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Reduction of *Escherichia coli,* as a surrogate for *Salmonella* spp., on the surface of grapefruit during various packingline processes

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ABSTRACT

The US Produce Safety Rule allows for use of water that does not meet its microbial standards if corrective measures are employed. This research was initiated to determine the suitability of nonpathogenic *Escherichia coli* as a surrogate for *Salmonella* during citrus washing, and to evaluate the removal of *E. coli* from grapefruit on two pilot packinglines (CREC and IRREC) as corrective measures. Whole grapefruit were inoculated with either *E. coli* or *Salmonella* and dried, and exposed to a variety of treatments on a lab-scale brush wash system. Individual processes were evaluated on the pilot packinglines with *E. coli* only. In all lab-scale brush wash system treatments, bacterial population reductions between *E. coli* and *Salmonella* were not significantly different ($P \leq 0.05$). On pilot packinglines, *E. coli* populations were reduced by 3.59 to > 5.11 log CFU/grapefruit at the CREC packingline, and by 3.30 to > 5.13 log CFU/grapefruit at the IRREC packingline. Treatment of fruit through complete packingline processing at both locations reduced *E. coli* populations to levels below the detection limit (< 1 log CFU/grapefruit). The studies indicate *E. coli* is an appropriate surrogate for *Salmonella* under tested conditions, and that standard citrus packingline processes can be used as a corrective measure.

1. Introduction

In 2011, the Food Safety Modernization Act (FSMA) was signed into law resulting in the Produce Safety Rule being released as a final rule in November 2015 (US FDA, 2015a). This rule impacts fresh citrus growers, harvesters, and some citrus packers. One of the most controversial aspects of the Produce Safety Rule are its requirements on the microbiological quality of production agricultural water that contacts the harvested surface of the fruit before harvest. It specifies testing requirements in terms of locations and numbers of samples collected per water source, and tests used to quantify levels of generic *Escherichia coli* in 100 mL of water. After a grower has received the required number of test results over the prescribed period of time, they are required to calculate a geometric mean (GM) and a statistical threshold value (STV). The calculated grower values must be equal to, or less than a GM of 126 CFU *E. coli*/100 mL and a STV of 410 CFU *E. coli*/100 ml. If either or both the calculated GM or STV of the production agricultural water does not meet the microbial quality standards, the grower may continue to use that agricultural water to contact the harvested surfaces of the fruit provided that they also use a corrective measure(s) as specified by the rule that will reduce the microbial population to at or below the prescribed GM and STV (US FDA, 2015a). Corrective measures include: (i) applying a preharvest time interval (in days) between the last application of water and harvest (up to 4 days, growers can assume a 0.5 log CFU/day reduction, i.e. up to a 2 log reduction); (ii) a postharvest intervention, such as commercial washing, where microbial removal rates have been documented; and/or (iii) a time interval (in days) between harvest and the product reaching a retail location, where microbial reductions over time are known (US FDA, 2015a). In all cases, the microbial reduction needed by the corrective measure(s) to meet the GM and STV microbial standards is dictated by the initial quality of the water used in the field and will not be the same for every grower/packinghouse. Other corrective measures that can be applied prior to water use in the field include (iv) treating the water to decrease microbial populations, and (v) discontinuing water use.

Pao and Brown (1998) demonstrated that standard washing and rinsing procedures for Valencia juice oranges using potable water reduced generic *E. coli* freshly inoculated at 4.8 log CFU/cm^2 onto the

