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PEER-REVIEWED ARTICLE

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A Bisulfate of Soda and Peroxyacetic Acid Solution Reduces *Salmonella* on Fresh-Cut Spinach

ABSTRACT

Fresh produce is commonly implicated in foodborne illness outbreaks, including outbreaks of *Salmonella* infection. Chlorine is commonly added to produce wash water to control pathogen cross-contamination in water and is moderately efficacious because of limitations associated with organic matter and pH requirements. This study was conducted to evaluate a bisulfate of soda-peroxyacetic acid (SP) wash for fresh-cut spinach inoculated with *Salmonella* at > 6 log CFU/g. An unwashed control was compared with produce washed with gentle agitation in SP (80 ppm of peroxyacetic acid plus 0.5% [w/v] bisulfate of soda), chlorine (150 ppm, pH 7.0), or tap water. Spinach was stored in microperforated retail display bags at 7°C, and *Salmonella* levels were enumerated on days 0, 1, 3, 5, and 10 on xylose lysine tergitol-4 agar plus a tryptic soy agar overlay. SP was the most effective wash, reducing *Salmonella* by 1.8 log CFU/g ($P < 0.05$) in comparison with the control. Washing with SP significantly reduced *Salmonella* populations on fresh-cut spinach and may serve as an effective alternative to chlorine washes.

INTRODUCTION

The implication of contaminated produce in foodborne illness outbreaks has been a major shift in foodborne illness source attribution *(4)*. Foodborne illness caused by leafy greens is a major public health concern, with 16 outbreaks of *Salmonella enterica* subsp. *enterica* infection in the United States traced to salad or leafy greens between 2004 and 2012 *(5)*. In an epidemiological study conducted by the Centers for Disease Control and Protection, Herman et al. *(17)* reported that contaminated leafy greens were implicated in 606 foodborne illness outbreaks between 1973 and 2012.

The use of produce washes as an intervention to control microbial contamination on produce has been extensively

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reported *(1, 2, 10, 16, 27, 28)*. Antimicrobials serve a critical role in preventing cross-contamination of noncontaminated produce by contaminated produce in wash water systems *(13)*. Chlorine is commonly used as a sanitizer for produce wash water systems; however, the efficacy of chlorine for reducing pathogen populations on produce and wash water is affected by multiple water- and produce-related parameters *(28, 32)*. Organic matter can impact the use of chlorine by lowering the residual concentration, thus reducing the ability of the chlorine to eliminate indicator and pathogenic microorganisms *(1)*. Peroxyacetic acid (PAA) has been suggested as an alternative to chlorine-based sanitizers because it is less affected by organic matter *(1)*.

PAA is composed of acetic acid and hydrogen peroxide *(22, 44)*. The production of reactive oxygen species is responsible for the primary antimicrobial effect of PAA on bacterial cells *(39, 44)*. These reactive oxygen species damage bacterial lipids and DNA *(39, 44)*. PAA denatures enzymes and proteins and increases permeability of the cell wall *(18, 39, 44)* and disturbs cell membranes and blocks transport and enzymatic systems *(24, 44)*.

When added to water, bisulfate of soda (BS) dissociates into ions of sodium, hydrogen, and sulfate, lowering the pH and creating an osmotic effect *(23)* that stresses bacterial cells. Slight declines in pH require enteric microorganisms to expend energy to regulate cytoplasmic pH to maintain conditions that are near neutral; however, this process stresses bacterial cells and will often lead to cell death *(19, 25)*. In 1998, the U.S. Food and Drug Administration *(41, 42)* identified BS as generally recognized as safe (GRAS), and BS is used as a food additive for many applications, including the prevention of browning in fresh-cut produce *(41)*.

A wash water solution containing PAA and BS would expose microorganisms to multiple mechanisms of antimicrobial action, which could result in microbial population reductions on produce greater than those achieved by traditional chlorine postharvest washes. Kim et al. *(21)* combined PAA and BS as a synergistic hurdle intervention to reduce *Listeria innocua* on whole apples postharvest. The efficacy of PAA and BS blends was compared with that of a water control and 150 ppm of chlorine at pH 6.5. The treatment with 3% BS and 60 ppm of PAA resulting in a 5.56-log reduction in *L. innocua* after 14 days of storage *(21)*. The reductions achieved by the blend of PAA and BS against *L. innocua* on apples suggested that more research investigating the efficacy of this blend in other postharvest applications was warranted. Therefore, the present study was conducted to quantify reductions of *Salmonella* populations on fresh-cut spinach after use of a blend of PAA and BS as a postharvest wash solution.

