Final Report
on

Reducing Microbiological Safety Risk on Blueberries through Innovative Washing Technologies

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Research Proposal

By

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Objective

The objective of this study was to determine the efficacy of ozonated water, FIT® produce wash, and EO water in killing or reducing *E. coli* O157:H7 attached to blueberries compared to common bleach solution and tap water.

Justification

Center for Disease Control and Prevention documented several outbreaks of foodborne infections associated with fruit salad, mixed fruits, strawberries, and blueberries in 2006 (CDC, 2006). Although blueberries are rarely implicated with outbreaks of foodborne illnesses, they are susceptible to microbial contamination like other types of fresh produce at any point during production, harvesting, transportation, and processing (FDA, 2008). Potential microbial contamination of blueberries includes untreated manure used for fertilization, contaminated irrigation water, infected workers, the presence of animals, unclean containers and tools used in harvesting, packing, transporting, or processing (FDA, 2008; Rodas et al., 2009).

Fresh produce is generally washed or sprayed with chlorinated water containing 50-200 ppm total active chlorine to reduce microbial contamination. At home or in food service kitchens, fruits and vegetables are usually washed with water. However, washing with chlorinated water may not be effective in reducing microorganisms on fruits and vegetables at high concentrations (Wu and Kim, 2007). Likewise, washing steps generally practiced at home or in restaurant kitchens have been shown to be ineffective in removing pathogenic bacteria from produce (Parish et al., 2003).

Several alternative home-use sanitizers such as ozonated water, FIT® Fruit & Vegetable Wash (HealthPro Brands, Inc., Cincinnati, OH), and electrolyzed oxidizing (EO) water generated through innovative technologies have shown promises to be effective in killing bacteria. Ozonated water has been proven to be effective in killing foodborne pathogens. Kim et al. (2006) reported that ozonated water (5 mg/l ozone) was capable of reducing various pathogenic bacteria by 99% within 1-min in vitro treatment and was as effective as 100 ppm chlorinated

water in reducing total microorganisms or coliform bacteria on fresh-cut lettuce. The native bacterial population on iceberg lettuce was reduced by 1.4 log CFU/g after treatment with ozonated water (5 mg/l ozone) for 5 min (Koseki and Isobe, 2006). Ozonated water (4 mg/l ozone) was as effective as 100 ppm chlorine solution in reducing the populations of mesophilic and psychrotrophic bacteria on fresh-cut lettuce (Akbas and Olmez, 2007). FIT® prototype wash is marketed in different products such as FIT® Fruit & Vegetable Wash, FIT® Antibacterial liquid and FIT® Antibacterial powder. In vitro tests, treatments of *Salmonella* and *Escherichia coli* O157:H7 with FIT® Antibacterial liquid or powder for 30 sec reduced the number of the pathogens by more than 6.0 log CFU/ml (Park et al., 2008). Extensive research conducted at the University of Georgia has demonstrated the efficacy of EO water in inactivating *E. coli* O157:H7, *Listeria monocytogenes, Salmonella, and Bacillus cereus* (Kim et al., 2000; Venkitanarayanan et al., 1999). Information on the efficacy of these noble home-use sanitizers in killing or reducing pathogens on fresh fruits is limited.

Methodologies

Preparation of inoculum

A mixture of five nalidixic acid-adapted *E. coli* O157:H7 strains was used as inoculum in this study. The five strains included CDC-658 (human feces, cantaloupe-associated outbreak), E-19 (calf feces isolate), F-4546 (human feces, alfalfa sprout-associated outbreak), H-1730 (human feces, lettuce-associated outbreak), and LJH-557 (apple cider isolate). Stock cultures of all strain were maintained on tryptic soy agar supplemented with 50 µg/ml nalidixic acid (TSAN) at 4ºC, occasionally (not more than 4 weeks) recultured in tryptic soy broth supplemented with 50 µg/ml nalidixic acid (TSBN) at 37ºC for 24 h, and transferred to new TSAN slants. The mixture of five bacterial strains was prepared according to the procedures of Pangloli et al. (2009) with modifications. After centrifugation at 2000 x *g* at 22ºC for 15 min, the supernatant was discarded and the cells were resuspended in 10 ml of 0.1% peptone water. Equal volumes (5 ml) of each strain were combined to obtain a cocktail inoculum containing cells approximately 8-9 log CFU/ml.

