Antimicrobial Activity of Apple, Hibiscus, Olive, and Hydrogen Peroxide Formulations against *Salmonella enterica* on Organic Leafy Greens

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ABSTRACT

*Salmonella enterica* is one of the most common bacterial pathogens implicated in foodborne outbreaks involving fresh produce in the last decade. In an effort to discover natural antimicrobials for use on fresh produce, the objective of the present study was to evaluate the effectiveness of different antimicrobial plant extract–concentrate formulations on four types of organic leafy greens inoculated with *S. enterica* serovar Newport. The leafy greens tested included organic romaine and iceberg lettuce, and organic adult and baby spinach. Each leaf sample was washed, dip inoculated with *Salmonella* Newport (10CFU/ml), and dried. Apple and olive extract formulations were prepared at 1, 3, and 5% concentrations, and hibiscus concentrates were prepared at 10, 20, and 30%. Inoculated leaves were immersed in the treatment solution for 2 min and individually incubated at 4°C. After incubation, samples were taken on days 0, 1, and 3 for enumeration of survivors. Our results showed that the antimicrobial activity was both concentration and time dependent. Olive extract exhibited the greatest antimicrobial activity, resulting in 2- to 3-log CFU/g reductions for each concentration and type of leafy green by day 3. Apple extract showed 1- to 2-log CFU/g reductions by day 3 on various leafy greens. Hibiscus concentrate showed an overall reduction of 1 log CFU/g for all leafy greens. The maximum reduction by hydrogen peroxide (3%) was about 1 log CFU/g. The antimicrobial activity was also tested on the background microflora of organic leafy greens, and reductions ranged from 0 to 2.8 log. This study demonstrates the potential of natural plant extract formulations to inactivate *Salmonella* Newport on organic leafy greens.

*Salmonella* is one of the leading pathogens causing foodborne illness in the United States. Fresh produce outbreaks due to this pathogen are becoming more prevalent (12). Between 1996 and 2008, 82 produce-related foodborne illness outbreaks occurred in the United States, 28 of which were linked to leafy greens, and included 949 illnesses and 5 deaths (27). These outbreaks included organic leafy greens such as spinach. According to the Centers for Disease Control and Prevention (3), of the estimated 3.6 million cases of foodborne illnesses caused by bacteria, nontyphoidal *Salmonella* was responsible for 1 million (11%) cases (25). In the United States, 228,744 hospitalizations were associated with foodborne illnesses; 64% of those were bacterial, with 35% of these caused by nontyphoidal *Salmonella* (25). Of the 2,612 deaths, 64% were caused by bacteria, and of these, 28% were associated with nontyphoidal *Salmonella* (25).

In 2000, more organic food than conventionally derived food was purchased in markets, with fresh produce being the top-selling item (6). Organic foods are cultivated naturally, following national standards overseen by the U.S. Department of Agriculture’s National Organic Program (26). Organic production is ecologically based, using specific pest management and crop rotation practices, and composting through use of manure (6). Organic foods are produced without the use of pesticides, antibiotics, and hormones (6). With limited treatment options for organic produce, there is a need for alternative processing methods such as washing and sanitizing to reduce surface microbial contamination.

Natural plant-derived compounds such as extracts, spices, and essential oils have been shown to have antimicrobial activity against foodborne pathogens. For example, plant extracts such as cinnamaldehyde and carvacrol showed antimicrobial activity against antibiotic-resistant and nonresistant *Campylobacter jejuni* in laboratory media (22). Citron essential oil was effective against *Salmonella Enteritidis, Escherichia coli*, and *Listeria monocytogenes* (1). Essential oil from *Satureja thymbra* (winter savory) also showed antimicrobial activity against biofilms containing *Staphylococcus simulans, Lactobacillus*...
fermentum, Pseudomonas putida, S. enterica, and L. monocytogenes (5). Studies have shown that many essential oils and their components such as carvacrol, cinnamaldehyde, those from garlic, and others have antimicrobial activity against S. enterica in microassay, edible films, apple juice, wine marinades, and on celery and oysters (7–10, 23, 24).

