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Use of Fulvic Acid Formulations as Flume-wash Treatments for Reduction of *Escherichia coli* O157:H7 on Organic Leafy Greens

ABSTRACT

Escherichia coli O157:H7 is a public health problem worldwide, with leafy greens as a major source of recent outbreaks. Standard organic produce-industry washes have been ineffective in reducing this problem. It is therefore crucial that effective alternatives for washing organic produce be developed. Fulvic acid (FA) is an organic acid reported to have antimicrobial and antifungal properties. The present study evaluated the antimicrobial efficacy of various FA formulations (I, II, III, IV) against *E. coli* O157:H7 as a flume-wash treatment for organic leafy greens. Organic baby and mature spinach, and romaine and iceberg lettuce, were inoculated with *E. coli* O157:H7 and washed with FA formulations at 1, 2, and 3% concentrations. Pathogen populations were determined on leafy greens stored at 4°C over a 3-day period. Treatment with all FA formulations at all three concentrations showed significant reductions ($P < 0.05$) in *E. coli* O157:H7 populations compared with the positive control. The most effective treatment was found to be FA-III at 3%, with 2.4 log CFU/g reduction on all leafy greens by day 3. Higher treatment concentrations caused higher reductions in pathogen populations. Fulvic acid could be used as an effective antimicrobial during flume washing and subsequent short-term storage of organic leafy greens.

INTRODUCTION

Consumption of organically grown fresh produce has increased dramatically in the United States (U.S.) during the past few decades *(18)*, and sales of such produce have gone up by almost 20% annually since 1990, with consumer sales of $28 billion in 2012 *(21, 48)*. This increase in produce consumption has been accompanied by a significant food safety challenge. Over the past decade, 13% of food-associated outbreaks have been linked to fresh fruits and vegetables *(17, 22, 37)*. Recently, organic fresh produce was also implicated in foodborne illness outbreaks caused by *E. coli* O157:H7, *Salmonella* and Hepatitis A virus *(10, 11, 12)*. *Escherichia coli* O157:H7 is an important foodborne

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pathogen with serious public health concerns. It is a common cause of hemolytic uremic syndrome (HUS), which can lead to kidney failure, predominantly among children and the elderly *(29, 38)*. More than 73,480 cases of foodborne illness and 61 deaths each year in the U.S. are linked to this pathogen *(30)*. From 1982 to 2002, 49 states reported 350 *E. coli* O157:H7 outbreaks, of which 52% were foodborne, and 21% of these foodborne outbreak cases were associated with fresh produce *(36)*.

Various factors can contribute to the presence of pathogens in fresh produce, such as direct contact with untreated manure or contaminated soil in the fields and poor quality of irrigation water *(44)*. Pathogen transfer can occur directly through animals, birds and insects at the pre-harvest *(40)* as well as the post-harvest level *(29)*. The fresh produce industry employs a disinfectant wash step during the post-harvest processing of fresh produce to keep the wash water from cross-contaminating fresh or fresh cut produce that is being washed. This step can also function as a means to reduce any contamination from human pathogens that may come in contact with the fresh produce. However, the U.S. Department of Agriculture-National Organic Program (USDA-NOP) limits the use of certain antimicrobials in handling or packing organic produce, and sanitation products must comply with specific regulations *(18, 43)*. Some of the USDA-NOP-approved disinfecting or sanitizing materials allowed in the wash water for organic fresh produce include chlorine (4ppm, residual), hydrogen peroxide, ozone, and peroxyacetic acid *(35)*. Several studies have shown that hydrogen peroxide is an effective surface sanitizer against foodborne pathogens on fresh fruits and vegetables *(4, 45, 49)*. However, its efficacy is limited to certain kinds of produce and pathogens *(4, 33, 41)*. For instance, 2% hydrogen peroxide treatment of cantaloupe cubes resulted in less than 1 log reduction of *Salmonella* population *(4)*. In a study by Denton et al. *(16)*, hydrogen peroxide reduced *E. coli* O157:H7 populations by less than 2 logs on baby spinach. Moreover, hydrogen peroxide treatment has shown to cause severe browning in shredded lettuce and mushrooms *(28, 32)*, and therefore may not be suitable for all produce types. Because there are few wash-treatment options for organic produce, it is necessary to seek alternative intervention strategies.

