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Research Note

Effect of Acidified Sodium Chlorite, Chlorine, and Acidic Electrolyzed Water on *Escherichia coli* O157:H7, *Salmonella,* and *Listeria monocytogenes* Inoculated onto Leafy Greens

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ABSTRACT

Recent foodborne outbreaks implicating spinach and lettuce have increased consumer concerns regarding the safety of fresh produce. While the most common commercial antimicrobial intervention for fresh produce is wash water containing 50 to 200 ppm chlorine, this study compares the effectiveness of acidified sodium chlorite, chlorine, and acidic electrolyzed water for inactivating *Escherichia coli* O157:H7, *Salmonella,* and *Listeria monocytogenes* inoculated onto leafy greens. Fresh mixed greens were left uninoculated or inoculated with approximately 6 log CFU/g of *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* and treated by immersion for 60 or 90 s in different wash solutions (1:150, wt/vol), including 50 ppm of chlorine solution acidified to pH 6.5, acidic electrolyzed water (pH 2.1 ± 0.2, oxygen reduction potential of 1,100 mV, 30 to 35 ppm of free chlorine), and acidified sodium chlorite (1,200 ppm, pH 2.5). Samples were neutralized and homogenized. Bacterial survival was determined by standard spread plating on selective media. Each test case (organism × treatment × time) was replicated twice with five samples per replicate. There was no difference ($P \geq 0.05$) in the time of immersion on the antimicrobial effectiveness of the treatments. Furthermore, there was no difference ($P \geq 0.05$) in survival of the three organisms regardless of treatment or time. Acidified sodium chlorite, resulted in reductions in populations of 3 to 3.8 log CFU/g and was more effective than chlorinated water (2.1 to 2.8 log CFU/g reduction). These results provide the produce industry with important information to assist in selection of effective antimicrobial strategies.

Several major outbreaks of foodborne illness linked to the consumption of fresh fruits and vegetables have increased their importance as a serious threat to public health. Most recently, outbreaks of *Escherichia coli* O157:H7 infection linked to the consumption of fresh spinach caused 204 illnesses and three deaths *(4).* The number and severity of foodborne illnesses associated with fresh produce has increased in recent times, and there appears to be a trend regarding the types of produce items implicated. Between 1998 and 2006, five commodity groups comprised 76% of produce-related outbreaks, namely (i) lettuce/leafy greens (30%), (ii) tomatoes (17%), (iii) cantaloupe (13%), (iv) herbs (basil, parsley, 11%), and (v) green onions (5%) *(2).* Pathogens of primary concern (up to 80% of produce-related outbreaks) appear to be *Salmonella, E. coli* O157:H7, *Listeria monocytogenes, Shigella,* and Norwalk-like viruses *(3).* Documentation of produce-related outbreaks due to *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* has prompted considerable research into interventions to reduce incidence of these pathogens on raw produce.

A wide range of sanitizers including: ozone, peroxyacetic acid, chlorine dioxide, chlorinated trisodium phosphate, oxidized water, and acidified sodium chlorite have been evaluated for their ability to reduce pathogenic bacteria on fresh produce. The most commonly used sanitizer used to treat fresh produce is water chlorinated at levels of 50 to 200 ppm; however, chlorine is rapidly inactivated by organic material and can even react with the organic material to form carcinogenic organochlorine compounds (i.e., chloramines, etc.) *(7).* Washing fresh produce with 50 to 200 ppm of chlorinated water generally results in approximately a 2-log reduction of microbial populations *(12)*; however, with the heightened concern regarding the public health affect of fresh produce, researchers have increased their efforts to evaluate alternative sanitizers for removing and inactivating pathogens on raw produce. Acidic electrolyzed water is generally recognized as safe that has been reported to be effective against pathogens on produce *(8, 13)* and is an economic alternative to chlorinated water. Acidified sodium chlorite has demonstrated efficacy against pathogens on fresh produce *(5, 6)* and even though applied at relatively high concentrations (1,200 ppm) and low pH (2.5 to 2.9) did not affect the appearance of cabbage leaves *(6).*

Chlorinated water may not provide the level of antimicrobial efficacy needed to increase the safety of leafy greens; thus, the effect of acidified sodium chlorite as an alternative to current interventions (primarily chlorinated water) used in the industry was evaluated for reducing pop-

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ulations of *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* inoculated onto leafy greens.