Antimicrobial activity of plant-derived compounds has also been studied in fresh produce. Clove extract has been shown to have antimicrobial activity against Salmonella Typhimurium and E. coli O157:H7 on fresh lettuce (15). The maximum reductions were 4 and 3.7 log on exposure to clove extract for 10 min for Salmonella Typhimurium and E. coli O157:H7, respectively (15). Tea tree and clove essential oils have exhibited antimicrobial effects against bacteria, yeasts, and molds on romaine lettuce leaves (20).

Studies have also indicated that organic produce might have a higher microbial load than conventionally grown produce (19, 28). E. coli was present in 22.2% of organic lettuce samples, as opposed to 12.5% of conventionally grown samples (19). Organically grown edible flowers (Viola tricolor) and sweet basil had higher levels of microbial contamination than conventionally grown sweet basil (28).

As part of an effort to discover new, natural antimicrobial treatments against foodborne pathogens on organic food, the objective of this study was to evaluate the effectiveness of different antimicrobial plant extract–concentrate formulations on four different types of organic leafy greens inoculated with Salmonella Newport.

MATERIALS AND METHODS

Bacterial culture preparation and media. S. enterica serovar Newport was used for this study. The culture was prepared by inoculating cryopreserved cells in tryptic soy broth (Difco, BD, Sparks, MD) and incubating overnight (18 to 22 h) at 37°C. For each experiment, fresh overnight culture was used. For inoculation, 10^6 CFU/ml cells were used, and these were prepared by diluting the overnight culture. Samples were stomached in buffered peptone water (Difco, BD). Survivors after each treatment were enumerated by serial dilution in 0.1% peptone water (Difco, BD) and plated on xylose lysine desoxycholate agar for Salmonella (XLD; Difco, BD), tryptic soy agar for total aerobic count (TSA; Difco, BD), and Pseudomonas isolation agar (Difco, BD). Plates were incubated at 37°C for 24 to 48 h and counted.

Food products. Four types of organic leafy greens were obtained from local grocery stores (Tucson, AZ): romaine lettuce, iceberg lettuce, adult bunched spinach, and ready-to-eat baby spinach.

Preparation of antimicrobial treatment solutions. Antimicrobial extracts of olive (CreAgri, Inc., Hayward, CA) and apple (Apple Poly, L.L.C., Morrill, NE) were evaluated for their effectiveness against Salmonella Newport. Concentrations of 1, 3, and 5% were prepared by mixing the powdered extract in sterile phosphate-buffered saline (PBS) until dissolved. Freshhibiscus calyces (roselle) that were fully mature and ready for harvest were obtained from the Southern University Agricultural Research and Extension Center (Baton Rouge, LA). Hibiscus concentrate was made as described previously (17), with some modifications (13), by blending the thoroughly washed, edible, mature calyces in sterile water (1:2 [wt/vol]). The resulting mixture was filtered through four layers of sterile cheesecloth, and the filtrate was boiled for 5 min. Aliquots of the concentrate were stored at −20°C until needed. Hibiscus antimicrobial treatment was prepared at 10, 20, and 30% concentrations in sterile PBS. Hydrogen peroxide (3%) was purchased from a local grocery–drug store.