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surface of oranges by about 2.4-logs. Addition of sodium o-phenylphenate (SOPP) or 200 ppm free chlorine did not further reduce *E. coli* populations, but wax application and drying at about 52 °C for 2 min further reduced populations by ca. 1 log. Immersion of Valencia oranges in various disinfectant solutions resulted in 1.8- to 3.1-log reductions in *E. coli* from midsection surfaces and 1.0-log reductions from stem scar areas (Pao and Davis, 1999a). Water immersion alone was almost as effective. Rapid hot water immersion (80 °C for 1 min or 70 °C for 2 min) proved even more effective, resulting in a 5-log reduction but this approach is not applicable to fresh market citrus as it injures the peel, causing unsightly blemishes and rapid subsequent deterioration (Ritenour et al., 2003). Combinations of washing and waxing reduced inoculated levels of *E. coli* on oranges by 3.4 log CFU/cm^2 (Pao and Davis, 1999a). During waxing, the synergistic effect of heat applied to dry the wax combined with alkaline pH (> 8.0) resulted in a 4.7-log reduction in *E. coli* on midsection Hamlin orange surfaces, but only a 1.0 log reduction in stem scar areas (Pao et al., 1999b). Since Pao et al. conducted these studies more than 15 years ago, numerous changes have occurred in the citrus packing industry, including a greater use of carnauba-based waxes, the use of waxes without morpholine (with antimicrobial properties), and increased use of peracetic acid on the line.

Some citrus packinghouses may fall under the Preventive Controls (PC) for Human Foods Rule (US FDA, 2015b) under FSMA. In its final form, the PC rule requires all food handling companies have a Food Safety Plan. A cornerstone of these plans is the validation of risk-based process preventive controls, or in the case of Florida citrus packinghouses processes similar to the postharvest interventions, or corrective measures, discussed above.

This research was initiated to determine the suitability of non-pathogenic *E. coli* as surrogate for *Salmonella* during citrus storage and washing, and to evaluate the removal of *E. coli* from grapefruit surfaces on two pilot packinglines. The outcomes of this research provide key data for industry use in complying with the Produce Safety Rule and establishing a Food Safety plan if they are required to comply with the PC for Human Foods Rule.

2. Materials and methods

2.1. Grapefruit

Red grapefruit were used in all experiments during two crop seasons and were obtained directly from various groves within central and east Florida. When necessary, grapefruit were stored at 4 °C for one or two days and brought to ambient temperature prior to the start of the experiment.

2.2. Strains and culture preparation

Salmonella strains, from the authors' culture collection, used included serovars Gaminara (CDC HO622; orange juice outbreak of 1995), Rubislaw (MDD26; orange juice outbreak of 1995), Typhimurium Typhimurium (ATCC 14028; orange juice outbreak of 1999), Hartford (MDD79, citrus processing facility involved in outbreak of 1995) and Muenchen (MDD30; orange juice outbreak of 1999), all previously isolated from orange juice or orange processing and packing facilities. *E. coli* strains were ATCC 25922 and 35218 as previously evaluated (Pao and Brown, 1998; Pao and Davis, 1999a; Pao et al., 1999b). All strains were adapted and confirmed to grow in appropriate media amended with 80 μg/mL rifampicin.

Prior to experimentation, frozen stock bacterial cultures were streaked onto tryptic soy agar supplemented with rifampicin (TSAR; 80 μg/mL; unless otherwise noted, all culture media used were from Difco, BD, Sparks, MD) and were incubated at 37 °C for 18 h. An isolated colony was transferred into tryptic soy broth supplemented with rifampicin (TSBR; 80 μg/mL) and was incubated at 37 °C for 24 h. At two consecutive 24-h intervals, single-loop (10 μL) transfers were made of each *E. coli* and *Salmonella* strain into TSBR. After overnight incubation, strains were collected by centrifugation at 3000 × *g* for 10 min (Allegra X-12, Beckman Coulter, Fullerton, CA). Cells were washed twice by first removing the supernatant and suspending the cell pellet in 10 mL of 0.1% peptone. Washed cells were suspended in 5 mL of 0.1% peptone. Strains were diluted and combined in equal volumes to a concentration of about 10^8 CFU/mL. Final concentrations of cocktail bacterial suspensions were verified by enumeration on TSAR (80 μg/mL rifampicin) and XLT4 (*Salmonella*) or MacConkey (*E. coli*) agar amended with 80 μg/mL rifampcin (XLT4R or MACR).

