

DUAL-PHASIC INACTIVATION OF *ESCHERICHIA COLI* O157:H7 WITH PEROXYACETIC ACID, ACIDIC ELECTROLYZED WATER AND CHLORINE ON CANTALOUPE AND FRESH-CUT APPLES

HUA WANG¹, HAO FENG^{1,3} and YAGUANG LUO²

¹*Department of Food Science and Human Nutrition
University of Illinois at Urbana-Champaign
Urbana, IL 61801*

²*Produce Quality and Safety Laboratory
USDA-ARS, Baltimore Avenue
Beltsville, MD 20705*

Accepted for Publication May 19, 2006

ABSTRACT

The effects of peroxyacetic acid (POAA), acidic electrolyzed water (AEW) and chlorine on inactivation of Escherichia coli O157:H7 on fresh-cut apples and cantaloupe rinds were investigated. Apple cylinders were dip-inoculated with E. coli O157:H7 and treated with sterilized water (control), chlorine, AEW or POAA for up to 8 min. Cantaloupe cylinders were spot-inoculated with E. coli O157:H7 to the rind and treated with sterilized water, AEW or POAA for up to 15 min. All sanitizer treatments showed a significantly ($P < 0.05$) higher inactivation than the control. The residual counts of E. coli O157:H7 on both fruits exhibited a dual-phasic reduction behavior, with a fast inactivation (D values: 0.8–5.0 min) in the first minute (phase I) of treatments followed by a much slower inactivation (D values: 14.6–59.8 min) in the remaining time (phase II). The dual-phasic inactivation seems to be related to fruit surface topography that determines the bacterial distribution.

INTRODUCTION

Fresh and fresh-cut produce have been implicated in several outbreaks of diseases caused by enterohemorrhagic *Escherichia coli* O157:H7 (DeWaal and Barlow 2002; Venkitanarayanan *et al.* 2002). *E. coli* O157:H7, a specific serotype that produces toxin and causes hemorrhagic colitis, is carried by ruminants in their gastrointestinal tracts and is shed in feces. The presence of

³ Corresponding author. TEL: 217-244-2571; FAX: 217-333-9329, EMAIL: haofeng@uiuc.edu

feces, irrigation water, insects, improperly composed manure, wild and domestic animals, as well as human handling have been identified as possible sources for preharvest contamination of produce with foodborne pathogens (Beuchat 1996). Bacteria can also be brought into contact with produce during postharvest handling (Burnett and Beuchat 2001). Fresh-cut produce or uncut produce with a rough surface provide favorable environments for microbial growth, and the removal of organisms from such porous and pitted surfaces can be difficult. It has been shown that *E. coli* O157:H7 can survive on wounds (Janes *et al.* 2002) and cut surfaces of apples (Janisiewicz *et al.* 1999; Gunes and Hotchkiss 2002), as well as on cantaloupe and watermelon rinds (Del Rosario and Beuchat 1995). The decontamination of produce with sanitizers is still a challenge to the produce industry as most sanitizers can only reduce the number of pathogenic organisms by less than 2-log cycles in industrial-scale operations (Hegenbart 2002).

Chlorine is the most widely used sanitizer for disinfecting fruits and vegetables in the food industry. However, chlorine depletes rapidly under conditions of high organic loading and can form potentially carcinogenic organochlorine compounds by reacting with trace amounts of organic material. Many new sanitizers such as Tsunami 100 (Ecolab, St Paul, MN) and acidic electrolyzed water (AEW) have thus been studied for use as alternatives to chlorine. Peroxyacetic acid (POAA) is the main active ingredient of Tsunami 100 and its application as a disinfectant for fruits, vegetables, meat and eggs has been approved by the U.S. Food and Drug Administration (FDA) for a maximum concentration of 80 mg/L in wash water (FDA[21CFR173] 2003). The sanitizing power of POAA is not affected by organic load, solution pH or temperature, and it can rapidly break down into water, oxygen and acetic acid. Gonzalez *et al.* (2004) reported that 80 mg/L of POAA was equally effective as 200 mg/L of chlorine for decreasing *E. coli* O157:H7 population on fresh-cut carrots, but had significantly greater efficacy than chlorine when treatments were conducted with simulated process water. AEW has shown a strong bactericidal effect against pathogens and spoilage microorganisms because of a combination of lethal factors including free chlorine (<80 mg/L), low pH (2.3~2.7) and high oxidation reduction potential (ORP) (1130~1160 mV) (Koseki *et al.* 2001, 2004; Wang *et al.* 2004). AEW has been approved as a bactericidal agent for use in restaurants in Japan and is currently used for the commercial sanitization of fertile hatching eggs in the U.S.A. (Russell 2004; Yoshida 2004). Although a bacterium is easily killed when directly exposed to a sanitizer, the inactivation becomes much more difficult when the bacterium is attached to the produce surface. The population reduction in *E. coli* O157:H7, *Listeria monocytogenes* and *Shigella* cell suspensions treated with AEW reached over 8.0 log cfu/mL in 1 min (Kim and Hung 2000). However, less than a 2.7-log-cfu/g reduction was achieved in a 15-min treatment with

