Ultrasound Enhanced Sanitizer Efficacy in Reduction of *Escherichia coli* O157 : H7 Population on Spinach Leaves

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ABSTRACT: The use of ultrasound to enhance the efficacy of selected sanitizers in reduction of *Escherichia coli* O157 : H7 populations on spinach was investigated. Spot-inoculated spinach samples were treated with water, chlorine, acidified sodium chlorite (ASC), peroxyacetic acid (POAA), and acidic electrolyzed water with and without ultrasound (21.2 kHz) for 2 min at room temperature. The effects of ultrasound treatment time and acoustic energy density (AED) were evaluated at an ASC concentration of 200 mg/L. The effect of ASC concentration, with a fixed AED of 200 W/L, was also examined. Microbial analysis indicated that ASC reduced *E. coli* O157 : H7 population by 2.2 log cycles over that of water wash, while the reduction from other sanitizers was about 1 log cycle. Ultrasonication significantly enhanced the reduction of *E. coli* cells on spinach for all treatments by 0.7 to 1.1 log cycle over that of washes with sanitizer alone. An increase in the ASC concentration enhanced the efficacy of the combined treatment of ASC and ultrasonication, especially at ASC concentrations of < 300 mg/L. Increasing the ultrasound treatment time from 0 to 4 min and AED from 0 to 500 W/L were both effective in increasing the effectiveness of the ASC and ultrasound combined treatments. In addition, *E. coli* O157 : H7 inoculated on the underside of spinach leaves (rough side) were more difficult to remove than those inoculated on the upper side (smooth side).

Keywords: acidified sodium chlorite, cavitation, *Escherichia coli*, sanitizer, ultrasound

Introduction

The 2006 outbreaks of *Escherichia coli* O157 : H7 infections due to consumption of bagged spinach reaffirmed the importance and challenge of produce microbial safety. In its entirety, the outbreak that began on August 19, 2006, comprised 199 reported cases of illness in 26 states, including 102 hospitalizations and 3 deaths (CDC 2006). Nearly the entire spinach industry shut down its operation within 24 h when U.S. Food and Drug Administration (FDA) issued a warning on spinach consumption on September 14, 2006. The economic losses for the spinach industry alone were estimated to be at least $75 million. More importantly, recurring produce-related outbreaks erode consumer confidence in fresh produce and could jeopardize the long-term development of the produce industry.

Currently, commercial operations rely on a wash treatment with antimicrobials as the only step to reduce microbial populations on fresh produce. However, chlorinated water containing 50 to 100 mg/L free chlorine can only achieve a 1- to 2-log CFU/g reduction in microbial population in an industrial scale operation (Sapers 1998). Even on a laboratory scale, the efficacy of decontamination for a model wash step is often less than satisfactory. A few wash studies conducted using spinach have demonstrated the insufficiency of produce washes. Pirovani and others (2001) treated fresh-cut spinach with chlorine (25 to 125 mg/L) and reported 2 to 3 log cycles reduction in total native microbial count. Izumi (1999) achieved a 1.8-log reduction in total microbial count on trimmed spinach leaves with a 4-min electrolyzed water wash. Obviously, more effective postharvest intervention technologies are needed for assuring the microbial safety of fresh and fresh-cut produce.

In recent years, several new sanitizers have been introduced for the purpose of improving fresh produce safety. Acidified sodium chlorite (ASC) has been approved by FDA for dip or spray operations for food items, including fresh and fresh-cut fruits and vegetables. The ASC has shown strong ability to control pathogens (*E. coli* O157 : H7, *Listeria monocytogenes*, *Salmonella* Poona, and so on) on fresh-cut carrots and cilantro (Gonzalez and others 2004; Cruz-Ruiz and others 2006; Allende and others 2009). Acidic electrolyzed water (AEW) and peroxyacetic acid (POAA) are also new sanitizers that have been tested in recent studies. AEW is a strong antimicrobial agent effective against pathogens and spoilage microorganisms (Wang and others 2004, 2006), and is more efficient than chlorinated water for inactivating *E. coli* O157 : H7, *S.* Enteritidis, and *L. monocytogenes* on selected fresh produce and meat products (Park and others 2001; Bari and others 2003). POAA (80 mg/L) was reported to be more effective in reducing populations of *L. monocytogenes* on lettuce than a chlorine solution of 100 mg/L (Beuchat and others 2004).

