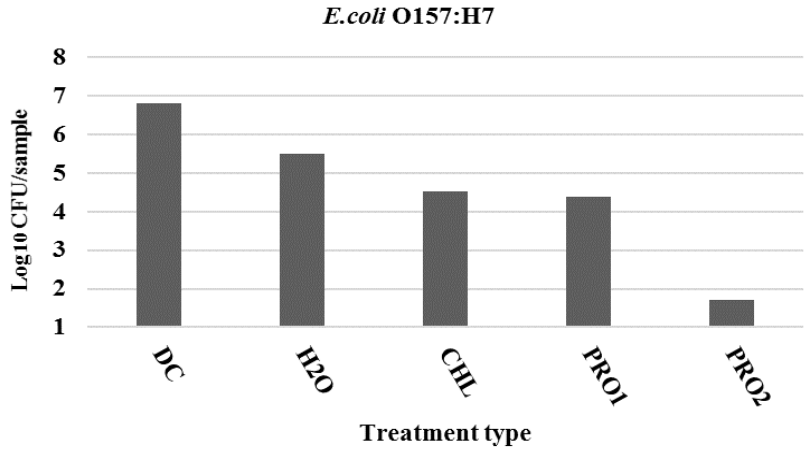

Fig. 1. E. coli O157:H7 survivors on 25g of parsley after immersion for 2.0 minutes in treatment solutions at 23 °C.

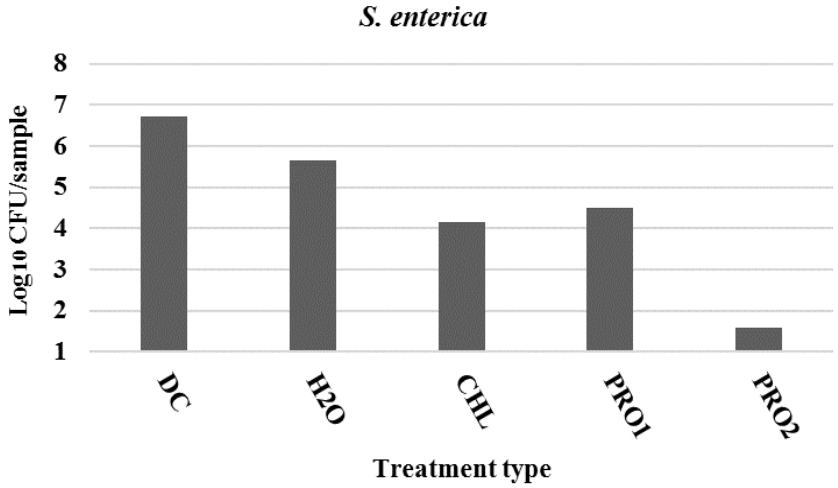

Fig. 2. S. enterica survivors on 25g of parsley after immersion for 2.0 minutes in treatment solutions at 23 °C

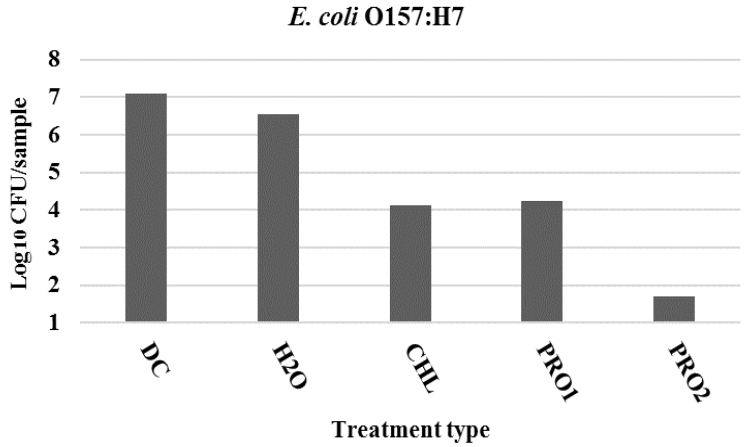

Fig. 3. E. coli O157:H7 survivors on 25g of green onion after immersion for 2.0 minutes in treatment solutions at 23 °C.

In contrast with DW solution, the CHL solution has been more efficient in pathogen destruction and reduced initial numbers of viable *E. coli* O157:H7 by 2.29 log and by 2.57 log in the initial viable count of S. *enterica* on parsley. On green onions, the CHL treatment resulted in a reduction of less than 3 log of viable cells for both pathogens. Viable *E. coli* O157:H7 were reduced by 2.97 log and viable *S. enterica* were reduced by 2.24 log. (Figures 3 and 4). Chlorine-based solutions are extensively used for surface sanitization in fresh produce industry because they are very inexpensive. According to federal regulations, two conditions must be met when sodium hypochlorite solutions are used for this purpose: i) the concentration of the sanitizer may not be more that 2,000 ppm (0.2%) hypochlorite, and ii) the fresh produce must undergo a rinse with potable water after the hypochlorite treatment (Code of Federal Regulations, Title 21, Part 173, 2017). Most processors will not use solutions with more than 200 ppm total chlorine to sanitize fresh produce unless the produce is very dirty. Washing of produce in sodium hypochlorite solutions for 1.0 minute or more is usually adequate for obtaining a good sanitizing effect depending on pH of the solutions. Hypochlorite solutions at pH 6.5 to 7.5 are considered safe, effective sanitizers because at pH less than 6.0 the solutions can be corrosive to equipment and at pH less than 5.0 they can give off harmful concentrations of chlorine gas. In the present study, the pH of the sodium hypochlorite solution was adjusted to 6.07 (using citric acid) to increase the formation of hypochlorous acid (HOCl) which is the active agent that kills microorganisms. Although chlorinated water (50 to 200 ppm chlorine) is routinely used as a surface sanitizer for fruits and vegetables, chlorine readily reacts with organic matter present on or released from fresh produce and causes a depletion of free chlorine. Consequently, the antimicrobial activity of CHL is reduced.