AEW when those bacteria were attached to produce surfaces and air-dried for 1 h (Park and Hung 2001) or 7 h (Stan and Daeschel 2003) after inoculation. The hydrophobic cutin, diverse surface morphologies and abrasions on the epidermis may have contributed to reduce the efficacy of a sanitizer on fruits and vegetables (Burnett and Beuchat 2001). Few studies have examined the inactivation behavior of a sanitizer with an emphasis on the effect of surface conditions of produce on the efficacy of microbial inactivation, especially for the inactivation of microbes on fresh-cut produce surfaces or on produce with rough surfaces. There are no documented studies on the inactivation of *E. coli* O157:H7 on surfaces of fresh-cut apples.

The main objective of this study was therefore to assess the reduction of *E. coli* O157:H7 population achieved with POAA, AEW or chlorine on cantaloupes and fresh-cut apples. The two types of produce were selected because their rough and porous surface characteristics provide more challenging conditions under which to evaluate the limitations of sanitizers for the inactivation of *E. coli* O157:H7.

MATERIALS AND METHODS

Preparation of Treatment Solutions

AEW was generated using an AEW generator (ROX-20TA, Hoshizaki, Nagoya, Japan) and collected from the anode outlet of the generator with sterile beakers. The pH and ORP of the AEW were measured with an AR15 pH and ORP meter (Accumet Research, Pittsburgh, PA) and the residual chlorine concentration of AEW was determined with a U.S. Environmental Protection Agency-approved chlorine colorimetric test kit (model PCT-DR, LaMotte Co., Chestertown, MD). POAA (80 mg/L; Tsunami 100, Ecolab) was prepared with reference to the manufacturer's instruction. The concentration of POAA was measured with a POAA test kit provided by the manufacturer. Chlorine solution was prepared with sodium hypochlorite (4–6% NaClO) and the pH was adjusted to 6.5 with 1.0 N HCl. The available chlorine was determined with the chlorine colorimetric test kit. Sterilized deionized water was used as a control.

Inoculum Preparation

A five-strain cocktail of *E. coli* O157:H7 (13B88, apple juice isolate; G5303, apple cider isolate; C7927, apple cider isolate; 204P, pork isolate; EDL933, human feces isolate) was obtained from Purdue University. Cultures were transferred three times to tryptic soy broth (pH 7.3; Difco Lab, Detroit, MI) by loop inoculation at successive 24-h intervals and incubated at 37C.

Twenty-four-hour bacterial cells were harvested by centrifugation ($10,000 \times g$) at 4°C for 10 min. The cell pellets were washed twice in salt peptone (0.85% NaCl, 0.1% Bacto Peptone) and resuspended in 10 mL of 0.1% peptone water. Equal volumes (2 mL) of five cultures were mixed to obtain a 10-mL inoculum containing approximately 10^8 cfu/mL of *E. coli* O157:H7.