Antimicrobials derived from organic acids have been shown to have antimicrobial activity against foodborne pathogens. For example, lactic, citric, acetic, and ascorbic acids have all shown inhibitory activity towards *E. coli* and *Listeria monocytogenes* on iceberg lettuce *(2)*. Fulvic acid is also an organic acid that is reported to have antimicrobial and antifungal properties *(38, 46)*. It is a mixture of several organic acids with acidic functional groups, primarily carboxylic acid and phenolic hydroxyl groups, which give it the capacity to react with free radicals, minerals and biological enzyme systems *(1, 3)*. Sherry et al. *(38)* reported that fulvic acid interacts nonspecifically with the bacterial cell membrane to cause membrane disruption. Antimicrobial activity of fulvic acid, measured by use of a macrobroth tube dilution assay, has been determined against eight pathogens, *Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa, E. coli, Streptococcus pyogenes, Klebsiella pneumonia, Proteus mirabilis* and *Candida albicans (46)*. Zhu et al. *(50)* demonstrated fulvic acid activity against *L. monocytogenes, S.* Typhimurium and *P. aeruginosa* on food contact surfaces. However, few studies have been conducted to determine its efficacy in the organic fresh produce industry as an antimicrobial produce wash. In the current study, the effectiveness of various formulations of fulvic acid during flume-tank washing and subsequent short-term storage of organic leafy greens was evaluated against *E. coli* O157:H7.

MATERIALS AND METHODS

Bacterial culture preparation

A cocktail of two *E. coli* O157:H7 strains (ATCC 43888, and ATCC 43895) was used in this study. A swab of frozen culture (-80˚C) of each strain was transferred to 10 ml tryptic soy broth (TSB; Bacto™, BD, Sparks, MD) and incubated at 37˚C for 18–24 hrs. The revived culture was then transferred to TSB (100 µl culture in 9.9 ml TSB), incubated at 37˚C for 18–22 hrs, and maintained at 4˚C on tryptic soy agar (TSA; Acumedia, Lansing, MI). One day prior to the experiment, one to two colonies from a TSA plate were inoculated into 9 ml TSB and incubated at 37˚C for 18–24 hrs. To obtain an overnight culture, 1 ml of the resulting culture was transferred to fresh 9 ml TSB and incubated at 37˚C for 18–20 hrs. On the day of the experiment, a cocktail (1:1) from the overnight cultures of the two *E. coli* O157:H7 strains was prepared and further diluted in buffered peptone water (BPW; Oxoid Ltd., Basingstoke, Hampshire, England) to obtain the dip inoculum (10^6 CFU/ml) for leafy greens.

Antimicrobial treatments preparation

The following fulvic acid (FA) formulations (GTX Technologies, Amarillo, TX) were tested: FA-I (GTX-GOLD-L 30%, pH 1.95), FA-II (GTX-GOLD-L 40%, pH 2.15), FA-III (GTX-PLATINUM 5%, pH 1.63), and FA-IV (Alkaline 30%, pH 8.08). These formulations were mixed (v/v) with sterile distilled water to prepare concentrations of 1%, 2%, and 3%. Hydrogen peroxide (3%) and sterile distilled water were used as industry-control treatments with each experiment. The pH of the fulvic acid formulations was measured before each experiment *(Table 1)*.

Organic leafy greens preparation

The organic leafy greens tested were mature and baby spinach and romaine and iceberg lettuce. Organic leafy greens were bought on the day of the experiment from a local store in Stillwater, OK, transported on ice, and stored at 4˚C until use. Running tap water (room temperature (RT); 23–

TABLE 1. pH values of fulvic acid formulations at 1, 2, and 3% concentration in distilled water

Treatment	Concentration (%)	pH
Fulvic acid-I	1	3.78
Fulvic acid-I	2	3.07
Fulvic acid-I	3	2.96
Fulvic acid-II	1	3.10
Fulvic acid-II	2	3.06
Fulvic acid-II	3	2.83
Fulvic acid-III	1	2.67
Fulvic acid-III	2	2.58
Fulvic acid-III	3	2.54
Fulvic acid-IV	1	6.66
Fulvic acid-IV	2	6.82
Fulvic acid-IV	3	7.25

25˚C) was used to wash the greens thoroughly for 2 minutes to remove any soil or organic matter. The outer leaves of romaine and iceberg lettuce and the core of iceberg lettuce were removed aseptically. The lettuce leaves were cut with sterile scissors into 2 X 2 inch pieces. Whole leaves of baby spinach (approximately 1.5 X 2.0 inches) were used. Bunched mature spinach samples were prepared by separating individual leaves, trimming off the stalks, and then cutting the leaves into 2 X 2 inch pieces, using aseptic techniques.