The efficacy of PRO-SAN on the numbers of viable pathogens depended on the concentration of the washing solutions. Immersion of parsley for 2.0 minutes in PRO1 and PRO2 reduced initial numbers of viable *E. coli* O157:H7 by 2.41 and 5.1 log, respectively and *S. enterica* has been reduced by 2.59 log (PRO1) and 5.02 log (PRO2) (Figures 1 and 2). Dipping of the green onions in PRO1 resulted in a reduction of *E. coli* O157:H7 by 2.85 log and of *S. enterica* by 1.91 log. Overall, dipping of parsley or green onions, for 2 minutes, in CHL or PRO1 resulted in similar reductions of both pathogens. Increasing the PRO-SAN solution concentration from 1% to 2% resulted in the killing of *E. coli* O157:H7 and *S. enterica* with approximately 5 log or more. Exposure of parsley to PRO2 for 2 minutes reduced the initial viable counts of *E. coli* O157:H7 by 5.39 log (Figure 3) and of *S. enterica* by 4.9 (Figure 4). Although both PRO-SAN solutions have a similar pH (Table 1), the noted difference between PRO1 and PRO2 efficacy is most probably due to the higher level of sodium lauryl sulfate (SLS) in the PRO2 solution. Sodium lauryl sulfate is a generally regarded as safe (GRAS) food additive when used between 10 to 5000 ppm. It is generally used as an emulsifier or a wetting agents in animal fats, vegetable oils, fruit juices and beverages, gelatin, marshmallows, egg whites (Code of Federal Regulations, Title 21, Part 172, 2017). Sodium lauryl sulfate is believed to cause membrane damage and protein denaturation in microorganisms when its activity is enhanced at pH below 4.0 (Dychdala, 1983) a fact which may explain the better killing of pathogens when a 2% PRO-SAN solution (PRO2) is used for washing the parsley and green onions.

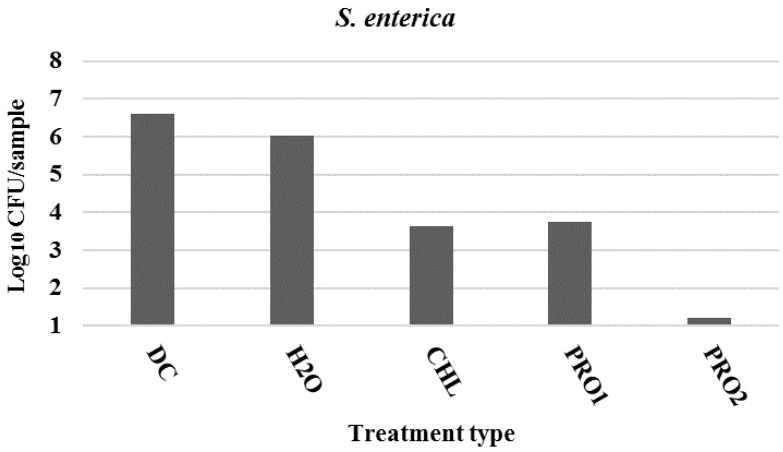

Fig. 4. S. enterica survivors on 25g of green onion after immersion for 2.0 minutes in treatment solutions at 23 °C.

Table 1. Description of codes, concentration and pH of solutions used for treating the surface of whole cucumbers

Treatment code	Description	Concentration	pH
DC	dry control	n/a	n/a
H2O	distilled water	n/a	6.52
CHL	sodium hypochlorite solution	150 ppm	6.07
PRO1	PRO-SAN	1.0% (v/v)	2.64
PRO2	PRO-SAN	2.0% (v/v)	2.38

3. CONCLUSIONS

The biodegradable wash PRO-SAN LC 2% (PRO2) have the best potential as a surface wash for parsley and green onions to destroy human enteric pathogens, such as *S. enterica* and *E. coli* O157:H7 and to enhance the microbial safety of these two popular ready-to-eat vegetable products. The use of PRO2 reduced the selected pathogens to a level which is comparable with essential oils such as carvacrol and cinamaldehide. From economic standpoint, and ease of formulation and application, PRO-SAN LC represents a better choice to be considered as a food safety intervention strategy. PRO-SAN 2% solutions offer the advantage of an effective, and safe rinse step to be used either in foodservice units, as an *in-house* intervention for controlling *S. enterica* and *E. coli* O157:H7, before the leafy greens are to be served to consumers, or in households, directly by the consumer.

Acknowledgments

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