Sample Preparation

Golden delicious apples and cantaloupes purchased from local supermarkets were sanitized with UV light for 20 min to inactivate naturally occurring microorganisms on the fruit surfaces. A sterilized brass cork borer (No. 9, 15.5-mm inside diameter) was used to prepare fruit plugs, and a sterile knife was then used to cut apple plugs into cylinders (15.5-mm diameter \times 35-mm length) without skin and was also used to cut cantaloupe plugs into cylinders (15.5-mm diameter \times 35-mm length) with rind. The cylinders were prepared from different locations of the same fruit and from multiple fruits. After preparation, the cylinders were pooled together, and seven cylinders from the pool were randomly selected and used as test samples. Each test sample of one kind of fruit was used for one experiment. The experiments were repeated three times.

Apple cylinders (250 g) without skin were dip-inoculated with the inoculum (2.5 L) described earlier for 15 min and the cantaloupe cylinders (250 g) were spot-inoculated with 20- μ L inoculum on the outer rind. All inoculated samples were air-dried for 1 h in a laminar flow biological hood (Labconco Purifier PCR Enclosure, Kansas City, MO) before treatment.

Treatment Procedures

Each test sample (60 g) of apple and cantaloupe cylinders was submerged in 600-mL treatment solutions of sterilized deionized water, AEW (pH 2.7, 1150-mV ORP and 68–70 mg/L of free chlorine), POAA (80 mg/L) or chlorine (only for apples, pH 6.5, 88 mg/L of free chlorine) at room temperature for up to 15 min with agitation at 240 rpm with an agitator (Thermolyne Cimarec, Dubuque, IA), respectively. The treated samples were removed from the solutions at 0.5, 1, 2, 3, 5, 8 and 15 min and immediately rinsed with Dey/Engley neutralizing broth (Difco Lab) for 5 s to neutralize the residual sanitizer on cylinder surfaces. The samples were then transferred to a sterile stomacher bag for microbiological analysis.

Bacteria Enumeration

Each fruit sample was combined with 50 mL of sterile 0.1% peptone solution in a 400-mL sterile stomacher bag (Fisher Scientific, Inc., Pittsburgh,

PA) and was blended with a Lab-Blender 400 (Cooke Laboratory Products, Alexandria, VA) for 4 min. The homogenate was filtered through sterile glass wool. A 100- μ L sample of each filtrate and its appropriate dilutions were plated in triplicate on Sorbital–MacConkey agar (SMAC, Difco Lab) supplemented with cefixime-tellurite (CT) (Oxoid Ltd, Basingstoke, Hampshire, U.K.). All CT–SMAC plates were incubated at 37C for 24 h. For each plate, two typical *E. coli* O157:H7 colonies were chosen and identified by an *E. coli* O157 latex test (Oxoid, Inc., Ogdensburg, New York, NY).

Scanning Electron Microscopy Examination

Freshly cut fruit surfaces were inoculated with *E. coli* O157:H7 as described earlier. The inoculated samples were air-dried for 1 h before washing with sterilized deionized water or POAA for 5 min. After the washing treatments, the samples were fixed in 2.5% (v/v) glutaraldehyde (E.M. grade) in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 h at refrigeration temperature and rinsed thrice with 0.1 M sodium cacodylate buffer every 10 min. The samples were postfixed in a 1% (v/v) osmium tetroxide (OSO₄) solution for 90 min at room temperature in a biosafety hood in the dark and rinsed with 0.1 M sodium-cacodylate buffer every 10 min. The samples were then dehydrated in a graded series of ethanol solutions (50, 70, 95 and 100%) and dried in a CO₂ critical-point drier (Samdri-DVT-3D, Tousimis Research Corporation, Rockville, MD). The dry samples were then mounted on aluminum stubs coated with a thin layer of gold–palladium by a Dest II TSC sputter coater and examined by environmental scanning electron microscopy (ESEM; Philips XL30 ESEM-FEG, FEI Company, Eindhoven, the Netherlands).

Statistical Analyses

All experiments were conducted in triplicate. Data were analyzed using the Statistical Analysis System software program (SAS Institute, Cary, NC). Fisher's least significant difference test was used to determine differences among means at $\alpha = 0.05$.