MATERIALS AND METHODS

Strains examined and preparation of inocula. Three strains of *Salmonella* (SM-1 [environmental isolate], ST-1 [isolated from a food-processing facility], and SA-1 [isolated from a food-processing facility]); three strains of *L. monocytogenes* (ATCC 19115 [human isolate], F2-145 [isolated from a food-processing facility], and F2-146 [isolated from a food-processing facility]); and two strains of *E. coli* O157:H7 (P-116 [isolated from a food-processing facility] and A00116855 [environmental isolate]) were used to create a cocktail inoculum of each target pathogen. Bacterial strains were available as frozen (−70°C) stock cultures in tryptic soy broth (Difco, Becton Dickinson, Sparks, Md.) with 25% glycerol and were activated by inoculating tryptic soy broth plus 0.6% yeast extract (Acumedia, Lansing, Mich.) and incubated at 35°C for 24 h. Cultures were then streaked on tryptic soy agar with 5% blood (Difco, Becton Dickinson) and incubated at 35°C for 24 h to screen cultures for purity. Colonies from overnight cultures of each strain were suspended in phosphate-buffered saline (pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl and 0.2 g of KCl in 1 liter of distilled water) to yield a suspension concentration of approximately 10^7 cells per ml. Equal volumes of each pathogen strain suspension were mixed to obtain pathogen cocktails with a concentration of approximately 10^7 cells per ml.

Preparation of leafy greens. Leafy greens were purchased from a local retail store and stored at 5°C for use within 72 h. Components of leafy greens included: organic baby lettuces (red and green romaine, red and green oak leaf, lollo rosa, tango), organic red and green chard, organic mizuna, organic arugula, organic frisée, and organic radicchio.

Inoculation of leafy greens. Leafy greens portions (10 g) were left uninoculated or individually spray inoculated (approximately 1 ml of a 10^7-CFU/ml suspension), with each target pathogen to obtain approximately 10^6 cells per g. Leafy green portions were spray inoculated in a biosafety hood (SterilGard Hood, The Baker Company, Inc., Sanford, Me.) and air dried for 2 h at 22°C in the same.

Treatment solutions. Leafy greens were treated with (i) sterilized tap water as a control (water); (ii) 50 ppm of chlorine solution (as NaOCl in sterilized tap water) acidified to pH 6.5 with citric acid; (iii) 50 ppm of chlorine solution (as NaOCl in sterilized tap water) acidified to pH 6.5 with sodium acid sulfate (pHase, Jones-Hamilton Co., Walbridge, Ohio); (iv) 1,200 ppm of acidified sodium chlorite (ASC; Sanova, Ecolab, St. Paul, Minn.) adjusted to pH 2.5; and (v) acidic electrolyzed water (AEW) at pH 2.1 ± 0.2, oxygen reduction potential of 1,100 mV, 30 to 35 ppm of free chlorine (Super Water Mini, Janix, Atsugi, Japan). Water was chlorinated using sodium hypochlorite (6.2%, NaOCl; Clorox, Clorox Co., Oakland, Calif.) mixed with sterilized tap water to achieve 50 ppm of free chlorine. The concentration of free chlorine was determined using a colorimeter (model DR890, Hach, Loveland, Colo.). The pH of solutions was measured using a digital pH meter (UltraBasic UB-10, Denver Instrument, Arvada, Colo.) with a glass pH electrode (Denver Instrument).

Treatment of leafy greens. Individual portions (10 g) of uninoculated and inoculated leafy greens were placed in stainless steel strainers. Strainers containing leafy greens were completely immersed and manually agitated in treatment solutions for 60 or 90 s to simulate product contact time in a two- and three-flume (immersion tank containing water for washing produce) treatment system, respectively. All treatment solutions were maintained at 22°C. The ratio of produce weight to treatment solution volume was 1:150. Uninoculated samples and samples inoculated with different pathogens were treated in separate containers to prevent cross-contamination. After submerging leafy greens in treatment solutions, they were removed and placed in Whirl-Pak bags (Nasco, Fort Atkinson, Wis.) containing 90 ml of Dey-Engley neutralizing broth (Acumedia) to neutralize the effect of residual sanitizer. The experiments were repeated twice with five samples in each experiment. Each treatment was therefore performed 10 times.