MATERIALS AND METHODS

Culture preparation

The inoculation cocktail was prepared from frozen stock cultures of *S. enterica* subsp. *enterica* serovars Anatum (Kansas State University TX 2006 C20), Montevideo (TX 2006 C7), Newport (TX 2006 F10), and Typhimurium (ATCC 14028) that had been stored in tryptic soy broth (TSB; Difco, BD, Franklin Lakes, NJ) with 15% glycerol at −80°C. The isolates of *Salmonella* serovars Anatum, Montevideo, and Newport were originally recovered from cattle at Kansas State University. All *Salmonella* isolates were revived from frozen by streaking onto tryptic soy agar (TSA; Difco, BD) at 37°C for 24 h and selecting a colony to transfer in 9 mL of TSB at 37°C for 24 h. Two tubes were prepared for each serotype (18 mL total). Following 24 h of incubation, 18 mL of each serotype was centrifuged at 5,200 × g for 15 min at 4°C (Allegra X-30R, Beckman Coulter, Brea, CA). The supernatant was discarded, and pellets were resuspended in 18 mL of buffered peptone water (BPW; Difco, BD). Resuspended cultures were combined in equal proportions to prepare an inoculum cocktail (75.33 mL), which was diluted in 8.7 L of BPW to achieve a starting titer of 7.0 log CFU/mL.

Spinach preparation and inoculation

Fresh, unwashed mature spinach *(Spinacia oleracea)* was purchased from a local wholesale produce supplier. Stems remaining from harvest were trimmed with sterile tools to represent fresh-cut spinach, and the product was held at 4°C until inoculation. Spinach was inoculated via complete product submersion as previously described *(14, 15)* to simulate a worst case contamination scenario that occurs from exposure to contaminated wash water. Postharvest washing may lead to pathogen internalization *(8)*, and this inoculation approach simulates such a contamination event, which allows for treatment efficacy to be tested under extreme conditions. The spinach (1,000 g) was completely submerged in 8.7 L of the *Salmonella* inoculum (ca. 7.0 log CFU/mL) inside a biosafety cabinet. Spinach leaves were stirred into the inoculum with a spatula, soaked for 30 min, then placed on four stainless steel trays with grate overlays in the biosafety cabinet for 30 min to facilitate drying. Spinach leaves were then turned over and left to dry for an additional 30 min, for a total 1 h of drying time. Although quality was not specifically assessed in this study, wilting and other product quality defects were not observed during this process.

Preparation of wash treatments

Three gallons (11.4 L) of each water washing treatment was prepared: a tap water wash containing BS and PAA (SP), a sodium hypochlorite (chlorine) wash, and tap water. SP was prepared by adding 4.6 mL of a commercial produce wash (Tsunami 100, Ecolab, St. Paul, MN) containing 15.2% PAA and 56.78 g of BS (0.50% [w/v] target concentration of BS; pHase, Jones-Hamilton Co., Walbridge, OH). A target concentration of 80 ppm of PAA was determined with the manufacturer's test kit (Ecolab). Kim et al. *(21)* evaluated 1 and 3% concentrations of BS with 60 ppm of PAA on

apples; however, the SP blend used in the present study was reduced to 0.50% (w/v) BS because of the more sensitive nature of fresh-cut spinach. Because the regulatory limit of 80 ppm of PAA *(43)* has been used as a postharvest spinach intervention *(32)*, this concentration was chosen for the SP blend in the present study. The chlorine wash was prepared by adding 19.4 mL of germicidal bleach (Clorox Professional Products Company, Oakland, CA). The pH was adjusted by adding 1 N HCl to achieve pH 6.5 to 7.0, which has been targeted by previous research *(32, 36)* and identified as an ideal range for maximizing concentration of the more bactericidal hypochlorous acid and minimizing production of the toxic chlorine gas *(11)*. Total chlorine was measured with an ultra-high-range chlorine meter (Hanna Instruments, Woonsocket, RI) with a target total chlorine concentration of 150 ± 10 ppm.