Antimicrobial activities of olive extract, apple extract, hibiscus concentrate, and hydrogen peroxide against Salmonella Newport on organic leafy greens. The leafy greens were each washed thoroughly with deionized water and weighed into 10-g samples. The leaf green samples were then exposed to UV (254 nm) radiation under a biohood for 30 min (15 min) on each side of the leaf to reduce normal microflora. The samples were each dip inoculated with Salmonella Newport (10^6 CFU/ml) for 2 min and then dried for 30 min under a biohood. Control samples were taken before and after drying to check for bacterial levels. The inoculated leaf samples were then immersed in the antimicrobial treatment solutions for 2 min, with gentle agitation. An experiment using a PBS control sample was conducted alongside treatment samples. Treated leaves were immediately placed into individual stomacher bags and incubated at 4°C. After incubation, samples were taken on days 0, 1, and 3 for enumeration of surviving bacteria. Leaf samples were stomached in 90 ml of buffered peptone water with a stomacher (Seward, Ltd., London, UK) at normal speed for 1 min and then serially diluted in 0.1% peptone water (Difco, BD) and plated on XLD. Colonies were counted after 24 h of incubation at 37°C. Experiments were repeated three times.

Antimicrobial activities of olive extract, apple extract, hibiscus concentrate, and hydrogen peroxide against background microflora on organic leafy greens. In order to assess the population of background microflora on the leafy greens, 10-g leaf samples were each taken before and after washing thoroughly in deionized water. Leaf samples were stomached in 90 ml of buffered peptone water at normal speed for 1 min and then serially diluted in 0.1% peptone water and plated on XLD, TSA, and Pseudomonas isolation agar. Colonies were counted after 24 to 48 h of incubation at 37°C.

Each antimicrobial rinse was also used to treat background microflora. Unwashed leaf samples were treated with the same concentrations of antimicrobials listed above, under the same processing conditions as described for Salmonella Newport. Samples were plated on TSA to evaluate overall effectiveness of the antimicrobial agent on any type of background microflora. Experiments were done in duplicates.

Statistical analysis. A split-plot design was used, with three replicates per treatment. The Salmonella Newport populations recovered after different antimicrobial treatments at each sampling period were converted to log CFU per gram, and analyzed by a two-way analysis of variance with PROC MIXED of SAS 8.2 software (SAS Institute Inc., Cary, NC) for interaction effects of the antimicrobial agent, concentration of the antimicrobial agent, and sampling period. In all cases, the level of statistical significance was P < 0.05.

RESULTS AND DISCUSSION

The results from this study show that the antimicrobial activity of the tested natural plant extract treatments against Salmonella Newport was both concentration and time
dependent. A dip treatment was chosen for this experiment because in earlier investigations, dip treatment has shown better effectiveness than spray treatment. In addition, dip treatment resembles conventional fresh produce wash operations, in which the produce is submerged in chlorinated water. Dip treatment has also shown the greatest reduction in bacteria in other studies (11, 20).

**Background microflora.** In order to quantitate the amount of background microflora and the effects of water wash, controls were taken on each type of leafy green, before and after washing in deionized water. There were varying amounts of background microflora (Table 1) for each batch of leafy greens tested, but total populations ranged from 0 to 8.0 log, depending on type of leafy green and media. After washing the samples, the levels dropped depending on the produce type, ranging from 0 to 7.0 log. In general, washing the leaves with deionized water reduced the background microbial load of the produce samples by at least 1 log or more on the various media tested (Table 1). It is important to note that colonies seen on XLD agar were not *Salmonella*, but likely coliforms (as identified by colony color).

During this study, an attempt to keep the produce brands consistent throughout the study was difficult because of the availability of specific brands of leafy greens. This could be a possible reason for the wide variations in the background microflora populations. The amount of background microflora is not surprising, because all of the leafy greens were produced organically. Wetzel et al. (28) recognized that organically grown sweet basil had higher levels of microbial contamination as well as varying types of microorganisms compared with conventionally grown basil. This suggests that the treatment restrictions that organic growers have to follow might contribute to the overall microbial content of the organic produce.

**Antimicrobial activities of olive extract, apple extract, hibiscus concentrate, and hydrogen peroxide against *Salmonella* Newport on organic leafy greens.**

Control samples taken after inoculation, and before and after drying showed inoculation levels on the leafy greens around $10^3$ CFU/g, with no loss of organisms after the drying process.