Preparation and inoculation of blueberries

Blueberries were purchased from a local grocery, stored at 4ºC for a maximum 2 days before used, and tempered to 22ºC before inoculation. Each blueberry sample consisted of 6 berries (ca. 10 $\pm$ 1 g). Blueberries were placed stem scar end up on the holes of 1-ml pipette tip trays. Each blueberry was spot inoculated with 10 µl (60 µl/sample) of mix culture (ca. 8-9 log CFU/ml). The inoculum suspension was applied with pipetter in a small drop onto 4-6 locations on the skin and stem scar of each blueberry. The inoculated blueberries were air-dried in a biosafety hood at 22ºC for 2 h to allow attachment of the pathogen. Each sample (6 inoculated blueberries) was then placed into a 24-oz Whirl-Pak bag, which was closed and stored at 4ºC for 22 $\pm$ 2 h to simulate handling of blueberries at home prior to consumption.

Preparation of sanitizers

Ozonated water was generated from tap water using a Lotus Sanitizing System (model LSR100, Tersano International SRL, Buffalo, NY) with spray bottle attachment according to the manufacturer's instructions with modification. Tap water for generating ozonated water was at room temperature (22ºC). At least three cycle batches were prepared to stabilize the machine before collecting ozonated water with relatively stable pH and oxidation-reduction potential (ORP) values to treat blueberries. Ozonated water was used within 15 min after produced before the ozone started to convert to oxygen according to the manufacturer's instruction. Care was

taken throughout the experiment to avoid splashing or shaking the ozonated water which would cause the ozone to lose. The pH and ORP values of ozonated water were measured using a dual channel ACCUMET meter (model AR50, Fisher Scientific). Ozone levels were determined by the Indigo method with high-range ozone Accu Vac Ampuls (Hach Co., Loveland, CO) using a portable colorimeter (model DR/890, Hach Co).

FIT solution was prepared by diluting 11.4 ml of liquid FIT® Antibacterial Fruit & Vegetable Wash containing levulinic acid (HealthPro Brands Inc., Cincinnati, OH) in 1 litter of tap water according to the manufacturer's instructions. The FIT solution was used to treat blueberries within 2 h after preparation. The pH and ORP values of FIT solution were determined according to the procedure described above.

Electrolyzed oxidizing (EO) water was generated by electrolyzing NaCl solution (0.075%) using a home-use generator (model BTM-3000, Bion-Tech Co., Ltd., Seoul Korea). Salt solution was electrolyzed for 20 min according to manufacturer's instructions to produce acidic EO water with ca. 30 mg/L free chlorine. The EO water was kept in a screw-cap bottle and used within 2 h. The pH and ORP values of EO water were determined according to the procedure described previously. Free chlorine levels were determined using the DPD-FEAS method (Hach Co., Loveland, CO).

Regular bleach (Everyday Living, Inter-American Products, Cincinnati, OH) containing ca. 6.0% sodium hypochlorite was purchased from a local supermarket. Bleach solution was prepared by diluting 1.7 ml of regular bleach in 998.3 ml deionized water to obtain solution of ca. 100 mg/l free chlorine. Bleach solution was kept in screw-cap bottle until used within 2 h. The pH, ORP, and free chlorine levels of bleach solution were determined according to the procedures described above.

Procedures for treating blueberries

The inoculated blueberries in Whirl-Pak bags were tempered to room temperature (22ºC) before treating with sanitizers. Each sample (6 blueberries) in a 24-oz Whirl-Pak bag was added and treated with 50 ml of tap water (control), ozonated water, FIT solution, EO water, or bleach solution. The bag containing blueberries and treatment solution was immediately closed and placed in a metal basket which was sit on a platform shaker (model Classic C10, New Brunswick, NJ) and shaken at 150 rpm for 1, 3, or 5 min. Placing bags with blueberries in metal basket on the shaker helped ensure berries moved freely during shaking to facilitate washing bacterial cells from the surfaces of blueberries. At the end of each treatment time, the wash solution was decanted into a 24-oz sterile Whirl-Pak bag and the blueberries were immediately added with 25 ml of Dey-Engley (DE) broth to stop reaction and subjected to microbiological analysis. Wash solution (25 ml) collected separately in an 18-oz Whirl-Pak bag was combined with 25 ml of double DE (dDE) broth and subject to microbiological analysis.