Antimicrobial treatment of organic leafy greens

Organic leafy green samples, prepared as just described, were weighed (400 g), transferred to a sterile plastic tub containing sterile distilled water and washed three times for 2 minutes each time, using gentle agitation. The leafy greens were then transferred to a bio-safety cabinet and exposed to UV (254 nm) light for 30 minutes (15 minutes on each side) to reduce background microflora. Immediately afterward, a 20-g sample was removed and placed in a 24-oz. Whirl-Pak™ bag (Nasco, Fort Atkison, WI, USA) as the negative (uninoculated) control. The remaining leafy greens were then dip inoculated with the *E. coli* O157:H7 cocktail (10^6 log CFU/ml) for 2 minutes *(9, 13, 26)* and placed for 30 minutes in the bio-safety cabinet to facilitate bacterial attachment *(6, 31, 42)*. A 20-g sample of inoculated leafy greens was transferred into a 24-oz. Whirl-Pak™ bag as the positive control, while the remaining greens were separated into 20-g samples for each antimicrobial treatment. Each of these samples was washed in the appropriate antimicrobial treatment (200 ml each) for 2 minutes, with gentle agitation. Additional neutralization step was performed for the treated samples with 180 ml Dey/Engley (D/E) neutralizing broth (Remel Inc., Lenexa, KS) for 1 minute. Immediately after washing, leafy greens were transferred to Whirl-Pak™ bags and stored at 4˚C for 3 days. On days 0, 1, and 3, a 5-g sample was removed for each treatment, including the negative and positive controls, to determine surviving *E. coli* O157:H7 populations. Each sample was transferred into a sterile Whirl-Pak™ bag and stomached (Seward, Ltd., London, UK) at 230 rpm for 1 minute with 45 ml of sterile BPW. Immediately following, samples were serially diluted in BPW and plated on Sorbitol MacConkey agar (SMAC; Remel Inc., Lenexa, KS). Colonies of *E. coli* O157:H7 (CFU/g) were counted after 18–24 hrs of incubation at 37˚C.

Statistical analysis

All experiments were performed 3 times. Surviving *E. coli* O157:H7 populations, recovered after different antimicrobial treatments at each sampling period, were converted to $\log_{10}$ CFU/g and mean values of the three replicates obtained. The limit of detection was 1 log CFU/g. Data were analyzed using PROC GLM (SAS v. 9.3 software; SAS Inst., Cary, NC, USA) to determine the analysis of variance (ANOVA) for main and interaction effects of treatments, wash times and storage times for all the leafy greens. Significance of differences between results was set at $P < 0.05$.

RESULTS

For all the organic leafy greens, significant reduction ($P < 0.05$) in *E. coli* O157:H7 population was observed during the 3-day storage period at 4˚C after treatment with fulvic acid formulations, while it remained at 4.5–5.0 logs CFU/g for the positive control.

Organic baby and mature spinach

The surviving *E. coli* O157:H7 population for the positive control on baby spinach leaves *(Table 2)* was 4.1–4.5 log CFU/g over a period of 3 days. However, when compared to the positive control, all fulvic acid formulations showed an immediate reduction in pathogen populations (1.2 to 2.2 log CFU/g) on baby spinach after the initial wash (day 0). This reduction was maintained over the 3-day storage period *(Table 2)*. Compared with the positive control, washing of baby spinach with FA-I or FA-II (1%, 2%, and 3%) reduced *E. coli* O157:H7 populations by 1.1 – 2.0 log CFU/g after 3 days of storage. The 3% concentration of all fulvic acid formulations proved to be the most effective in reducing pathogen populations on organic baby spinach leaves. However, those treated with FA-III at 3% showed the highest reduction (2.4 log CFU/g) after a 3-day storage. Fulvic acid-IV at all 3 concentrations reduced pathogen populations by approximately 1.9 log CFU/g on baby spinach, after the 3-day storage period. The 3% hydrogen peroxide treatment showed results similar to those of some of the fulvic acid treatments, with a 1.9 log CFU/g reduction in *E. coli* O157:H7 after 3 days of storage. However, unlike the fulvic acid treatments, washing with water alone did not result in an immediate reduction in pathogen population on day 0.