Ultrasonic waves in the frequency ranging from 20 to 100 kHz have long been used as an industrial surface-cleaning tool. The application of ultrasound for produce surface decontamination, however, is relatively new. Seymour and others (2002) conducted an ultrasound and chlorinated water combined treatment and obtained an additional log cycle reduction of *S.* Typhimurium on iceberg lettuce compared with a chlorinated water alone wash. Scouten and Beuchat (2002) found that ultrasound in combination with 1% calcium hydroxide enhanced the decontamination efficacy on alfalfa seeds inoculated with *S. enterica* and *E. coli* O157 : H7. Huang and others (2006) reported up to 1 log cycle additional

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reduction of *S. enterica* and *E. coli* O157 : H7 on apples in an ultrasound and chlorine dioxide combined treatment, whereas no obvious increase in log reduction was observed for *E. coli* O157 : H7 inoculated on lettuce. Ajlouni and others (2006) also found no effect of ultrasound on the inactivation of natural flora on Cos lettuce in a 20-min sanitizer wash (0.02% peracetic acid, 4 mg/L hydrogen peroxide, 2% acetic acid, 100 mg/L chlorinated water) except with 200 mg/L chlorinated water. The lack of effectiveness reported by Huang and others (2006) and Ajlouni and others (2006) might be attributable to the nonuniform ultrasound distribution in the ultrasonic cleaning baths due to standing wave formation and blockage of produce leaves to ultrasound propagation in the wash liquid.

There is a need to further examine the function of ultrasound, when combined with a sanitizer, in a washing operation for decontamination of fresh produce. This study was therefore undertaken to determine the effect of selected new sanitizers with or without ultrasound on the reduction of *E. coli* O157 : H7 population inoculated on the surface of spinach.

Materials and Methods

Preparation of treatment solutions

ASC was prepared from 10% sodium chloride solution and 5% citric acid solution. The concentration of sodium chlorite was determined by a method developed by Alcide Corporation (Redmond, Wash., U.S.A.). Acidic electrolyzed water (AEW; 80 mg/L) was generated using an AEW generator (ROX-20TA, Hoshizaki, Nagoya, Japan) and collected from the anode of the generator with sanitized beakers. The pH and oxidation reduction potential (ORP) of the AEW were measured with an AR15 pH and ORP meter (Accumet Research, Pittsburgh, Pa., U.S.A.), and the residual chlorine concentration was determined using an EPA-approved chlorine colorimetric test kit (Model PCT-DR, LaMotte Co., Chestertown, Md., U.S.A.). POAA (80 mg/L; Tsunami 100; Ecolab, St. Paul, Minn., U.S.A.) was prepared according to the manufacturer's instruction. The concentration of POAA was measured with a test kit provided by the manufacturer. Chlorine solution (200 mg/L) was prepared with a concentrated food-grade bleach and the pH was adjusted to 6.5 with 1.0 M HCl. The available chlorine was determined with a chlorine test kit.

Inoculum preparation

In this experiment, a nalidixic acid-resistant derivative of *E. coli* O157 : H7, strain 87-23 (nonpathogenic) was adopted, which was obtained from the former Produce Quality and Safety Laboratory, USDA-ARS (Beltsville, Md., U.S.A.). The cells in tryptic soy agar (TSA) slant were transferred 3 times to tryptic soy broth (pH 7.3, Difco Laboratories, Detroit, Mich., U.S.A.) by loop inoculation at successive 24-h intervals followed by incubation at 37 °C. Bacterial cells were harvested, after 24 h of growth, by centrifugation (6000 × *g*) at 4 °C for 10 min. The cell pellets were washed twice in peptone water (0.85% NaCl, 0.1% Bacto Peptone), and resuspended in 10 mL of peptone water. The final concentration of *E. coli* O157 : H7 in the inoculum, determined by plating serial dilutions on TSA containing 50 μg/mL nalidixic acid and incubating at 37 °C for 24 h, was approximately 10^9 CFU/mL.

Inoculation of spinach leaves

Baby spinach were obtained from a local wholesale market, kept at 4 °C, and used within 3 d. Fresh spinach leaves were spot-inoculated on upper surfaces with 100 μL per leaf of the inoculum and air-dried for 60 min in a laminar flow biological hood (Labconco Purifier PCR Enclosure, Kansas City, Mich., U.S.A.) before treatments.