Microbiological analyses

Populations of *E. coli* O157:H7 on blueberries before and after treatment and in wash solutions after treatment were determined. The blueberries with DE broth in Whirl-Pak bags were shaken at 150 rpm for 2 min on a platform shaker, while wash solutions with dDE broth were pummeled at normal speed for 2 min in a stomacher (Stomacher 80, Seward, London, UK). DE broth and wash solution with dDE broth were serially diluted in 0.1% peptone water (if necessary) and plated in duplicate onto sorbitol MacConkey agar containing 50 µg/ml nalidixic acid and 0.1% sodium pyruvate (SMACNP) and tryptic soy agar supplemented with 50 µg/ml nalidixic acid and 0.1% sodium pyruvate (TSANP) using a spiral plater (WSAP 2, Microbiology International, Frederick, MD) to enumerate populations of *E. coli* O157:H7. Undiluted samples

were spread plated onto SMACNP and TSANP in quadruplicate to enumerate the pathogen in samples with very low populations. SMACNP and TSANP plates were incubated at 37ºC for 24 h before counting colonies by a Colyte Colony Counter (model 7510/SYN, Microbiology International).

To detect the presence of low numbers of survivors that would not be detected by direct plating, 25 ml of double strength modified tryptic soy broth supplemented with 50 µg/ml nalidixic acid and 0.1% sodium pyruvate (dmTSBNP) was added to each bag containing blueberries and DE broth. For bags containing wash solutions with dDE broth, 50 ml of dmTSBNP was added to each bag. The enrichment broth samples were incubated at 37ºC for 24 h. When counts for the respective samples were negative by direct plating, the enrichment broth was streaked onto SMACNP and TSANP plates to detect the presence of the pathogen at low number.

Presumptive positive colonies (10 to 20 per treatment) were randomly selected from SMACNP and TSANP plates for confirmation by biochemical test using lactose and 4-methylumbelliferyl-β-D-glucuronide (MUG) and serological test using the O157 spot dry agglutination test kit (Oxoid). Colonies were picked up by sterile 2.1-mm diameter of wooden applicators and spot inoculated onto MacConkey agar plates supplemented with MUG (0.1 g/l). The plates were incubated at 4ºC for 24 h. Colonies positive for lactose (pink color) and negative for MUG (non fluorescent) were subjected to the O157 agglutination test for final confirmation.

Data analysis

Experiments were replicated three times and each replicate consisted of two samples for each treatment. Data were subjected to analysis of variance with a randomized block design, block on replication. Statistical analysis was performed with the SAS Mixed Procedures using SAS Software Release 8.2 (SAS Institute Inc., Cary, NC). Significant differences among means were determined by the least square means method with *P* value for differences (PDIFF) option (Saxton, 1998).

Results

Sanitizer properties

The properties of sanitizers used to treat blueberries are presented in Table 1. Tap and ozonated water had neutral or near neutral pH, FIT solution and EO water were in acidic pH, and bleach solution had alkaline pH (9.6). Ozonated and EO water had relatively high ORP values (1009 – 1163 mV) which could be an important factor in inactivating bacterial cells. The ORP values of tap water, FIT solution, and bleach solution (704 – 786 mV) were in the ranges where most aerobic bacteria can grow (Su et. al., 2007). Tap water also had trace amounts of chlorine compared to EO water and bleach solution, while ozonated water had ozone level of 1.5 mg/l.

Table 1. Properties of sanitizers used to treat blueberries

Sanitizer type	pH	ORP (mV)	Free chlorine (mg/l)	Ozone (mg/l)
Tap water	7.30	720	1.0	N/A
Ozonated water	7.09	1009	N/A	1.5
FIT solution	3.16	704	N/A	N/A
EO water	2.63	1163	31.1	N/A
Bleach solution	9.64	786	105.8	N/A

Reduction of pathogen on blueberries

E. coli O157:H7 counts were averaged from non-selective agar media (TSANP) and selective media (SMACNP). The purpose of using non-selective media was to facilitate resuscitation of injured cells due to desiccation or exposure to sanitizers. Selective media helped eliminate background microorganisms (non-target microorganisms) that might not be resistant to nalidixic acid.