All fulvic acid formulations significantly ($P < 0.05$) reduced *E. coli* O157:H7 populations on organic mature spinach throughout the storage period at 4˚C *(Table 3)*. The initial concentration of *E. coli* O157:H7 in positive control leaves was 4.3 log CFU/g. When mature spinach was washed with fulvic acid formulations, an immediate reduction of up to 3.1 log CFU/g in *E. coli* O157:H7 populations was observed, compared to the positive control, on day 0. By day 3, further reductions between 1.5 to 1.9 log CFU/g in pathogen population were observed for all fulvic acid treatments at all concentrations. As was observed for organic baby spinach, the 3% concentration of all the fulvic acid formulations was the most effective in reducing *E. coli* O157:H7 populations on organic

TABLE 2. *Escherichia coli* O157:H7 populations on organic baby spinach following fulvic acid treatments

Treatments	Concentration (%)	Surviving *E. coli* O157:H7 population* (log_{10} CFU/g)			*E. coli* O157:H7 log_{10} reduction**		
		Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
PC	-	4.3 ± 0.9^{a}	4.5 ± 0.8^{a}	4.1 ± 0.8^{a}			
W	-	3.5 ± 0.7^{a}	3.4 ± 1.0^{b}	2.6 ± 0.9^{b}	0.7	1.0	1.4
HP	-	1.9 ± 0.4^{b}	1.9 ± 0.6^{c}	2.2 ± 0.6^{b}	2.4	2.5	1.9
FA-I	1	2.7 ± 0.7^{c}	2.9 ± 0.4^{b}	3.0 ± 1.1^{b}	1.6	1.6	1.1
FA-I	2	2.1 ± 0.1^{bc}	2.8 ± 0.7^{bc}	2.5 ± 0.6^{b}	2.2	1.7	1.6
FA-I	3	2.8 ± 0.6^{c}	2.4 ± 0.6^{bc}	2.1 ± 0.5^{b}	1.4	2.0	2.0
FA-II	1	2.6 ± 0.5^{c}	2.6 ± 0.4^{bc}	2.2 ± 0.8^{b}	1.7	1.8	1.9
FA-II	2	2.8 ± 0.4^{c}	2.2 ± 0.2^{bc}	2.4 ± 0.4^{b}	1.4	2.2	1.7
FA-II	3	2.5 ± 0.4^{c}	2.2 ± 0.8^{bc}	2.1 ± 0.2^{b}	1.7	2.2	2.0
FA-III	1	2.8 ± 0.6^{c}	2.6 ± 0.5^{bc}	2.3 ± 0.2^{b}	1.5	1.8	1.7
FA-III	2	2.4 ± 0.6^{c}	2.1 ± 0.3^{bc}	2.2 ± 0.3^{b}	1.9	2.4	1.9
FA-III	3	2.5 ± 0.7^{c}	2.3 ± 0.3^{bc}	1.7 ± 0.2^{b}	1.8	2.1	2.4
FA-IV	1	3.1 ± 0.5^{a}	2.4 ± 0.1^{b}	2.3 ± 0.1^{b}	1.2	2.0	1.8
FA-IV	2	3.1 ± 0.4^{a}	2.7 ± 0.1^{b}	2.3 ± 0.2^{b}	1.2	1.7	1.8
FA-IV	3	2.9 ± 0.4^{a}	2.7 ± 0.3^{bc}	2.2 ± 0.1^{b}	1.3	1.8	1.9

PC: Positive Control; W: Water; HP: Hydrogen Peroxide; FA: Fulvic Acid. *Values represent the average of three replications. Standard deviation (±) for surviving *E. coli* O157:H7 population (log_{10} CFU/g) follows mean value. Letters *a, b, c* provide evidence of significant difference, where different letters represent statistical significance ($P < 0.05$) between treatments for the same sampling day. **Calculations based on PC values of surviving populations on day 0, 1, and 3.

TABLE 3. *Escherichia coli* O157:H7 populations on organic mature spinach following fulvic acid treatments