Application of wash treatments

A 250-g portion of inoculated, unwashed spinach was set aside as a control. For each wash treatment, 250 g of inoculated spinach was placed in slotted containers (8.6 by 24.1 by 19 cm; InterDesign, Solon, OH) and covered with foil to prevent escape of the product and aerosol production during washing. The covered containers were then submerged in the wash treatment and exposed to gentle manual agitation for 2 min to simulate the typical time that large-scale commercial washing systems wash spinach before packing. Samples subjected to the chlorine treatment were then briefly submerged in a subsequent tap water rinse with gentle agitation for 10 s to remove residual chlorine from the product. This action simulated a final wash that some processors use to remove residual chlorine. Following the wash treatment, the spinach was spun dry (centrifuged) in a salad spinner (26 cm diameter; Prepworks, Kent, WA) to remove excess liquid. The salad spinner was placed inside a biohazard bag and operated under the biosafety cabinet, with the opening of the biohazard bag directed to the inside of the biosafety cabinet to control aerosol release. The salad spinner cord was then pulled 10 times to standardize the centrifugation process. Following centrifugation, samples (including the control) were immediately removed for pathogen enumeration and then packaged in retail display packages (26.4 by 16.6 cm; 50 g of spinach) specifically designed for fresh-cut spinach: 100-ga oriented polypropylene/70-ga oriented polypropylene, 18.875 in. [48 cm] roll width, four lanes of continuous perforations spaced 0.30 in. [0.12 cm] apart, and perforation flow rate of 50 + 10 standard cm^3/s (American Packaging Corp., Columbus, WI). Packages were stored at 7°C to mimic retail storage conditions *(33)*. The ratio of spinach weight to packaging size was chosen to mimic commercial packaging (~1 g spinach per 8.7 cm^2 of package). Air within the package was not released prior to sealing, and the atmosphere within the package was not modified.

***Salmonella* enumeration**

Salmonella populations were enumerated on day 0 and throughout the shelf life (days 1, 3, 5, and 10) by homogenizing 25 g of spinach (Stomacher 400 Circulator, Seward, Bohemia, NY) with 225 mL of Dey Engley neutralizing broth (BBL, BD) for 60 s at 230 rpm. Samples were diluted in 0.1% peptone water (Difco, BD), spread plated onto xylose lysine tergitol-4 agar (XLT-4; Remel, Lenexa, KS) with a TSA overlay (XLT4 + TSA), and incubated at 37°C for 24 h. TSA is a nonselective medium that provides a favorable environment for the growth of injured cells *(47)*. The thin agar layer method *(47)* was used to enumerate injured cells by spread plating on XLT-4 + TSA.

Statistical analysis

Three replications were completed, and data were analyzed using the MIXED model procedure of Statistical Analysis Software (v. 9.4, SAS Institute, Cary, NC). Data for each media were analyzed separately. Significance was determined at $P \leq 0.05$ for the main effects (treatment and day), the treatment × day interaction, and all comparisons between treatments.

RESULTS

The main effects of treatment ($P < 0.0001$) *(Fig. 1)* and sampling day ($P = 0.0008$) *(Fig. 2)* were significant. The treatment × sampling day interaction was not significant ($P > 0.05$); therefore, these data are shown in *Figure 3* for informational purposes only, are not considered statistically relevant, and are not discussed in detail. Data are presented and discussed for the significant main effects of treatment and sampling day only.

All treatments were significantly different from one another *(Fig. 1)*, with the largest population of *Salmonella* (6.7 ± 0.14 log CFU/g) recovered from control (inoculated, unwashed) spinach. SP treatment resulted in the largest (1.8 log CFU/g) and most significant $P < 0.0001$ reduction compared with control. SP was significantly more effective for reducing *Salmonella* populations than were water $P < 0.0001$ and chlorine $P = 0.0270$.