Treatment procedures

Twenty-five grams of inoculated spinach leaves were loaded into a 1 L jacket glass beaker. The beaker contained 500 mL of one of the following 5 treatment solutions: sterilized deionized water, chlorinated water (200 mg/L), AEW (pH 2.7, ORP 1150 mV, and free chlorine 45 mg/L), POAA (80 mg/L), and acidified sodium chlorite (200 mg/L). A metal net was used to hold the leaves in the solutions to make sure that all leaves were at a location below the ultrasonic probe during a treatment. An ultrasonic probe system, Ultrasonic IL 1000-6/2 unit (Ultrasonic Technique—Inlab Ltd., St. Petersburg, Russia) was used in conjunction with the sanitizers. The ultrasonic probe (31 mm in diameter) was placed 50 mm from the bottom of the beaker and was set to a frequency of 21.2 kHz with an input acoustic energy density (AED) of 200 W/L. The treatment time was 2 min for all the experiments, during which a magnetic stir bar was used to agitate the leaves. The treated spinach leaves were dried by a manually operated salad spinner (OXO and Good Grips, Elmira, N.Y., U.S.A.) for 1 min, and then transferred to a sterile stomacher bag for microbiological analysis. The washing solutions were also sampled for microbiological analysis. The AED was measured using a calorimetric method as described by Baumann and others (2005).

Another study was conducted to determine the effect of different concentrations of ASC solution in combination with ultrasound on reduction of *E. coli* O157 : H7 inoculated onto spinach leaf surfaces. The ASC solutions were tested at concentrations of 100, 200, 300, 400, and 500 mg/L with an AED fixed at 200 W/L, and distilled water was used as the control.

A third test compared the effect of different input AED of ultrasonic treatment with 200 mg/L of ASC on the inactivation of *E. coli* O157 : H7 inoculated on spinach leaf surfaces at input AED of 0, 100, 200, 300, 400, and 500 W/L.

A final study was performed to determine the effect of inoculation position on the removal of *E. coli* O157 : H7 from spinach leaves. In this test, fresh spinach leaves were spot-inoculated on either the upper side or the under side of each leaf with 100 μL of inoculum per leaf (10^8 *E. coli* O157 : H7 cells), and air-dried for 120 min in a laminar flow biological hood (Labconco Purifier PCR Enclosure, Kansas City, Mich., U.S.A.) before application of a sanitizing treatment. The inoculated samples were treated with 200 mg/L ASC and 21.2 kHz ultrasonication (AED: 0, 200, and 400 W/L).

Microbiological analysis

Twenty-five grams of spinach leaves were macerated in 225 mL 0.1% (w/v) sterile peptone water for 2 min with a stomacher blender (Lab-Blender 400, Cooke Laboratory Products, Alexandria, Va., U.S.A.). The homogenate was filtered through sterile glass wool, serially diluted in peptone water, plated (100 μL in triplicate) on trypticase soy agar (TSA, Difco Laboratories) containing 50 μg/mL nalidixic acid, and incubated at 37 °C for 24 h before enumeration.

Statistical analysis

Three replications for each treatment were performed. The 1st experiment was conducted with a 6 × 2 factorial design with 6 levels of sanitizer treatment used with or without ultrasound; other parameters such as treatment time, sanitizer concentration, ultrasound power setting, and inoculation arrangement were held constant. The 2nd and 3rd experiments were run as single factor designs using concentration, treatment time, and AED as the single

factor, respectively. The last experiment was conducted as a 2 × 3 factorial design with 2 inoculation options and 3 AEDs. For the different experiments, treatment effects including different sanitizers, treatment times, ASC concentrations, input AEDs, and leaf inoculation positions were compared using the GLM procedure of SAS (SAS Inst. Inc., Cary, N.C., U.S.A.). The Fisher's LSD test was used to determine differences among means at $\alpha = 0.05$.

Results and Discussion

Effects of ultrasonication in combination with sanitizers on reduction of *E. coli* O157 : H7 population on spinach surfaces

The population reductions of *E. coli* O157 : H7 spot-inoculated on the upper surface (smooth surface) of spinach by ultrasound and sanitizer combined treatments are presented in Table 1. Among the sanitizer only washes, the treatments with ASC (200 mg/L) for 2 min reduced *E. coli* population by 2.1-log over that of water wash, while the reductions from other sanitizers were about 1 log cycle.