Treatments	Concentration (%)	Surviving *E. coli* O157:H7 population (log_{10} CFU/g)			*E. coli* O157:H7 log_{10} reduction		
		Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
PC	-	5.8 ± 1.2[a]	4.7 ± 0.4[a]	4.3 ± 0.6[a]			
W	-	3.3 ± 0.5[b]	3.4 ± 0.3[b]	3.0 ± 0.5[b]	2.5	1.3	1.3
HP	-	2.2 ± 0.1[c]	3.0 ± 0.8[c]	2.7 ± 0.3[c]	3.6	1.7	1.6
FA-I	1	2.8 ± 0.1b[c]	2.5 ± 0.3[c]	2.8 ± 0.1[bc]	3.0	2.2	1.5
FA-I	2	2.9 ± 0.1b[c]	2.5 ± 0.4[bc]	2.7 ± 0.1[bc]	2.9	2.1	1.5
FA-I	3	2.9 ± 0.3b[c]	2.6 ± 0.1[bc]	2.7 ± 0.1[bc]	2.9	2.1	1.5
FA-II	1	2.9 ± 0.3b[c]	2.8 ± 0.1[bc]	2.7 ± 0.7[c]	2.9	1.9	1.6
FA-II	2	2.9 ± 0.6b[c]	2.9 ± 0.2[bc]	2.7 ± 0.2[bc]	2.8	1.8	1.6
FA-II	3	2.6 ± 0.1[bc]	2.6 ± 0.2[bc]	2.5 ± 0.2[c]	3.1	2.1	1.8
FA-III	1	2.8 ± 0.2[bc]	2.8 ± 0.2[bc]	2.7 ± 0.2[bc]	3.0	1.8	1.5
FA-III	2	2.9 ± 0.5[bc]	2.7 ± 0.2[bc]	2.4 ± 0.2[c]	2.9	1.9	1.9
FA-III	3	2.7 ± 0.2[bc]	2.5 ± 0.1[bc]	2.6 ± 0.3[c]	3.1	2.2	1.7
FA-IV	1	2.9 ± 0.4[bc]	2.9 ± 0.1[bc]	2.6 ± 0.2[c]	2.8	1.7	1.6
FA-IV	2	3.0 ± 0.4[bc]	3.0 ± 0.3[bc]	2.4 ± 0.2[c]	2.8	1.7	1.9
FA-IV	3	2.7 ± 0.4[bc]	2.7 ± 0.5[bc]	2.6 ± 0.1[bc]	3.1	1.9	1.7

PC: Positive Control; W: Water; HP: Hydrogen Peroxide; FA: Fulvic Acid. *Values represent the average of three replications. Standard deviation (±) for surviving *E. coli* O157:H7 population (log_{10} CFU/g) follows mean value. Letters *a, b, c* provide evidence of significant difference, where different letters represent statistical significance ($P < 0.05$) between treatments for the same sampling day. **Calculations based on PC values of surviving populations on day 0, 1, and 3.

mature spinach. Water and hydrogen peroxide control treatments also reduced *E. coli* O157:H7 populations by 1.3 and 1.6 log CFU/g, respectively, after 3 days of storage.

Organic romaine and iceberg lettuce

Initial *E. coli* O157:H7 populations for the positive control on organic romaine lettuce was 4.4 logs CFU/g *(Table 4)*. Compared with results for the positive control, the highest reduction (2.4 log CFU/g) in *E. coli* O157:H7 population was observed on day 3 for romaine lettuce washed with 3% FA-III, followed by 2.3 log CFU/g reductions with 3% FA-I and 1% FA-III. Wash treatments of FA-I at 1% and 2% concentrations reduced *E. coli* O157:H7 populations by 2.2 and 1.9 log CFU/g, respectively, after 3 days of storage. Fulvic acid-II at all 3 concentrations resulted in about 2.1 log CFU/g reduction in pathogen populations by the end of storage. By day 3, populations of *E. coli* O157:H7 on romaine lettuce leaves treated with FA-IV at 1%, 2% and 3% concentrations were decreased by 2.0, 1.4 and 1.6 log CFU/g, respectively. The industry controls, water and hydrogen peroxide, showed similar results on day 3 to the results with fulvic acid treatments, exhibiting 2.0 and 2.6 log CFU/g reduction in *E. coli* O157:H7, respectively.

All fulvic acid formulations significantly ($P < 0.05$) reduced *E. coli* O157:H7 populations on organic iceberg lettuce throughout the storage period at 4˚C *(Table 5)*. The initial concentration of *E. coli* O157:H7 on the positive control for organic iceberg lettuce was 4.2 log CFU/g *(Table 5)*. Washing iceberg lettuce with FA-III at 2% and 3% produced an immediate reduction (day 0) of 2.3 log CFU/g in pathogen population. All the other fulvic acid formulations at all three concentrations produced reductions of 1.0 to 1.7 log CFU/g by day 3 on iceberg lettuce. Water and hydrogen peroxide reduced *E. coli* O157:H7 populations by 1.4 and 3.0 log CFU/g, respectively, on iceberg lettuce after 3 day storage at 4˚C.