The number of *E. coli* O157:H7 recovered and reduced before and after treatment of blueberries is presented in Table 2. The number of the pathogen recovered from untreated blueberries was approximately 5 log CFU/g. Thus, the number of cells died during air-drying under laminar hood for 2 h and storage at 4ºC for 20 – 24 h was nearly 2 log CFU/g.

The number of *E. coli* O157:H7 cells reduced after treatment with sanitizers varied with the type of sanitizers and treatment time (Table 2). Based on the pathogen reduction, the most effective sanitizer in inactivating the pathogen on blueberries was bleach solution (ca. 100 mg/l free chlorine), which reduced *E. coli* O157:H7 by 4.4 – 4.8 log CFU/g followed by EO water (3.9 – 4.4 log CFU/g), FIT solution (3.3 – 4.6 log CFU/g), ozonated water (2.3 – 3.5 log CFU/g),

Table 2. Population of *E. coli* O157:H7 recovered from blueberries and solutions after treatment

Treatment [a]	Treatment time (min)	On blueberries [b]		Treatment solutions (log CFU/ml)
		Recovery (log CFU/g)	Reduction (log CFU/g)	
Untreated blueberries		4.98 A		
Tap water	1	3.11 B	1.87 I	3.60
	3	2.65 BC	2.33 HI	3.72
	5	2.32 CD	2.66 GH	3.80
Ozonated water	1	2.70 BC	2.28 HI	1.42
	3	1.93 DE	3.05 FG	0.93
	5	1.49 EF	3.49 EF	0.95

FIT solution	1	1.64 E	3.34 F	3/6 [c]
	3	1.02 FGH	3.96 CDE	ND [d]
	5	0.35 IJ	4.63 AB	ND
EO water	1	1.08 FG	3.90 DE	ND
	3	0.79 GHI	4.20 BCD	ND
	5	0.54 IJ	4.44 AB	ND
Bleach solutions	1	0.57 HIJ	4.41 ABC	1/6 [c]
	3	0.24 J	4.74 A	ND
	5	0.17 J	4.81 A	ND

[a] Blueberry samples were treated in 50 ml of tap water, ozonated water, FIT solutions containing levulinic acid, electrolyzed oxidizing (EO) water (ca. 30 ppm free chlorine), or bleach solutions (ca. 100 ppm free chlorine) with continuous shaking (150 rpm).
[b] Mean values not followed by the same letter are significantly different ($P \leq 0.05$).
[c] Not detected by direct plating (detection limit, 0.3 log CFU/ml), but three or one of six samples were positive by enrichment.
[d] ND, not detected by direct plating and enrichment.

and tap water (1.9 – 2.7 log CFU/g). Increasing treatment time from 1 to 5 min significantly increased the reduction of the pathogen in most cases (Table 2) except bleach solution. Five min treatment with FIT and EO water achieved 4.6- and 4.4-log reduction, respectively, which were not significantly different from the reductions achieved by washing with bleach solution for 1, 3, and 5 min (4.4, 4.7, and 4.8 log CFU/g). Treatment of blueberries in ozonated water for 3 to 5 min inactivated significant number of *E. coli* O157:H7 (3.1 – 3.5 log CFU/g). However, the reductions were lower than those achieved by FIT, EO water, and bleach solution for each respective treatment time.

EO water was the only solution completely inactivated *E. coli* O157:H7 in the treatment solution after treatment and hence can eliminate cross-contamination during blueberry washing. The pathogen was not detected in solutions by direct plating after treatment of blueberries in FIT and bleach solutions for 1 min; however, three and one of the six samples were positive for the pathogen by enrichment, respectively. Increasing treatment time in FIT and bleach solution to 3 or 5 min also achieved complete elimination of *E. coli* O157:H7 in solution. After treatment, ozonated water still had 0.93 to 1.42 log CFU/ml survival *E. coli* O157:H7, whereas tap water had 3.6 to 3.8 log CFU/ml survivors.

Conclusions

Application of bleach or other home-use sanitizers can reduce the risk of *E. coli* O157:H7 that may present on blueberries. Based on the inactivation of the pathogen on blueberries and in solution, the best methods to reduce microbiological risk on blueberries include treatment with FIT solution for 5 min, with EO water, or with bleach solution for 3 and 5 min. The choice of sanitizers will depend on the available resources and time constrain.

Impact Statement

Information generated from this study demonstrated consumer friendly sanitizers are available for consumers to use at home to wash blueberries before consumption to ensure food safety.

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