2.3. Inoculation

Each grapefruit was inoculated by spotting 100 μL of either *E. coli* or *Salmonella* cocktails (8 log CFU/mL) which were freshly prepared onto the equator or stem scar surface (7 log CFU/grapefruit) and dried under ambient temperatures (21 ± 2 °C for surrogate studies; 24 ± 2 °C for pilot plant studies) for 2 h prior to storage or fruit treatment. Following 2 h of storage at ambient temperatures, all fruit were visibly dry.

2.4. Surrogate studies

2.4.1. Storage survival

Survival of *Salmonella* and *E. coli* on grapefruit surfaces was evaluated by storing the inoculated fruit at 20 ± 1 °C and sampling fruit for survived *Salmonella* and *E. coli* populations on days 0, 3, 7, and 14. Three replicates of two grapefruit each were performed (n = 6 fruit).

2.4.2. Lab-scale brush wash treatments

Two fruit wetting and six fruit washing treatments were applied using a lab-scale spray and brush washing system to grapefruit inoculated at either the equator or stem scar. The pilot wash system was a laboratory-scale wash system (Agri Machinery Inc., Orlando, FL, U.S.A.), installed in a purifier class II biosafety cabinet (Labconco Corporation, Kansas, MO, U.S.A.). It is composed of three spray nozzles (Spraying Systems Co., Wheaton, Ill, U.S.A.) located 13 cm above nylon brushes (46 by 12 cm). The system rotated at 180 rpm and the sprayer operated at 12 psi with a 21.4 mL/s flow rate (Chang and Schneider, 2012). Details of the treatments are provided under the fruit treatment section below (section 2.6; Table 1, treatments 1 to 7). Each treatment had three replicates of four fruit each (n = 12 fruit). Control fruit were inoculated but not treated, or treated but not inoculated.

2.5. Pilot plant packinglines

Five pilot plant trials took place at each of two locations (University of Florida, Citrus Research and Education Center [CREC], Lake Alfred, and Indian River Research and Education Center [IRREC], Fort Pierce, Florida) during different times (early-, mid- and late-season) of the 2015/2016 and 2016/2017 commercial harvest seasons. Both packinglines at CREC and IRREC were set up as simulated standard commercial citrus packingline systems. Standard commercial citrus packinglines in Florida include a dry dump, pre-wash fruit wetting, fruit washing, pre-wax drying, waxing, and drying.

2.5.1. CREC packingline

The packingline at CREC consists of fruit loading, fruit wetting, debris eliminating, and rinsing PCV roller conveyor, nylon wash brush rollers (Central Florida Sales and Service, Inc., Auburndale, FL), pre-wax dryer over steel roller conveyor (Beshaco, Inc., Vero Beach, FL) and a fruit sizing and grading system (Colour Vision System, Vero Beach, FL). The fruit waxing over nylon brush rollers and final dryer over the steel roller conveyor (Beshaco, Inc., Vero Beach, FL) were set as a separate line. The washing rollers rotated at 150 rpm, and waxing roller's rotating speed was at 80 rpm. The temperatures of dryers were at

54–60 °C.

2.5.2. IRREC packingline

The packingline system at IRREC consisted of wetting and debris elimination PVC roller conveyor, wash and rinse nylon brush rollers, pre-wax dryer over brush rollers, wax sprayer over brush rollers, final fruit dryer over steel roller conveyor (JBT Food Tech, Lakeland, FL), a sizing and grading system (Colour Vision System, Vero Beach, FL) and fruit collecting system. The running speeds of washing, pre-wax drying and waxing brush rollers were at 140 rpm. Temperatures of pre-wax dryer and final dryer were at 37–40 °C, and 41–43 °C, respectively, which were lower than those of the CREC packingline.