DISCUSSION

The current study evaluated the efficacy of various fulvic acid formulations at different concentrations against *E. coli* O157:H7 on four types of organic leafy greens during flume-

TABLE 4. *Escherichia coli* O157:H7 population on organic romaine lettuce following fulvic acid treatments

Treatments	Concentration (%)	Surviving *E. coli* O157:H7 population (log_{10} CFU/g)			*E. coli* O157:H7 log_{10} reduction		
		Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
PC	-	4.4 ± 0.5^{a}	4.3 ± 0.2^{a}	4.2 ± 0.2^{a}			
W	-	2.5 ± 0.2^{b}	2.2 ± 0.1^{b}	2.2 ± 0.3^{b}	1.8	2.1	2.0
HP	-	1.2 ± 0.7^{c}	2.1 ± 0.5^{bc}	1.5 ± 1.0^{c}	3.1	2.2	2.6
FA-I	1	2.5 ± 0.6^{b}	2.6 ± 0.4^{bc}	1.9 ± 0.2^{c}	1.8	1.7	2.2
FA-I	2	2.5 ± 0.5^{b}	2.2 ± 0.7^{bc}	2.3 ± 0.3^{bc}	1.8	2.1	1.9
FA-I	3	2.5 ± 0.4^{b}	1.9 ± 0.3^{bc}	1.9 ± 0.1^{c}	1.8	2.3	2.3
FA-II	1	2.3 ± 0.2^{b}	2.1 ± 0.1^{bc}	2.1 ± 0.7^{b}	2.0	2.2	2.1
FA-II	2	2.7 ± 0.4^{b}	2.1 ± 0.6^{bc}	2.2 ± 0.3^{b}	1.7	2.2	2.0
FA-II	3	2.2 ± 0.5^{b}	2.1 ± 0.9^{bc}	2.0 ± 0.7^{c}	2.1	2.1	2.1
FA-III	1	2.5 ± 0.3^{b}	2.1 ± 0.9^{bc}	1.9 ± 0.4^{c}	1.8	2.2	2.3
FA-III	2	2.3 ± 0.4^{b}	1.9 ± 0.4^{bc}	1.9 ± 1.2^{c}	2.0	2.4	2.2
FA-III	3	2.3 ± 0.4^{b}	1.5 ± 0.2^{c}	1.7 ± 0.3^{c}	2.0	2.8	2.4
FA-IV	1	2.8 ± 0.3^{b}	2.6 ± 0.7^{bc}	2.2 ± 0.2^{b}	1.6	1.6	2.0
FA-IV	2	3.2 ± 0.8^{b}	2.8 ± 0.5^{b}	2.8 ± 0.7^{b}	1.1	1.5	1.4
FA-IV	3	2.8 ± 1.0^{b}	2.3 ± 0.6^{bc}	2.6 ± 0.6^{b}	1.6	2.0	1.6

PC: Positive Control; W: Water; HP: Hydrogen Peroxide; FA: Fulvic Acid. *Values represent the average of three replications. Standard deviation (±) for surviving *E. coli* O157:H7 population (log_{10} CFU/g) follows mean value. Letters *a, b, c* provide evidence of significant difference, where different letters represent statistical significance ($P < 0.05$) between treatments for the same sampling day. **Calculations based on PC values of surviving populations on day 0, 1, and 3.

tank washing and subsequent 3-day refrigerated storage. Significant reductions in *E. coli* O157:H7 populations were observed when tested leafy greens were washed with fulvic acid. The results from the present study are similar to those of other studies that have evaluated the antimicrobial efficacy of fulvic acid. An *in vitro* study by Van Rensburg et al. *(46)* revealed that fulvic acid exhibited antibacterial activity against *S. aureus, P. aeruginosa, E. coli, S. pyogenes, P. mirabilis* and *C. albicans.* Zhu et al. *(50)* also demonstrated the antimicrobial potential of fulvic acid against *L. monocytogenes*, *S.* Typhimurium and *P. aeruginosa* on food contact surfaces.