Microbiological analysis. Uninoculated leafy greens were analyzed for *E. coli* O157:H7, *Salmonella, L. monocytogenes,* and total bacterial populations. Uninoculated portions (10 g) of leafy greens ($n = 10$) in Whirl-Pak bags were enriched with 90 ml of prewarmed (42°C) tryptic soy broth and incubated at 35°C for 24 h for detection of *E. coli* O157:H7 and *Salmonella* or 90 ml of *Listeria* enrichment broth (Difco, Becton Dickinson) and incubated at 35°C for 48 h for detection of *L. monocytogenes.* After enrichment, samples were analyzed via PCR using buffer specific for *E. coli* O157:H7 and *Salmonella* (ES6 buffer, Molecular Epidemiology, Inc., Seattle, Wash.) or *L. monocytogenes* (L4 buffer, Molecular Epidemiology) according to methods described by Stopforth et al. *(14).* Separate portions (10 g) of uninoculated leafy greens ($n = 10$) were homogenized (Seward Stomacher Lab System, Norfolk, UK) for 60 s in 90 ml of sterile 0.1% buffered peptone water (Fisher Scientific, Houston, Tex.), serially diluted, and plated onto aerobic plate count Petrifilm (APC; 3M, St. Paul, Minn.) and incubated at 35°C for 24 h for enumeration of total bacterial populations.

Treated and untreated (control) samples were homogenized for 60 s. Original and serially diluted homogenates (100 μl) were surface plated onto sorbitol MacConkey agar (Difco, Becton Dickinson) with cefixime-tellurite stock solution (2.5 mg/liter; Dynal, Inc., Lake Success, N.Y.) for selective enumeration of *E. coli* O157: H7, onto XLT-4 agar (Hardy Diagnostics, Santa Maria, Calif.) for selective enumeration of *Salmonella* populations, and onto modified Oxford medium (Acumedia) for selective enumeration of *L. monocytogenes.* Plates were incubated at 35°C for 24 h for the selective enumeration of *E. coli* O157:H7 and *Salmonella* and for 48 h for *L. monocytogenes.* After incubation, colonies were enumerated and recorded.

Statistical analysis. Two replicate experiments were conducted with five samples per test case in each replicate. Test cases represented each pathogen subjected to each treatment solution for each treatment time ($n = 10$ for organism × treatment × time). Microbiological data were converted to log CFU per gram before being analyzed. Data from the two replicate experiments were tested using Levene's test for homogeneity of variances. The variances between the two replicate experiments did not differ ($P \geq 0.05$), and hence the data was pooled from the two replicate experiments to obtain a set of 10 observations for each test case. Values for the mean log and standard deviation of each set of bacterial counts were calculated on the assumption of a lognormal distribution of microorganisms. For each pathogen, the data (log CFU/g) were evaluated using a 5 × 2 (treatment × time) factorial design. Data were analyzed using ANOVA procedures with Duncan's multiple comparison tests of SAS (SAS Institute, Inc., Cary, N.C.) to determine significant differences. Differences were reported at a significance level of $\alpha = 0.05$.

TABLE 1. *Survival of* E. coli *O157:H7,* Salmonella, *and* L. monocytogenes *on leafy greens exposed to different sanitizers for 0, 60, and 90 s*

Inoculated pathogen	Length of treatment (s)	Survival, log CFU/g (SD), by sanitizer[a]				
		Water	CAW	SAW	ASC	AEW
E. coli O157:H7	0	6.3 AZ[b] (0.1)	6.3 AZ (0.1)	6.3 AZ (0.1)	6.3 AZ (0.1)	6.3 AZ (0.1)
	60	5.3 AY (0.2)	3.9 BY (0.3)	4.0 BY (0.2)	3.0 CY (0.8)	3.9 BY (0.3)
	90	5.3 AY (0.2)	4.0 BY (0.2)	3.8 BY (0.3)	2.7 CY (0.5)	3.8 BY (0.2)
Salmonella	0	5.9 AZ (0.2)	5.9 AZ (0.2)	5.9 AZ (0.2)	5.9 AZ (0.2)	5.9 AZ (0.2)
	60	5.3 AY (0.2)	3.7 BY (0.2)	3.7 BY (0.2)	2.3 CY (0.5)	3.6 BY (0.3)
	90	4.8 AX (0.2)	3.1 BX (0.4)	3.8 BY (0.2)	2.1 CY (0.3)	3.4 BY (0.3)
L. monocytogenes	0	5.7 AZ (0.3)	5.7 AZ (0.3)	5.7 AZ (0.3)	5.7 AZ (0.3)	5.7 AZ (0.3)
	60	5.1 AY (0.4)	3.5 BY (1.2)	3.7 BY (0.4)	2.7 CY (0.5)	3.7 BY (0.4)
	90	4.3 AY (0.3)	3.6 BY (0.8)	4.0 ABY (0.2)	2.7 BCY (0.6)	3.2 BY (0.8)

[a] Sanitizers tested included CAW, 50 ppm of chlorine solution acidified to pH 6.5 with citric acid; SAW, 50 ppm of chlorine solution acidified to pH 6.5 with sodium acid sulfate; ASC, acidified sodium chlorite at 1,200 ppm and pH 2.5; AEW, acidic electrolyzed water (pH 2.1 ± 0.2; oxygen reduction potential 1,100 mV).