RESULTS

Table 1 shows the *E. coli* O157:H7 population reduction on the fresh-cut apples treated with POAA, AEW, chlorine solution or water (control). Samples treated with POAA, AEW or chlorine exhibited a significantly ($P < 0.05$) higher reduction in *E. coli* O157:H7 population than the control at all six sampling times. The POAA treatment achieved the highest reduction (0.76 log) in survival count of *E. coli* O157:H7 in the first 30 s compared with

TABLE 1.
ESCHERICHIA COLI O157:H7 POPULATION REDUCTION ON INOCULATED FRESH-CUT
 APPLES AT DIFFERENT TREATMENT TIMES

Treating time (min)	Bacterial population reduction (log cfu/cm ²)*			
	Water	POAA	AEW	Chlorine
0.5	0.17 ± 0.01 a† x‡	0.76 ± 0.31 b x	0.52 ± 0.09 b x	0.60 ± 0.18 b x
1	0.20 ± 0.04 a x	1.21 ± 0.08 b y	0.61 ± 0.03 c x	0.64 ± 0.08 c x
2	0.21 ± 0.05 a x	1.23 ± 0.10 b y	0.66 ± 0.20 c x	0.75 ± 0.09 c xy
3	0.30 ± 0.05 a x	1.42 ± 0.13 b y	0.77 ± 0.11 c xy	0.85 ± 0.05 c y
5	0.37 ± 0.17 a xy	1.51 ± 0.09 b y	0.88 ± 0.24 c y	0.99 ± 0.11 c y
8	0.38 ± 0.00 a xy	1.53 ± 0.31 b y	1.08 ± 0.02 c y	1.05 ± 0.08 c y

* Initial counts of *E. coli* O157:H7 on fresh-cut apples were 6.28 ± 0.02, 6.23 ± 0.06, 6.40 ± 0.21 and 6.36 ± 0.19 log cfu/cm² tissue (mean ± SD), respectively.

† Data followed by first different letters in each row are significantly different among the treatments at each sampling time by a least significant difference (LSD) of $P < 0.05$.

‡ Data followed by second different letters in each column are significantly different among the treatment times of one treatment by an LSD of $P < 0.05$.

POAA, peroxyacetic acid; AEW, acidic electrolyzed water.

other treatments. At 1 min, the bacterial population reduction from the POAA treatment increased to 1.2 log, which was significantly higher than ($P < 0.05$) that from other two sanitizers (AEW, 0.61 log and chlorine, 0.64 log). After 1 min, a slowdown in inactivation can be observed for all three sanitizers, but the POAA treatment was still significantly higher in bacterial population reduction than the AEW and chlorine treatments.

The *E. coli* O157:H7 population reduction on cantaloupe surfaces treated with POAA, AEW or water (control) is shown in Table 2. An increase in the bacterial population reduction with treatment time was observed for all treatments. The POAA and AEW treatments showed a significantly ($P < 0.05$) higher *E. coli* O157:H7 survival count reduction than the control at the seven sampling times (0.5, 1, 2, 3, 5, 8 and 15 min). The POAA treatment resulted in a 0.77-log reduction at 1 min, which was significantly higher ($P < 0.05$) than that by the AEW treatment (0.45 log). After the first minute, a significant difference in the bacterial population reduction between the POAA and AEW treatments was not found at any sampling time except at 3 min. After the maximum treatment time (15 min), the accumulated bacterial population reduction for the POAA and AEW treatments reached 1.15 and 1.10 log, respectively.

It is shown from Tables 1 and 2 that the sanitizing treatments seemed to be more effective in removing *E. coli* O157:H7 cells from fresh-cut apple and cantaloupe surfaces in the beginning of a treatment. To examine the inactivation behavior of the sanitizers, the data were plotted in Figs. 1 and 2,

TABLE 2.
 ESCHERICHIA COLI O157:H7 POPULATION REDUCTION ON INOCULATED CANTALOUPE SURFACES AT DIFFERENT TREATMENT TIMES

Treating time (min)	Bacterial population reduction (log cfu/cm ²)*		
	Water	POAA	AEW
0.5	0.00 ± 0.00 a† x‡	0.77 ± 0.04 b x	0.44 ± 0.11 c x
1	0.22 ± 0.00 a y	0.77 ± 0.00 b x	0.45 ± 0.07 c x
2	0.14 ± 0.11 a y	0.89 ± 0.09 b x	0.71 ± 0.06 b y
3	0.24 ± 0.00 a y	0.95 ± 0.22 b xy	0.63 ± 0.11 c xy
5	0.26 ± 0.02 a y	1.00 ± 0.01 b xy	0.88 ± 0.01 b y
8	0.25 ± 0.06 a y	0.97 ± 0.03 b xy	0.86 ± 0.11 b y
15	0.43 ± 0.06 a z	1.15 ± 0.02 b xy	1.10 ± 0.13 b y