Salmonella population variability was observed throughout the shelf life, with the largest population recovered on day 3 (6.2 ± 0.16 log CFU/g) and a significant increase ($P = 0.0003$) of 0.9 log CFU/g recorded between days 0 and 3 *(Fig. 2)*. In comparison to day 3, a significant decline in *Salmonella* populations was observed on days 5 (0.8 log CFU/g; $P = 0.0005$) and 10 (0.5 log CFU/g; $P = 0.0219$). *Salmonella* populations at the end of 10 days were not significantly different from those on day 0 ($P = 0.1256$).

Although the treatment × sampling day interaction was not significant ($P > 0.05$) *(Fig. 3)*, these data generally indicate that *Salmonella* populations increased by nearly 1 log CFU/g between days 1 and 3. In general, a decrease in *Salmonella* populations was observed on days 5 and 10, with *Salmonella*

FIGURE 1.

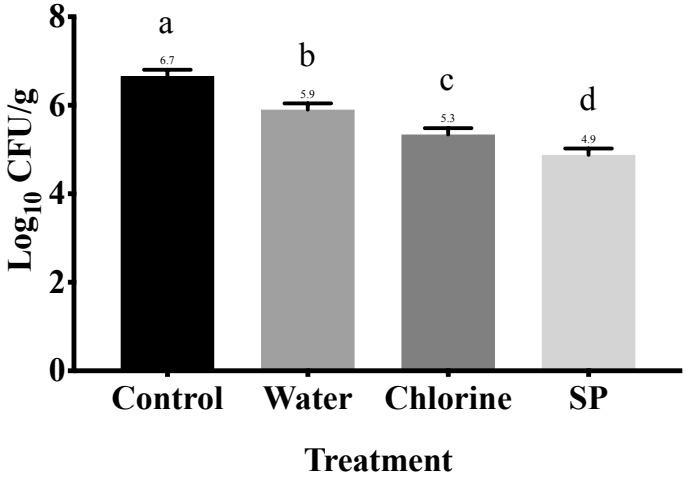

FIGURE 1. ***Salmonella*** **populations on spinach subjected to postharvest washing treatments and sampled throughout a 10-day storage period (7°C). Populations were determined by enumerating on xylose lysine tergitol-4 agar overlaid with tryptic soy agar. Treatment was a significant variable ($P < 0.0001$), and the treatment × day interaction was not significant ($P > 0.05$). Therefore, data for each treatment are not shown according to sampling day.**

a,b,c,d Denotes treatments that differ significantly ($P \leq 0.05$).
Error bars indicate standard error of the mean.

FIGURE 2.

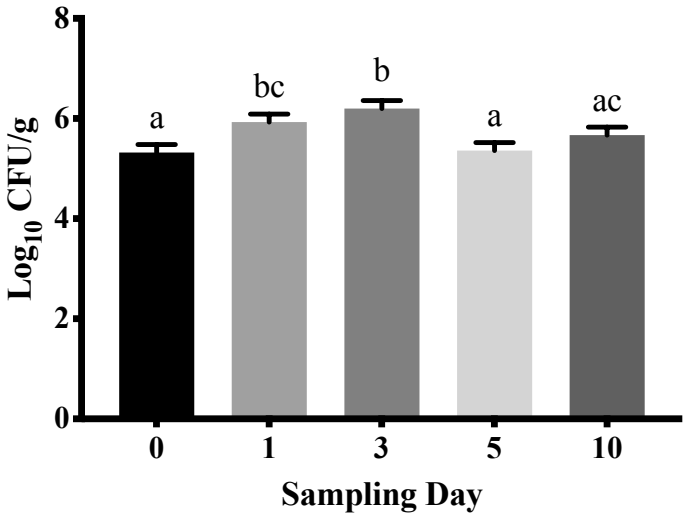

FIGURE 2. ***Salmonella*** **populations by sampling day on spinach subjected to postharvest washing and sampled throughout a 10-day storage period (7°C) by plating on xylose lysine tergitol-4 agar overlaid with tryptic soy agar. Day was a significant variable ($P = 0.0008$), and the treatment × day interaction was not significant ($P > 0.05$). Therefore, data are shown only by day and not according to treatment.**

a,b,c Denotes treatments that differ significantly ($P \leq 0.05$).
Error bars indicate standard error of the mean.

FIGURE 3.