Leaves treated with olive extract showed the greatest reduction by day 3 (Fig. 1). On day 0, there was an immediate, statistically significant reduction in *Salmonella* population for all three concentrations in all produces, as compared with the PBS control ($P < 0.05$). On day 0, there was a 1.43-log reduction for 1% olive extract treatment, and 2.3- and 1.7-log reductions in the 3 and 5% treatments, respectively. The *Salmonella* Newport populations were further reduced by day 1, with 1.9-, 1.88-, and 2.9-log reductions for 1, 3, and 5% olive treatments, respectively. Compared with the PBS control, 5% olive treatment showed a 2.7-log reduction in *Salmonella* Newport population by day 3 on romaine lettuce. Olive rinses of lower concentrations, 1 and 3%, showed around 2.5- and 2.7-log reductions, respectively, by day 3. Treatments were statistically significant from the PBS control for all days ($P < 0.05$). The 5% treatment was statistically significant from all other treatments ($P < 0.05$).

Iceberg lettuce showed a similar trend for day 0 and day 1. Reductions were 1.5, 2.8, and 2.5 log for 1, 3, and 5% olive treatments for day 0, respectively, and 1.9, 2.7, and 3.0 log for 1, 3, and 5% olive treatments by day 1, respectively. By day 3, iceberg leaves treated with 1 and 3% olive each had 2- and 2.3-log reductions, respectively; a 2.9-log reduction was seen for the 5% dip (Fig. 1). Adult spinach and baby spinach showed similar results. On day 0, there were ca. 1-log reductions for all three concentrations. By day 1, 1% treatment continued to show ca. a 1-log reduction, and 3% olive rinses showed 1.5- and 1.7-log reductions for adult and baby spinach, respectively. The 5% olive rinse showed ca. a 2-log reduction. The reduction remained constant for the 1% rinse, while the 3% rinse showed 1.6- and 2-log reductions at day 3 for adult and baby spinach, respectively. By day 3, adult spinach and baby spinach exposed to the 5% rinse showed 1.5- and 2.2-log reductions, respectively (Fig. 1). All treatments showed significant reductions on day 0, with 5% rinse showing results statistically different from all other treatments ($P < 0.05$).

Hibiscus concentrate was only used to treat three types of organic leafy greens, as supply of the hibiscus was limited. A reduction of 1 log for *Salmonella* Newport population was seen for all three leafy greens by day 3, using a 30% hibiscus treatment (Fig. 2). In all three leafy greens, using a 30% rinse, statistically significant reductions were seen on days 0, 1, and 3 ($P < 0.05$) compared with the control. However, the lower concentration rinses did not have statistically significant reductions on any of the treatment days ($P < 0.05$).

In previous studies, it was found that antibacterial activity of roselle (hibiscus) was both concentration dependent and heat resistant (17). Liu et al. (17) also determined that roselle showed inhibitory effects against multidrug resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter*.

### TABLE 1. Background microflora populations before and after washing organic leaves with deionized water

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Romaine</th>
<th>Iceberg</th>
<th>Adult spinach</th>
<th>Baby spinach</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before wash</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XLD*</td>
<td>3.0–7.0</td>
<td>1.9–7.0</td>
<td>4.1–7.0</td>
<td>3.8–7.0</td>
</tr>
<tr>
<td>TSA</td>
<td>4.9–8.0</td>
<td>4.3–8.0</td>
<td>6.7–8.0</td>
<td>5.5–8.0</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>3.5–4.5</td>
<td>0–6.1</td>
<td>4.6–7.0</td>
<td>4.2–7.0</td>
</tr>
<tr>
<td><strong>After wash</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XLD</td>
<td>3.0–5.3</td>
<td>0–4.1</td>
<td>3.6–7.0</td>
<td>3.5–4.7</td>
</tr>
<tr>
<td>TSA</td>
<td>3.0–6.1</td>
<td>2.0–6.4</td>
<td>5.8–6.8</td>
<td>5.7–6.9</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>2.5–5.3</td>
<td>0–5.0</td>
<td>5.0–7.0</td>
<td>4.2–5.9</td>
</tr>
</tbody>
</table>