2.5.3. Pilot packingline experimental design

The study consisted of thirteen treatments using *E. coli* (inoculated on fruit equator) as a surrogate for *Salmonella*. Individual processes evaluated on the pilot packinglines are listed below and represent the most commonly used commercial treatments in Florida. Each treatment had three replicates of 10 fruit each. *E. coli* was enumerated from pooled samples of 10 fruit. Background bacterial populations were also enumerated from the same samples. Control fruit were inoculated but not treated or treated but not inoculated.

2.6. Fruit treatments

Thirteen different common Florida citrus packinghouse fruit treatments were tested individually, and combined into a complete packingline process, to evaluate the reductions of inoculated *E. coli* and background bacterial populations. Treatments including: pre-wash fruit wetting (2 min, spraying on fruit rotating on PVC rollers); fruit washing (1 min wash followed by 5s ground or municipal water rinse over brush rollers); pre-wax drying of washed fruit; waxing and drying of washed and dried fruit; and the complete configuration, are described and numbered 1–13 in Table 1.

All sanitizers, alkaline detergents, additive and wax formulations were commercially used in citrus packinghouse and purchased from JBT Corporation (Lakeland, FL) and prepared according to manufacturer's instructions. The formulations evaluated include sanitizers: 200 ppm free chlorine (Freshgard 71, 10% sodium hypochlorite) adjusted to pH 7.0 using 10% HCl; 85 ppm PAA (VigorOX: peroxyacetic acid 15%, Hydrogen peroxide 10%); PAA (VigorOX) plus an acidic detergent with a pH of less than 4 (XA-15 additive); 2% SOPP (Freshgard 5, 24% sodium o-phenylphenate); and an alkaline detergent (Fruit Cleaner 395, pH 13). When dilution was necessary, untreated well water that had tested negative for generic *E. coli* in 100 mL (CREC packinghouse) or municipal water (IRREC packinghouse) was used. Free chlorine and PAA concentrations were confirmed using LaMott test strips (LaMotte Company Chestertown, MD). Waxes used included: shellac based wax (Sta-Fresh 590HS, high gloss); carnauba based wax without morpholine (Endura-Fresh 9000); carnauba based wax with morpholine (Sta-Fresh 2109LM). All wax products were ready to use coatings for the postharvest application of citrus fruits, no modifications or additions were made.

Table 1
Common Florida citrus packinghouse fruit treatments evaluated individually, and combined into a complete packingline process, to determine the reductions of inoculated *E. coli* and background bacterial populations on grapefruit.

Packingline process	Treatment Number	Treatment
Pre-wash fruit wetting (2 min, spraying on fruit rotating on PVC rollers)		
	1	Ground or municipal water
	2	200 ppm free chlorine, pH 7.0 (adjusted with 10% HCl)
Fruit washing (1 min wash followed by 5s ground or municipal water rinse over brush rollers)		
	3	Ground or municipal water
	4	85 ppm peroxyacetic acid (PAA)
	5	85 ppm PAA + acid detergent (XA-15 additive)
	6	Alkaline detergent (AD)
	7	AD + 2% sodium-o-phenylpenate (SOPP)
Pre-wax drying of washed fruit (Treatment 3 used to wash fruit prior to inoculation and drying)		
	8	pre-drying (1 min)
	9	pre-drying (2 min)
Waxing and drying of washed and dried fruit (Treatments 7 and 9 prior to inoculation and waxing)		
	10	Coated with shellac based wax
	11	Coated with carnauba based wax without morpholine
	12	Coated with carnauba based wax with morpholine
Complete configuration		
	13	Treatments 2, 7, 9 and 12