The fulvic acid formulations tested in the current study were found to be more effective than other organic acids evaluated in similar studies *(2, 13)*. Park et al. *(34)* revealed 0.8–1.4 log CFU/g reductions in *E. coli* O157:H7 populations on lettuce washed for 1 minute with propionic, acetic, lactic, malic or citric acids at 1% or 2% concentrations. In the current study, washing for 2 minutes with 1% and 2% concentrations of fulvic acid formulations gave an immediate reduction (day 0) of 1.4–2.0 and 1.1–2.3 log CFU/g, respectively, on romaine or iceberg lettuce. It is possible that longer washing time (2 minutes) in our experiments resulted in higher reductions of pathogen population on the leafy green surface. However, in a study by Francis and O'Beirne *(20)*, treatment of iceberg lettuce with 1% citric acid for 5 minutes resulted in 1.5 log CFU/g reductions in *E. coli* and *L. innocua* counts. It is therefore clear that fulvic acid may be more effective in reducing *E. coli* O157:H7 populations on leafy greens than other organic acids. Refrigerated storage (for 3 days) also resulted in further reduction of surviving *E. coli* O157:H7 population in the present study, which suggests the lingering antimicrobial effect of fulvic acid.

Higher concentrations of fulvic acid were particularly effective in reducing *E. coli* O157:H7 populations in the current study. More specifically, the 3% concentration of all fulvic acid formulations brought about higher reductions in *E. coli* O157:H7 populations than the 1% concentration. These findings are supported by studies conducted with fulvic acid or other organic acids *(2, 46, 50)*. In a study by Choi et al. *(13)*, when compared to lower concentrations (0.25, 0.5, or 1%), 2% concentrations of malic, lactic and citric acid were more

TABLE 5. *Escherichia coli* O157:H7 population on organic iceberg lettuce following fulvic acid treatments

Treatments	Concentration (%)	Surviving *E. coli* O157:H7 population (log_{10} CFU/g)			*E. coli* O157:H7 log_{10} reduction		
		Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
PC	-	4.2 ± 0.3^{a}	4.0 ± 0.1^{a}	3.7 ± 0.3^{a}			
W	-	3.2 ± 0.6^{b}	2.4 ± 0.2^{b}	2.2 ± 0.2^{b}	1.0	1.5	1.4
HP	-	2.0 ± 0.1^{c}	1.4 ± 0.9^{c}	0.7 ± 0.5^{c}	2.2	2.5	3.0
FA-I	1	2.3 ± 0.3^{c}	2.4 ± 0.5^{b}	2.3 ± 0.1^{b}	1.8	1.5	1.3
FA-I	2	2.2 ± 0.2^{c}	2.4 ± 0.2^{b}	1.9 ± 0.3^{d}	2.0	1.6	1.7
FA-I	3	2.1 ± 0.1^{c}	2.0 ± 0.2^{d}	2.2 ± 0.4^{b}	2.1	1.9	1.4
FA-II	1	2.3 ± 0.3^{c}	2.1 ± 0.1^{d}	2.4 ± 0.3^{b}	1.9	1.8	1.2
FA-II	2	2.4 ± 0.2^{c}	2.0 ± 0.2^{d}	2.2 ± 0.3^{b}	1.8	1.9	1.4
FA-II	3	2.1 ± 0.2^{c}	2.0 ± 0.3^{d}	2.5 ± 0.5^{b}	2.0	1.9	1.2
FA-III	1	2.6 ± 0.5^{c}	2.2 ± 0.5^{d}	2.2 ± 0.3^{b}	1.5	1.8	1.4
FA-III	2	1.8 ± 0.5^{d}	1.9 ± 0.1^{c}	1.3 ± 0.1^{c}	2.3	2.1	2.4
FA-III	3	1.8 ± 0.7^{d}	1.4 ± 0.4^{c}	1.6 ± 0.5^{c}	2.3	2.6	2.0
FA-IV	1	2.8 ± 0.4^{bc}	2.7 ± 0.5^{b}	2.7 ± 0.3^{b}	1.4	1.2	1.0
FA-IV	2	2.9 ± 0.3^{b}	2.8 ± 0.3^{b}	2.6 ± 0.3^{b}	1.2	1.2	1.1
FA-IV	3	2.6 ± 0.1^{bc}	2.7 ± 0.1^{b}	2.0 ± 0.1^{d}	1.5	1.3	1.7

PC: Positive Control; W: Water; HP: Hydrogen Peroxide; FA: Fulvic Acid. *Values represent the average of three replications. Standard deviation (±) for surviving *E. coli* O157:H7 population (log_{10} CFU/g) follows mean value. Letters *a, b, c* provide evidence of significant difference, where different letters represent statistical significance ($P < 0.05$) between treatments for the same sampling day. **Calculations based on PC values of surviving populations on day 0, 1, and 3.

effective in reducing *E. coli* O157:H7 populations. Zhu et al. *(50)* also demonstrated that a higher concentration (2.5%) of fulvic acid resulted in greater reductions in *S.* Typhimurium populations. These results indicate that higher concentrations of organic acids, including fulvic acid, would need to be used to obtain higher log reductions of the pathogen on leafy greens.