* Data followed by first different letters in each row are significantly different among the treatments at each sampling time by a least significant difference (LSD) of $P < 0.05$.
 † Data followed by second different letters in each column are significantly different among the treating times of one treatment by an LSD of $P < 0.05$.
 ‡ Initial counts of *E. coli* O157:H7 on cantaloupe surfaces were 6.82 ± 0.03 , 7.01 ± 0.01 and 6.80 ± 0.14 log cfu/cm² tissue (mean ± SD), respectively.
 POAA, peroxyacetic acid; AEW, acidic electrolyzed water.

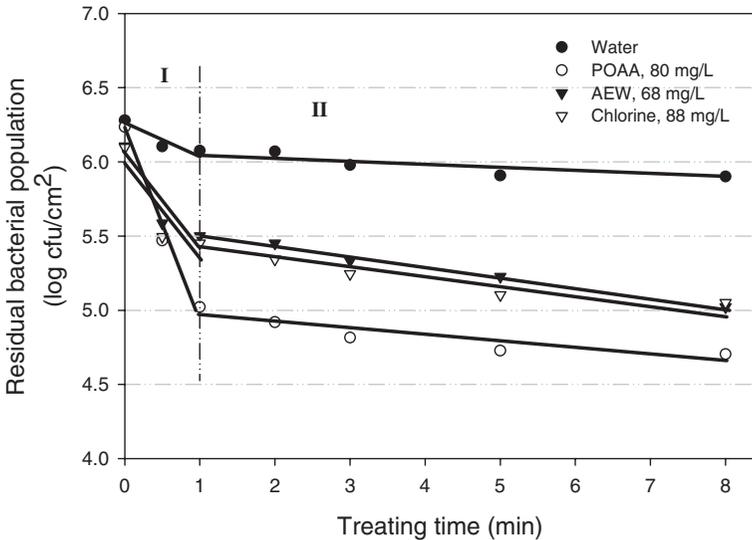


FIG. 1. EFFECT OF SANITIZER AND TREATMENT TIME ON THE INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON APPLE TISSUES
 Phases I and II correspond to the two linear phases on an inactivation curve, a fast reduction phase in the first minute and a slow one for the remaining 7 min, in a dual-phasic linear kinetic model.
 POAA, peroxyacetic acid; AEW, acidic electrolyzed water.

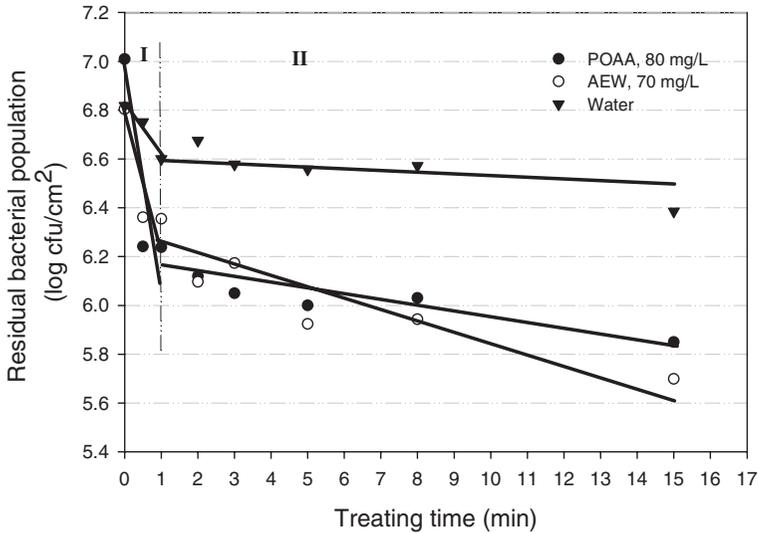


FIG. 2. EFFECT OF SANITIZER AND TREATMENT TIME ON THE INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON CANTALOUPE SURFACES

Phase I and II correspond to the two linear phases on an inactivation curve, a fast reduction phase in the first minute and a slow one for the remaining 14 min, in a dual-phasic linear kinetic model.