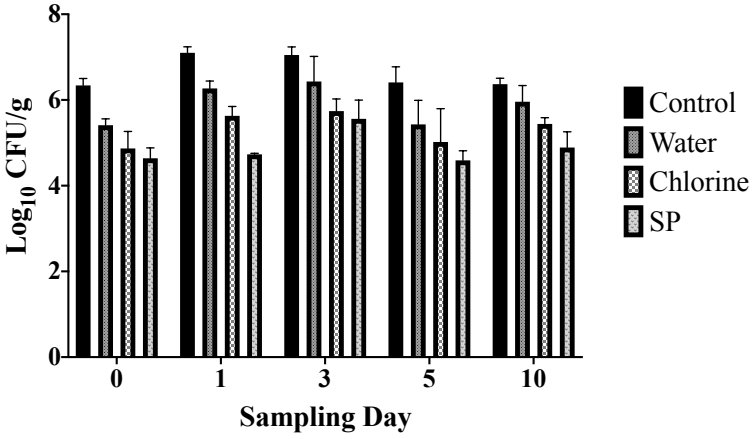

FIGURE 3. ***Salmonella*** **populations on spinach subjected to postharvest washing treatments during each sampling day of a 10-day storage period (7°C). Populations were determined by enumerating on xylose lysine tergitol-4 agar overlaid with tryptic soy agar. The treatment × day interaction was not significant ($P > 0.05$); therefore, these data are shown for illustrative and informational purposes only.**

Error bars indicate standard error of the mean.

populations on day 10 the same as those on day 0. These trends are similar to findings for the main effect of day *(Fig. 2)*.

DISCUSSION

The SP blend was evaluated in comparison to water, chlorine, and an inoculated control to determine efficacy as a novel postharvest wash intervention to reduce *Salmonella* on fresh-cut spinach. SP was significantly the most effective treatment, reducing *Salmonella* populations by 1.8 log CFU/g compared with the control. *Salmonella* populations varied within ±1 log CFU/g of day 0 populations throughout the shelf life.

The interventions used in this study could have resulted in cell injury rather than cell death for a portion of the *Salmonella* population, which can lead to challenges when enumerating populations on selective media that contain chemicals, inhibitory ingredients, and other substances that may be too harsh for injured cells to grow *(47)*. The resultant enumeration may not be an accurate representation of the surviving *Salmonella* population. XLT-4 + TSA was used for enumeration in this study to account for injured *Salmonella* populations.

Neal et al. *(32)* subjected *Salmonella*-inoculated spinach to 2% L-lactic acid at 55°C and achieved a 2.7-log reduction. However, application of calcium hypochlorite (200 mg/L), PAA (80 mg/L), and chlorine dioxide gas resulted in reductions of <1.0 log CFU/g, and a water wash achieved a 0.7-log reduction. In the present study, the water wash achieved a similar 0.8-log reduction, and SP surpassed the efficacy of 80 mg/L PAA, significantly reducing *Salmonella* populations by 1.8 CFU/g. Puerta-Gomez et al. *(36)* inoculated spinach with *Salmonella* at 10^5 CFU/g then washed it in water or 200 ppm of chlorine for 5 min, achieving 0.54- and 1.17-log reductions, respectively. In the present study, larger reductions were observed with a shorter contact time (2 min); water reducing *Salmonella* by 0.8 log CFU/g, and only 150 ppm of chlorine achieving a 1.4-log reduction. By washing with water and chlorine, Puerta-Gomez et al. achieved a 1.71-log reduction. Unlike that double washing technique, the SP wash was applied as a single washing step that produced similar results, reducing *Salmonella* by 1.8 log CFU/g on XLT-4 + TSA. From a feasibility perspective, it may be more advantageous to use a single SP wash that achieves similar reductions rather than multiple washes used in sequence.