Of all the fulvic acid formulations tested, FA-III was proven to be most effective. Authors of earlier studies have suggested that the inhibitory activity of organic acids is pH dependent *(5, 7)*. Organic acids have optimal inhibitory activity at low pH values, which favor the uncharged, un-dissociated molecular state responsible for the bactericidal activity. It is also well known that foodborne pathogens are more susceptible to acidic conditions and require slightly higher pH values for optimal growth *(8, 14)*. In this study, we found that among the four fulvic acid formulations tested, FA-III had the lowest pH value (2.54), which could explain the greater reductions in pathogen populations with this particular formulation. In contrast, the pH of FA-IV was found to be alkaline (7.25), which could explain the lower log reductions observed with this formulation. However, many other factors, such as the molecular structure, chain length, degree of branching, and the ratio of un-dissociated forms of organic acid can also affect the antimicrobial activity of organic acids *(19)*.

Although the extent of *E. coli* O157:H7 inactivation varied among the different fulvic acid formulations, their immediate effect was more pronounced on organic mature spinach leaves than on other leafy greens. The formulations were least effective on romaine lettuce leaves. This difference could be accounted for by the different type of leafy greens surfaces that were tested in the study. A study by Zhu et al. *(50)* suggested that the efficacy of fulvic acid may vary based on the type of food contact surface. In their study, *S.* Typhimurium populations were reduced to undetectable levels on plastic surfaces, in contrast to stainless steel surfaces, where a reduction of up to 2.8 log CFU/coupon was observed. Similarly, in the present study, variations in the efficacy of fulvic acid formulations were observed on the different types of leafy greens tested. Higher reductions in pathogen populations were observed on mature spinach (3.1 log CFU/g) and iceberg lettuce (2.3

log CFU/g) than on romaine lettuce (2.1 log CFU/g) on day 0. Previous studies with organic compounds *(16)*, and plant extracts *(9, 31)* have also suggested differences in efficacies of antimicrobial wash treatments with different leafy greens surfaces. Romaine lettuce leaves have rough underside surfaces, which could have provided protection to pathogens against surface sanitizers in the current study. Several studies have shown that rough surfaces of leafy greens provide grooves and crevices *(47)* where bacteria tend to aggregate and hide so that they are not exposed to sanitizer washes *(25)*. Additionally, the rough surface provides more contact points to facilitate firmer attachment by the bacteria *(23, 27)*. Rough surfaces of lettuce leaves could have protected pathogen cells and prevented their exposure to sanitizers.

In this study, hydrogen peroxide (3%) was used as an industry control for washing organic produce. The treatment of organic leafy greens with hydrogen peroxide produced reductions of 1.7–3.0 log CFU/g in *E. coli* O157:H7 populations during storage at 4˚C. However, an increase in surviving pathogen populations was observed by the end of storage period in hydrogen peroxide-treated organic baby and mature spinach leaves *(Tables 2 and 3)*. These results are consistent with the data reported by the authors in previous studies *(9, 16)* carried out with organic leafy greens and plant-derived antimicrobials. Buddhini et al. *(9)* and Denton et al. *(16)* observed up to 1.5 log CFU/g increases in *E. coli* O157:H7 populations on organic baby spinach and romaine lettuce leaves treated with hydrogen peroxide by the end of a 3-day storage. These results suggest that hydrogen peroxide does possess antimicrobial properties but may not be able to maintain long-term antimicrobial effects against *E. coli* O157:H7 in certain produce types. According to a report by Juven and Pierson *(24)*, hydrogen peroxide serves as an oxidative agent to exhibit bactericidal and inhibitory activity. It can also generate cytotoxic oxidizing molecules such as those containing hydroxyl radicals. Residual levels of hydrogen peroxide used to wash fresh produce may also depend on the presence or absence of peroxidase in the produce item. This could explain the differences observed in the inhibitory activity of hydrogen peroxide on different produce types in the current study.

CONCLUSIONS

This study showed that fulvic acid formulations effectively reduced *E. coli* O157:H7 populations on organic baby and mature spinach and on romaine and iceberg lettuce. Fulvic acid-III at 3% concentration was found to be the most effective flume-tank wash treatment of all the tested formulations. It therefore has the potential to be used as an alternative antimicrobial wash treatment for organic leafy greens. However, future studies must include sensory analysis of the organic leafy greens washed with fulvic acid formulations, to determine consumer acceptability.

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