POAA, peroxyacetic acid; AEW, acidic electrolyzed water.

where the slope of the linear portion on an inactivation curve was used to estimate the inactivation rates. Two linear portions can be identified on both figures for all the treatments. Apparently, there is a dual-phasic inactivation behavior for all the sanitizers used on both fruits. In the first minute of a treatment (phase I), a fast inactivation was observed, which was followed by a much lower inactivation in the remaining minutes (phase II). The inactivation rates together with D values for both fruits are listed in Tables 3 and 4.

In Table 3, the POAA treatment reduced *E. coli* O157:H7 population at a rate of $1.3 \log \text{ cfu/cm}^2\text{-min}$ (D value = 0.8 min), while that of AEW, chlorine and water treatments were 0.6, 0.6 and $0.2 \log \text{ cfu/cm}^2\text{-min}$ (D values = 1.7, 1.6 and 5.0 min) in phase I, respectively. In phase II, the rate of inactivation reduced by 6.7- to 30-fold compared with that in phase I. Similar to the fresh-cut apples, POAA was the most effective sanitizer in the inactivation of *E. coli* O157:H7 on cantaloupes, as shown by an inactivation rate of $0.4 \log \text{ cfu/cm}^2\text{-min}$ in phase I (Table 4). *E. coli* O157:H7 inactivation rates were 15- (AEW) to 20-fold (POAA) faster in phase I than in phase II.

TABLE 3.
D VALUES AND INACTIVATION RATES AT ROOM TEMPERATURE FOR INACTIVATION
OF *ESCHERICHIA COLI* O157:H7 ON APPLE TISSUES

Treatment	Phase I* (0–1 min)		Phase II* (1–8 min)	
	D value (min)	Inactivation rate (log cfu/cm ² ·min)	D value (min)	Inactivation rate (log cfu/cm ² ·min)
Water	5.0	0.2	36.4	0.03
Chlorine	1.6	0.6	17.9	0.06
AEW	1.7	0.6	14.6	0.07
POAA	0.8	1.2	23.4	0.04

* Phases I and II correspond to the two linear phases on an inactivation curve, a fast inactivation phase and a slow one, in a dual-phasic linear kinetic model.
POAA, peroxyacetic acid; AEW, acidic electrolyzed water.

TABLE 4.
D VALUES AND INACTIVATION RATES AT ROOM TEMPERATURE FOR INACTIVATION
OF *ESCHERICHIA COLI* O157:H7 ON CANTALOUPE

Treatment	Phase I* (0–1 min)		Phase II* (1–15 min)	
	D value (min)	Inactivation rate (log cfu/cm ² ·min)	D value (min)	Inactivation rate (log cfu/cm ² ·min)
Water	4.6	0.2	59.8	0.02
AEW	1.7	0.6	25.7	0.04
POAA	2.3	0.4	45.5	0.02

* Phases I and II correspond to the two linear phases on an inactivation curve, a fast inactivation phase and a slow one, in a dual-phasic linear kinetic model.
POAA, peroxyacetic acid; AEW, acidic electrolyzed water.

DISCUSSION

Compared with AEW and chlorine, POAA exhibited a higher efficacy to reduce *E. coli* O157:H7 population, although it may not be always statistically significant, on both fruits and at all sampling times. Beuchat *et al.* (2004) reported that there was no significant difference between *L. monocytogenes* population reductions on lettuce when treated with chlorine (100 mg/L) and POAA (80 mg/L). However, they noticed that free chlorine depleted rapidly at high lettuce/solution ratios (<1/10, w/v), especially for shredded lettuce. With the fruit/solution ratio used in this study (1/10, w/v), it is possible that a rapid depletion of free chlorine took place during washing of fresh-cut apples because more organic materials such as fruit juices may have leaked out from the cut surfaces and helped to diminish the efficacy of chlorine. AEW also