The application of ultrasound to a sanitizer wash treatment significantly ($P < 0.05$) increased *E. coli* O157 : H7 count reduction compared with a sanitizer only treatment. The additional reduction due to ultrasonication was in the range of 0.7 to 1.1 log cycle, with the highest additional reduction observed in the water wash + ultrasound treatment and the lowest in the Tsunami-100 + ultrasound treatment. The highest *E. coli* count reduction over nontreated spinach samples was 4 log cycles that was achieved in the ASC and ultrasound combined treatment. The *E. coli* O157 : H7 population reduction by ASC was significantly ($P < 0.05$) greater than other sanitizers. There was no significant ($P < 0.05$) difference among chlorinated water, AEW, and Tsunami-100 treatments.

The enhanced reduction in microbial population from produce surfaces by ultrasound may stem from the cavitation activities. When ultrasonic waves pass through a liquid, millions of microscopic cavities are produced which go through cycles of growth and contraction, ending with an implosion (Feng and Yang 2005). The collapse of the cavities generates localized high shear and waterjets pointing at produce surfaces, which may help to remove or dislodge cells from the produce. Ultrasound is also known to increase interface mass transfer by decreasing the thickness of boundary layers. This will help to maintain the concentration of a sanitizer on the produce surface at a level close to that in the bulk solution in a washing tank, allowing an improved inactivation of microorganisms on the produce surface.

Some previous studies have reported a less effective reduction of natural flora or human pathogen population from produce surfaces when ultrasound was applied to a produce wash (Ajlouni and others 2006; Huang and others 2006). It might be caused by the ultrasonication system and/or the operational procedure used in those disinfect treatments. There are a few key factors that have to be considered when applying ultrasound to a produce wash operation. For instance, dissolved gas in a washing solution is known to decrease the cavitation activity in a cleaning operation (Awad 2009). Therefore, degassing is essential for any ultrasonic cleaning applications. Moreover, the acoustic field distribution in an ultrasonic treatment chamber or tank is not uniform, mainly due to a standing wave formation. The nonuniform ultrasound field distribution and hence the nonuniform cavitation will result in variations in microbial inactivation activities at different locations in a washing tank. Consequently, during a wash treatment, those produce leaves that have received a good dose of ultrasound treatment and thus have a low microbial count would be easily cross-contaminated by neighboring leaves that have received less ultrasound treatment and hence have a high microbial population due to an un-even acoustic field distribution. A good understanding of the underlining principles of power ultrasound, as well as a good design in wash system and operation procedure is indispensable for fully unleashing the power of ultrasound in produce decontamination applications.

The recovery of *E. coli* O157 : H7 cells in the washing solutions after a treatment is shown in Table 2. With the exception of the tap water wash, the population of *E. coli* O157 : H7 in other sanitizer solutions was below the detectable level. The population of *E. coli* O157 : H7 recovered from the water wash solution without an ultrasonic treatment was significantly less than that with an ultrasonic treatment. It is thus evidenced that an ultrasound only treatment at AED of 200 W/L for 2 min only helped to remove *E. coli* cells from produce surface and did not kill the cells. It has been reported that inactivation of *E. coli* in drinking water by ultrasound alone at an AED of 900 W/L yielded 1.2 log inactivation in 2 min (Wong 2002). At a lower AED of 180 W/L, less than 1 log inactivation of *E. coli* was achieved within the first 20 min of sonication, but significant inactivation (greater than 7.5 log) was achieved from 30 to 60 min. To effectively inactivate *E. coli* cells suspended in a liquid, a higher AED or longer treatment time may be required (Ugarte and others 2007).

Effects of ASC concentration on reduction of *E. coli* O157 : H7 population on spinach surfaces

The ASC was used for evaluating the effect of sanitizer concentration on *E. coli* population reduction during an ultrasound treatment. It can be seen from Figure 1 that the *E. coli* count reduction for an ASC and ultrasound combined treatment increased significantly when the concentration of ASC increased from 0 mg/L to 300 mg/L ($P < 0.05$). However, there was no significant difference

Table 1—The reduction of *E. coli* O157 : H7 inoculated on the surface of spinach with ultrasound in combination with selected sanitizers for 2 min.