[b] Means in the same row with different letters (A, B, and C) are different ($P < 0.05$). Means in the same column for each pathogen with different letters (Z, Y, and X) are different ($P < 0.05$).

RESULTS AND DISCUSSION

E. coli O157:H7, *Salmonella,* and *L. monocytogenes* were not recovered from any of the uninoculated raw leafy green samples. The mean total bacterial populations on uninoculated leafy green samples were 7.1 ± 0.3 log CFU/g (data not shown). The measured inoculum level for *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* cocktails were 7.5, 7.2, and 7.2 log CFU/ml, respectively (data not shown).

Water had the lowest decontaminating effect on leafy greens inoculated with pathogens, resulting in reductions of 0.6 to 1.4 log CFU/g (Table 1). This is consistent with previous research that found wash water resulted in an average of a 1-log reduction in pathogens on produce surfaces *(12).* Use of chlorine to decontaminate leafy greens resulted in pathogen reductions of 1.7 to 2.8 log CFU/g (Table 1) regardless of the type of acidulant used (citric acid or sodium acid sulfate). Chlorine used at concentrations less than 200 ppm has not been particularly effective at reducing pathogens on leafy green components such as lettuce *(9).* Furthermore, increased time of application with chlorine does not appear to increase the antimicrobial effect of the sanitizer *(1).* This was confirmed by the results of this study indicating that the immersion of leafy greens in chlorine, and for that fact any of the sanitizers tested, for longer periods (60 versus 90 s) did not increase the antimicrobial activity of the sanitizer (Table 1).

Chlorine is the most widely used sanitizer in the food industry; however, there is a need to find alternative sanitizers that are as or more effective in decontaminating raw produce. In this study, AEW was as effective ($P \geq 0.05$) as chlorine in decontaminating leafy greens, resulting in pathogen reductions of 2.0 to 2.5 log CFU/g (Table 1). Chlorine and AEW have similar effectiveness since the hypochlorous acid and associated free chlorine are the major contributors to both chlorine and AEW's sanitizing activity *(11).* The results of this study suggest that AEW can effectively be used as an alternative to chlorine with the following advantages: (i) it is effective against a broad spectrum of organisms, (ii) there is no need to use chemicals to generate sanitizer, (iii) compound reverts to normal water without releasing harmful gases such as chlorine, and (iv) following initial capital investment, operational costs of generating sanitizer is minimal (since only salt is needed to generate the sanitizer).

There is limited research *(5, 6, 11)* evaluating the effect of ASC on fresh produce and especially leafy greens. The results of this study indicate that ASC was the most effective sanitizer when compared with chlorine, tap water, and acidic electrolyzed water, resulting in pathogen reductions of 3.0 to 3.8 log CFU/g (Table 1). ASC is approved by the U.S. Food and Drug Administration *(15)* as a ''secondary direct food additive permitted in food for human consumption,'' specifically as an antimicrobial intervention for raw agricultural commodities. The advantages of using ASC over chlorine are that it is (i) more effective, (ii) not inactivated by organic material, and (iii) does not form chlorinated organic compounds. It should be noted that there is a concern with using highly acidic sanitizers in food-processing facilities since they are corrosive to equipment.

The effectiveness of sanitizers used in this study decreased in the following order: ASC > chlorine = AEW > water. Under the conditions of this study, AEW was as effective as chlorine in reducing pathogens on leafy greens, and it may be a more suitable alternative since it does not readily form chlorinated organic compounds, does not require chemicals to generate sanitizer, and in the long run would be more economical as a widely used (product and equipment) sanitizer in processing facilities. ASC was the most effective sanitizer in this study and may be the most suitable alternative for the fresh produce processing since it has higher efficacy than conventional chlorine has, is not inactivated by organic material (as is chlorine and acidic electrolyzed water), and does not form chlorinated organic compounds. Furthermore, use of ASC did not adversely affect physical appearance of the leafy green leaves. These

results provide the produce industry with data to make informed decisions regarding the selection of antimicrobial interventions for their processes and serves as literature to support selection of interventions for their hazard analysis critical control points plans.

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