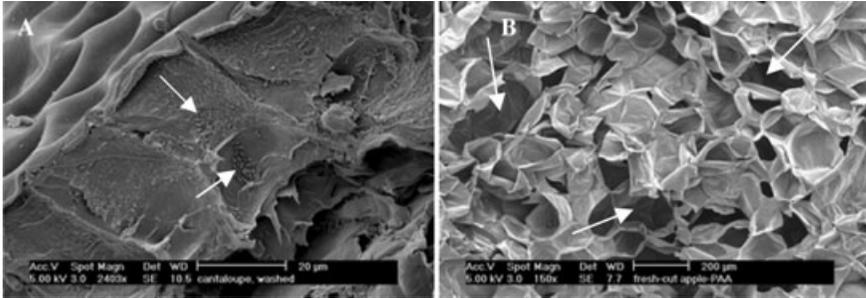


FIG. 3. SCANNING ELECTRON MICROSCOPY IMAGES OF FRESH-CUT APPLE AND CANTALOUPE SURFACES

(A) Cantaloupe surface. (B) Fresh-cut apple surface.

achieved a higher microbial reduction than the chlorine solution in phase II (Fig. 2). Because AEW had 20 mg/L less free chlorine concentration than chlorine solution did, the additional inactivation of AEW may be attributed to the low pH (≤ 2.7) and high ORP (1170 mV).

All treatments on both fruits, including water wash, displayed a similar pattern in *E. coli* O157:H7 population reduction. A treatment reduced the microbial population swiftly at first, but the reduction became much slower after what was approximately the first minute. Considering the rough surface of cantaloupes and porous surface of fresh-cut apples, it is reasonable to hypothesize that the accessibility of the microbes to the sanitizing agents is different in the two stages, which may be caused by the surface morphology of the two fruits used. During the first minute, the fast reduction of *E. coli* O157:H7 may be attributed to a removal and inactivation of the loosely attached bacterial cells on the shallow areas where the sanitizer or water can reach through a mechanical washing action. SEM images in Fig. 3A,B revealed that the numerous cavities on cantaloupe and fresh-cut apple surfaces can provide shelters that harbor *E. coli* O157:H7 cells, out of reach of sanitizers during a washing treatment. Therefore, bacterial inactivation rates should be expected to decrease after most bacteria distributed on the shallow surface sites are removed or inactivated. Figure 3A shows colonies of residual *E. coli* O157:H7 cells in cantaloupe surface cavities after a 5-min washing. In the case of fresh-cut apples (Fig. 3B), cell internalization may take place, as demonstrated by Fatemi *et al.* (2006), who recorded a 1.4-mm penetration of *E. coli* O157:H7 cells into the fresh-cut apple tissue 8 h after inoculation. The internalized cells are difficult to remove.

It is worth noting that the fast inactivation expressed in *D* values in phase I on fruit surfaces is still lower than the inactivation of the same microorganism treated with the same sanitizer if the microorganism is freely suspended. For

instance, Kim *et al.* (2000) reported a complete inactivation of freely suspended *E. coli* O157:H7 (8.88 log) after 30 s at a cell inoculum-to-AEW ratio of 1:100, which corresponded to a *D* value of <0.06 min. Venkitanarayanan *et al.* (1999) reported a 7-log reduction of a five-strain mixture of *E. coli* O157:H7 when exposing a 1-mL cell inoculum to 9 mL of AEW for 5 min, which corresponded to a *D* value of <0.72 min. Although the *D* values were influenced by the differences in the cell inoculum-to-AEW ratio, when bacterial cells were directly exposed to the sanitizer, the inactivation rate was still faster than when bacteria were attached to the produce surface. Rodgers *et al.* (2004) also reported significantly smaller *D* values determined in an aqueous model system than those obtained from the produce inactivation tests.

The two-stage washing behavior during a disinfection treatment of fresh-cut apples and cantaloupes may have an important practical application. It can be used to determine the optimal washing time for a sanitation treatment, as washing in the second stage is ineffective and should be avoided.

ACKNOWLEDGMENTS

This research was supported in part by the Illinois Agricultural Experiment Station. We thank Dr. Arun K. Bhunia of Purdue University for providing *E. coli* O157:H7 strains.

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