	Population reduction (log CFU/g sample)[a]	
Treatment	Sanitizer	Ultrasound (200 W/L) + sanitizer
H_2O	1.0 ± 0.4a[b]	2.1 ± 0.4b
Chlorinated water (200 mg/L)	2.0 ± 0.8b	3.1 ± 0.9c
AEW (80 mg/L)	2.2 ± 0.9b	3.1 ± 1.1c
POAA (80 mg/L)	2.2 ± 1.3b	2.9 ± 1.3c
ASC (200 mg/L)	3.1 ± 0.6c	4.0 ± 0.8d

[a]Initial counts of *E. coli* O157 : H7 on the spinach were 7.1 ± 0.8 log CFU/g sample (mean ± standard deviation).
[b]Data followed by different letters in the same column are significantly ($P < 0.05$) different among the treatments (LSD = 0.559, df = 81).

Table 2—The recovery of *E. coli* O157 : H7 from the washing solution.

	Population (log CFU/mL washing solution)	
Treatment	Sanitizer	Ultrasound (200 W/L) + sanitizer
H_2O	2.6 ± 1.0a[a]	3.2 ± 0.9b
Chlorinated water (200 mg/L)	NDc[b]	NDc
AEW (80 mg/L)	NDc	NDc
POAA (80 mg/L)	NDc	NDc
ASC (200 mg/L)	NDc	NDc

[a]Data followed by different letters in the same column are significantly ($P < 0.05$) different among the treatments (LSD = 0.319, df = 81).
[b]ND = not detectable.

in log reduction when the concentration of ASC was between 300 and 500 mg/L ($P < 0.05$). As a result, an ASC concentration of > 300 mg/L may not be needed for a produce sanitation operation. Cruz and others (2006) also reported that there was no obvious difference in the reduction of total coliforms on shredded carrots when the ASC concentration was at 250 and 500 mg/L.

Effects of sonication time and power density on removal of *E. coli* O157 : H7 from spinach leaves

Figure 2 shows *E. coli* population reductions for different ultrasound treatment times when the ASC concentration was 200 mg/L. A significant increase in log reduction over time can be observed, except for the data at 0.5 and 1 min. The survival count reduction for treatment time of 0.5 and 1 min also increased with time but the increase was not significant. Increasing ultrasound treatment time would thus result in a higher *E. coli* reduction from spinach surfaces, provided the quality of the spinach remains acceptable. There should be an optimal sonication time at which a maximal log reduction can be achieved without compromising the produce quality. A similar report was published by Huang and others (2006) with an observation that the reduction of *E. coli* O157 : H7 inoculated on lettuce by an ultrasound and chlorine dioxide (40 mg/L) combined treatment was enhanced when the treatment time increased from 3 to 6 min.

As cavitation activities are related to acoustic energy dissipation, the effect of acoustic energy consumed during an ultrasonic treatment on *E. coli* population reduction was evaluated. Generally, increasing acoustic energy density (AED) led to an increase in microbial count reduction (Figure 3). A close look at Figure 3 revealed that, the log reduction of *E. coli* O157 : H7 with an AED of 300 W/L was significantly higher than that from an AED of 100 W/L or 200 W/L, but was less than that of 500 W/L ($P < 0.05$). There was no significant difference between 300 and 400 W/L treatments ($P < 0.05$). Interestingly, the 100 W/L treatment was significantly less effective compared to the nonultrasound treatment ($P < 0.05$). The ultrasound treatment at the low AED of 100 W/L may have helped the break-up of cell clumps that produced an increased *E. coli* count in the subsequent plating counting.