Although internalization was not a specific focus of this study, the submersion inoculation approach may have facilitated *Salmonella* internalization or adherence to protective sites within the leaves that prevent access to sanitizers. *Salmonella* is capable of growing to moderately high populations on or within plants *(7–9, 20, 38, 46)* probably due to utilization of plant nutrients *(8, 48)*. Sampling day was a significant main effect, and *Salmonella* populations varied during the shelf life *(Fig. 2)*. *Salmonella* remained viable and varied within ±1 log CFU/g of day 0 populations during storage *(Figs. 2 and 3)*. Observed growth may have been due to access to plant nutrients and storage at 7°C. These data indicate that internal and external pathogen contamination remains on fresh-cut spinach after washing, which emphasizes the importance of pathogen control in the preharvest setting and effective postharvest interventions to prevent cross-contamination and internalization in fresh-cut spinach wash water.

The use of natural extracts to eliminate *Salmonella* on spinach has also been investigated. A Sporan and acetic acid combination and a cinnamaldehyde solution each achieved larger reductions (1.29 and 1.06 log CFU/g, respectively) of *Salmonella* on spinach than did water and chlorine treatments *(49)*. In comparison, the novel SP wash described in the present study reduced *Salmonella* on spinach by up to 1.8 log CFU/g. Orue et al. *(35)* found that *Salmonella* populations on spinach were reduced by 1.3 log CFU/g with chlorine, 1.7 log CFU/g with Citrol (a disinfectant made from grapefruit), 2.0 log CFU/g with lime extract, and 1.2 log CFU/g with oregano extract. These reductions are similar to those obtained in the present study. Olive extract, hibiscus concentrate, and apple extract have also been used to eliminate *Salmonella* on spinach, often with efficacy increasing during storage time (up to 3 days) *(30)*. Rada et al. *(37)* reported *Salmonella* reductions of 2.91 and 2.21 log CFU/g when mature spinach was treated with combinations of 0.1% cinnamon leaf oil with 3.0% olive extract and 0.1% oregano with 3.0% olive extract, respectively. Although some natural extracts have efficacy that is similar or superior to that of SP, organoleptic properties may be altered by the use of natural extracts, and these alterations should be evaluated in future comparison studies with SP. Although the impact of SP on organoleptic properties was not the focus of the present study, off-odors were not detected from SP spinach throughout the shelf life.

Consumer studies have shown that purchasers of fresh produce are willing to pay a premium when the likelihood of foodborne illness is reduced by 50% *(50)*. Thus, research into effective chemical washes such as the SP blend evaluated here is warranted. Chlorine, a chemical effective for reducing microbial populations in plant systems *(2)*, is frequently used in the produce industry for water washes; however, increasing numbers of foodborne illness outbreaks have challenged assumptions about the efficacy of these chlorine washes *(34)*. Chlorine interacts with organic material, which reduces the antimicrobial activity *(1, 3, 6, 14, 31)* and may lead to cross-contamination between batches of produce in wash tanks *(29, 31, 40)* and subsequent outbreaks of foodborne illness *(31, 45)*. The questionable efficacy and environmental and health risks of chlorine have prompted producers to look elsewhere for antimicrobial interventions *(12, 34)*, especially when considering the generation of disinfection by-products, which can cause health problems *(26)*. The SP wash of GRAS

BS *(42)* and the regulatory limit of 80 ppm of PAA *(43)* was significantly more effective than chlorine for reducing the microbial load of the enteric pathogens *Salmonella* on spinach. Therefore, washing spinach in SP may alleviate concerns about chlorine while providing an effective way to reduce the microbial load of spinach at levels similar to or better than achieved with chlorine.

CONCLUSIONS

These data provide initial insight into the efficacy of SP as a postharvest wash to reduce *Salmonella* on fresh-cut spinach. SP was very effective in this application, suggesting that SP warrants further investigation to address the limitations not addressed in the present study. Future research should focus on the impact of SP on product quality and sensory attributes. In the present study, the worst case scenario inoculation method used may have led to *Salmonella* internalization and access to internal plant nutrients; however, the data suggest that *Salmonella* remained viable and only varied ±1 log CFU/g during storage at 7°C. *Salmonella* viability during storage may differ based on inoculation method, and other methods such as spray or spot inoculation require further investigation. Future research efforts could also focus on optimization of SP efficacy by evaluating multiple concentrations of SP and multiple washing steps. SP efficacy might be enhanced in combination with other antimicrobial washing techniques. The antimicrobial efficacy achieved in the present study suggests that SP may be a promising novel postharvest intervention.

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