*Samples plated on XLD were not identified as *Salmonella* spp., and colonies were typical of coliforms.*
baumannii. Olaleye (18) also found antimicrobial effects of roselle (MICs of 0.30 ± 0.2 to 1.30 ± 0.2 mg/ml) against S. aureus, Bacillus stearothermophilus, Micrococcus luteus, Serratia marcescens, Clostridium sporogenes, E. coli, K. pneumoniae, Bacillus cereus, and P. fluorescence in laboratory media. In a study by Chao and Yin (4), roselle aqueous extract was shown to have antimicrobial effects (3- to 4-log reduction) against L. monocytogenes, E. coli O157:H7, S. aureus, and B. cereus in ground beef and apple juice. In the present study, the effect of hibiscus was limited on fresh produce. This could be attributed to differences in the type of food, type of bacteria, and methods used.

Unlike olive extract, apple extract did not show an immediate reduction of Salmonella Newport, but showed significant reductions by day 3 (Fig. 3). On day 0, only baby spinach and adult spinach showed significant reductions ($P < 0.05$). On day 1, the same trend occurred. Significant reductions of 1.7 and 1 log were seen for baby spinach and adult spinach, respectively ($P < 0.05$). By day 3, a 1-log reduction was observed in romaine lettuce for Salmonella Newport. A reduction of 0.7 log was observed in iceberg lettuce. Baby spinach and adult spinach both showed a 1.7-log reduction in Salmonella Newport population by day 3. By day 3, a 5% apple treatment showed statistically significant reductions, in both baby and adult spinach ($P < 0.05$) compared with the control.

It has been shown that apple extract containing apple polyphenols enhances heat inactivation of E. coli O157:H7 in ground beef as well as reduces the biological activity of Staphylococcus enterotoxin A (14, 21). Polyphenols found in red and white wine, in combination with other natural antimicrobial agents such as those found in garlic juice and oregano leaves, were found to have high antimicrobial activity against strains of B. cereus in wine marinades (9). These findings suggest that fruit polyphenols can potentially be added as antimicrobials in food applications, including leafy greens.

In order to compare these organic rinses against the industry standard, a hydrogen peroxide control was also tested. Inactivation of Salmonella Newport by hydrogen peroxide was not time dependent; the majority of reduction was observed on day 0 for each leafy green (Fig. 4). The same observation was made for all four types of leafy greens; hydrogen peroxide showed a log reduction on day 0 and maintained around the same number of survivors over the course of 3 days. As shown in this study, hydrogen peroxide does have antimicrobial effects; however, it might not be as effective as alternative natural plant extracts against S. enterica.
In general, romaine and iceberg lettuce showed better reductions in response to the antimicrobial treatments than spinach. In addition, the olive rinse had the greatest reduction compared with the other treatments. In adult spinach, most treatments were not statistically different compared with one another. However, 5% apple rinse had statistically higher reductions than hydrogen peroxide ($P < 0.05$). In baby spinach, 5% olive and 5% apple treatments showed statistically significant reductions compared with other treatments ($P < 0.05$). In iceberg lettuce, 5% olive rinse and 30% hibiscus showed statistically significant reductions, compared with other treatments ($P < 0.05$). In romaine lettuce, 5% olive rinse had the greatest significant reduction compared with other treatments ($P < 0.05$). Previous studies have investigated the reduction of Salmonella Typhimurium on fresh lettuce leaves under different exposure times to clove extracts (15). Their results showed that there was increased reduction of Salmonella Typhimurium in leaves treated with 10% clove for 10 min (3 log) than leaves treated for 1 min (1 log) (15). Belletti et al. (1) showed 2-log reductions of Salmonella Enteritidis in fruit salads that were exposed to 600 ppm citron essential oil for 9 days.