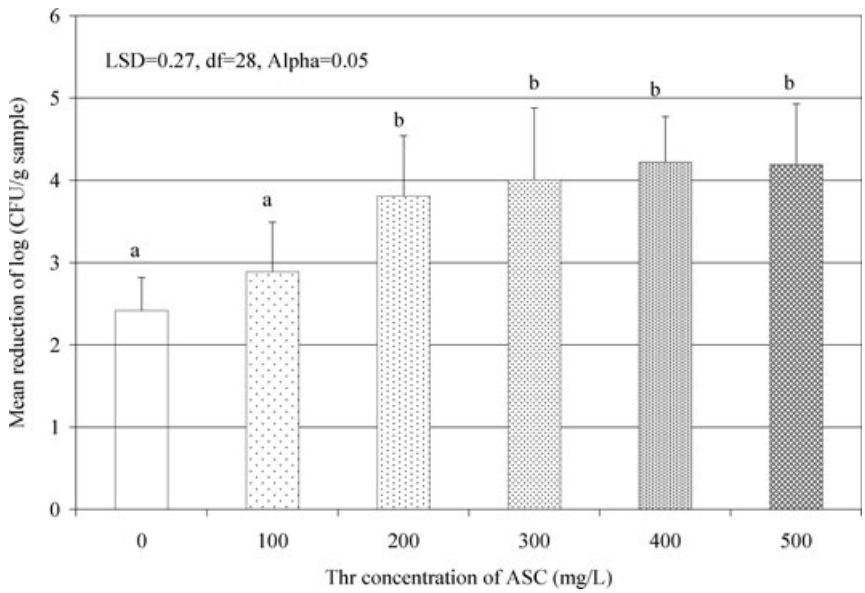

Figure 1—The effect of ASC concentration on the inactivation of *E. coli* O157 : H7 inoculated on spinach surface. Treatment time = 2 min; AED = 200 W/L.

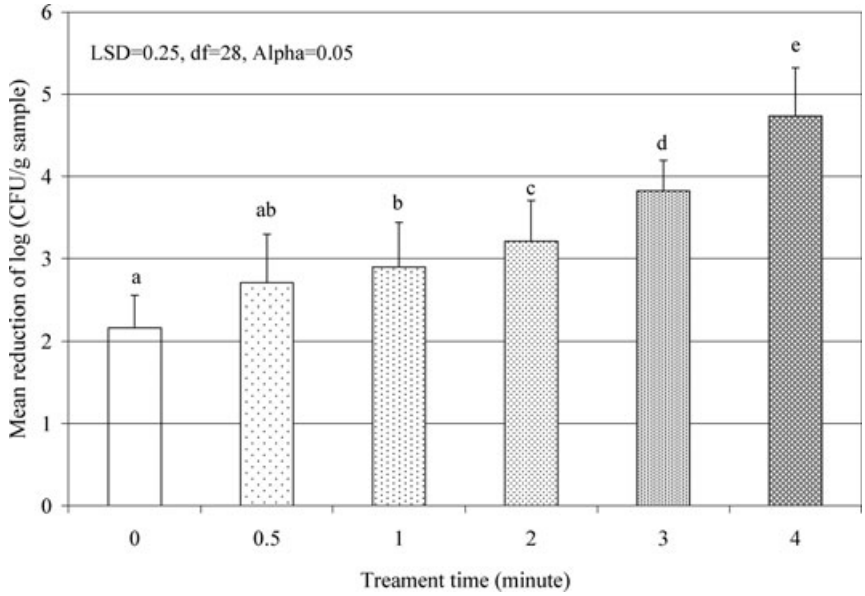

Figure 2—The effect of ultrasound treatment time on the inactivation of *E. coli* O157 : H7 inoculated on spinach surface. ASC concentration = 200 mg/L; AED = 200 W/L.

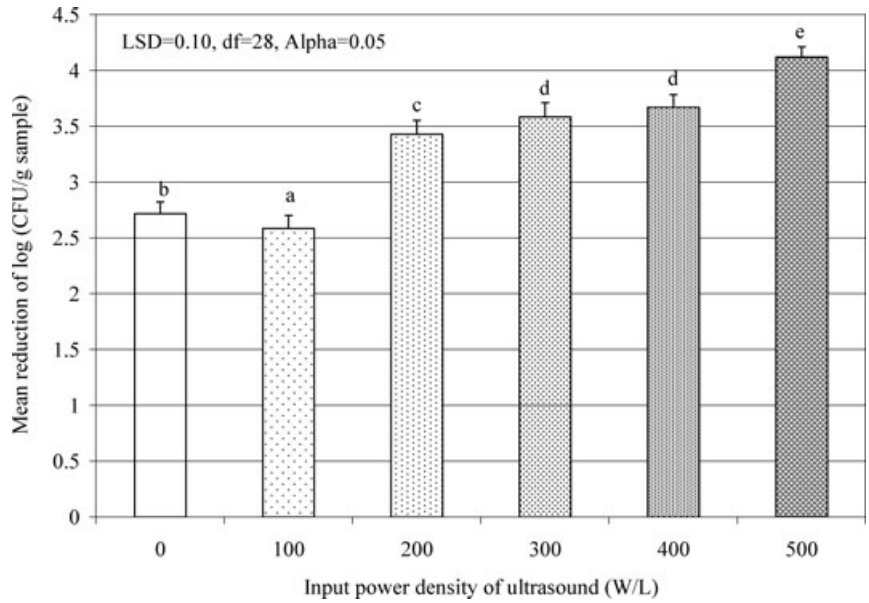

Figure 3—The effect of AED on the inactivation of *E. coli* O157:H7 inoculated on spinach surface. Treatment time = 2 min; ASC concentration = 200 mg/L.

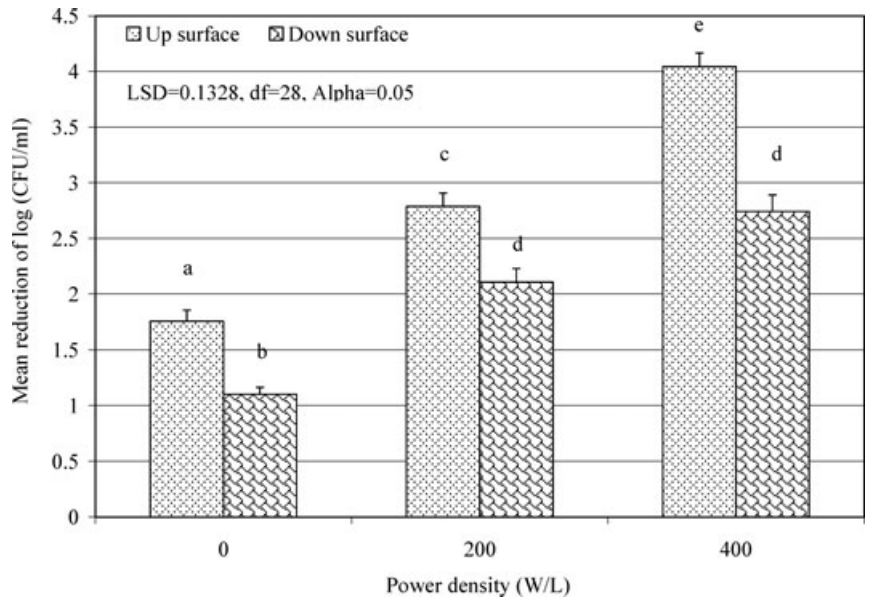

Figure 4—The effect of inoculation position on reduction of *E. coli* O157:H7 from surface of spinach under different AED. ASC concentration = 200 mg/L; treatment time = 2 min.

Effects of inoculation location on removal of *E. coli* O157:H7 from spinach leaves

The effect of inoculation location on the resistance of *E. coli* O157:H7 against a washing treatment was investigated. The results (Figure 4) showed that the treatments (AED: 0, 200, and 400 W/L) were significantly more effective for removal of *E. coli* O157:H7 inoculated on the upper smooth surface of spinach leaves than that on the under-side surfaces ($P < 0.05$). The rougher underside surfaces of the spinach seemed to have provided protection to the microbes harbored on the surface and hence reduced the efficacy of the treatment. A good understanding of the produce surface characteristics and their impact on the retention and removal of bacteria may help to develop more effective means for surface decontamination. With a newly developed surface roughness measurement method, Wang and others (2009) investigated the effect of surface roughness on removal of *E. coli* O157:H7 from fruit and metal surfaces treated with sanitizers or sonication. A linear increase of residual bacteria population with increased surface roughness was observed. To effectively remove attached bacterial cells from produce with a rough surface, an extended treatment time and more effective decontamination methods would have to be used.

Conclusions

The ultrasound treatment with an AED of 200 W/L significantly enhanced the removal of *E. coli* O157:H7 cells from spinach surfaces in all treatments. An increase in the concentration of ASC enhanced the efficacy of ASC and ultrasonication combined treatments. Increasing the treatment time of ultrasonication from 0 to 4 min and AED from 0 to 500 W/L both significantly improved the efficacy in the survival count reduction ($P < 0.05$). In addition, *E. coli* O157:H7 inoculated on the down surface of spinach leaves (rough side) was more resistant to the combined treatment of ASC and ultrasonication.

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