**Antimicrobial activities of olive extract, apple extract, hibiscus concentrate, and hydrogen peroxide against background microflora on organic leafy greens.**

The effects of antimicrobial treatments against the background microflora of organic leafy greens are shown in Table 2. Duplicate samples from the same batch were used because of the varying amounts of natural microflora seen from different batches of leafy greens. The highest concentration was chosen for each treatment in order to yield maximum results. As seen in Table 2, on day 3, maximum reduction of ca. 1.6 log was observed for romaine lettuce rinsed in 5% olive extract. In addition, with 5% olive and apple extracts, reductions of 0.3 to 0.7 log were seen for adult spinach and iceberg lettuce on day 3 compared with day 0. With the 30% hibiscus rinse, a reduction of 0.6 log was achieved for iceberg lettuce on day 3 compared with day 1. Compared with day 0, no additional reduction was seen on any produce treated with 3% hydrogen peroxide on day 3.

Comparing day 3 treatments to their respective PBS controls, reductions of 1.0, 2.5, 1.6, and 0.4 log were observed for romaine lettuce, iceberg lettuce, adult spinach, and baby spinach treated with 5% olive extract, respectively (Table 2). With 5% apple extract, reductions of 0.4 and 0.9 log were seen for romaine and iceberg lettuce, comparing day 3 to the control. With 30% hibiscus rinse, reductions of 0.3, 1.0, and 1.0 log were achieved for romaine lettuce, iceberg lettuce, and adult spinach, respectively, on day 3 compared with the control. Compared with the control, 1.5-, 2.2-, 2.4-, and 0.9-log reductions were seen in romaine lettuce, iceberg lettuce, adult spinach, and baby spinach treated with 3% hydrogen peroxide on day 3. These results generally mimic the findings shown in Figures 1 through 3, in that the plant extract rinses are time dependent, having the greatest bacterial reduction by day 3.

Ponce et al. (20) showed that tea tree and clove essential oils had antimicrobial effects on the native...
microflora–spoilage organisms of romaine lettuce leaves. These results suggest that these plant extract rinses would also be effective against background microflora. The results of this study were consistent with those of Ponce et al. (20), who achieved ca. a 2-log reduction in background microflora after 7 days in lettuce treated with antimicrobials encased in a lactose capsule. The lower reductions achieved in case of background microflora in our study compared with Salmonella Newport could be attributed to the possible presence of spore-forming bacteria that could have contaminated the leafy greens from the soil. It is also possible that some of the natural microflora could be less susceptible to these antimicrobials. Some other antimicrobials such as green tea, rosemary, and certain wine marinades have been

FIGURE 3. Survival of Salmonella Newport on organic (A) romaine lettuce, (B) iceberg lettuce, (C) adult spinach, and (D) baby spinach treated with 1, 3, and 5% apple extract. Values plotted at each sampling point are an average of three replicates. Error bars represent standard deviations from the means.

FIGURE 4. Survival of Salmonella Newport on organic (A) romaine and iceberg lettuces and (B) adult and baby spinach treated with 3% hydrogen peroxide. Values plotted at each sampling point are an average of three replicates. Error bars represent standard deviations from the means.
shown to inhibit and/or reduce spore-forming bacteria such as *B. cereus* (9, 16). This aspect merits further study with leafy greens.

In conclusion, this study demonstrates the potential of edible, natural plant extracts from olives, apples, and hibiscus as rinses to inactivate *Salmonella* Newport on organic romaine and iceberg lettuce, and adult and baby spinach. These extracts can therefore be used in the organic fresh produce industry as antimicrobial treatments to decontaminate fresh leafy greens. The activities of the natural plant extracts were greater than that of hydrogen peroxide over a 3-day period. Whether these compounds are as effective against other foodborne pathogens in leafy greens and other foods, and are acceptable from a sensory standpoint still needs further investigation.

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