2.7. Sample processing and enumeration

After treatment or storage in lab scale or pilot packingline experiments, fruit and Dey/Engley (DE, Thermo Fisher Scientific, Waltham, MA) buffer (10 mL/fruit) were placed in sterile bags (1.6 L, Thermo Fisher Scientific, Waltham, MA) and individual fruit was shaken (30s), rubbed (1 min), and shaken (30s) by hand. Serial dilutions were made in 0.1% peptone water and surface plated (0.1 mL) in duplicate onto TSAR and XLT4R (for lab scale tests for detecting *Salmonella*), or MACR (in pilot scale tests for detecting *E. coli*). In addition to selective and differential agars, uninoculated control samples were plated onto nonselective medium tryptic soy agar (TSA) to quantify background microflora. To increase the limit of detection, an additional 1 mL of the lowest dilution was plated onto four plates each (0.25 mL/plate) of TSAR and XLT4R or MACR media. Plates were incubated for at 35 ± 2 °C for 24 h, and bacterial colonies were counted by hand to determine population levels. The bacterial population levels were expressed in log CFU/grapefruit.

2.8. Analysis of data

The experiments studying *E. coli* as surrogate for *Salmonella* were conducted in duplicate. Each treatment for the trials examining reduction on grapefruit of both *E. coli* and total aerobic plate counts were conducted in triplicate on five separate occasions at each of two packinglines over two fruit harvest seasons. Bacterial population reductions due to specific different treatments were calculated based on treatment log CFU/grapefruit minus control log CFU/grapefruit. Statistical analysis for all treatments at both facilities was conducted concurrently as conducted utilizing a full factorial model in JMP Pro 13.2.0 (SAS Institute, Cary, NC); all treatments at both facilities were compared to each other. Analysis of variance including Tukey's Honest Significant Difference test indicated significant differences among treatments ($P \leq 0.05$).

3. Results and discussion

Previous work by Pao et al. (1998, 1999a, 1999b) is the foundation of the 5-log surface reduction of *Salmonella* for fresh citrus juice under the Juice Hazard Analysis Critical Control Point regulation (US FDA, 2001), no mention is made to any experimental work demonstrating *E. coli* as an appropriate surrogate for *Salmonella* in citrus washing applications. Initial laboratory studies focused on validating generic *E. coli* as a surrogate for *Salmonella; E. coli* was selected based on previously published work (Pao and Brown, 1998; Pao and Davis, 1999a; Pao et al., 1999b). A suitable surrogate is nonpathogenic, easy to prepare and enumerate, and exhibits similar behavior to the pathogen in both its survival on the fruit and its resistance to process treatments (Busta

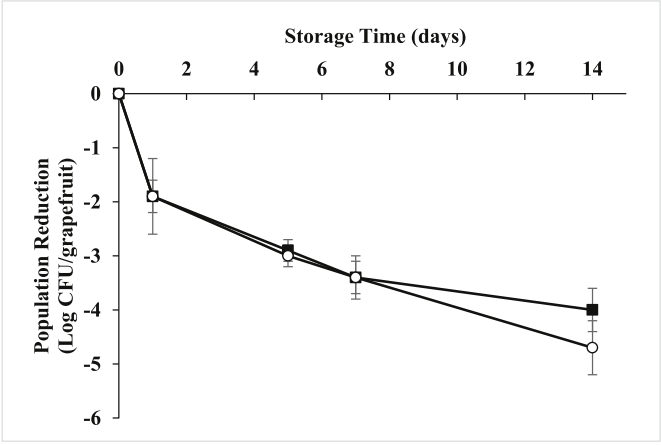

Fig. 1. Average log reductions and standard deviation (vertical bars) of *Salmonella* spp. (open circles) and *E. coli* (black squares) populations inoculated onto the surface of grapefruit and held at 20 °C for 14 days (n = 6 grapefruit).

et al., 2003). Current work used overnight broth cultures of five *Salmonella* isolates and *E. coli* strains ATCC 25922 and 35218, washed and diluted in peptone, and a spot inoculation technique, as recommended by Lang et al. (2004a, 2004b). Pao and Brown (1998) used ATCC 25922, mixed the inoculum with sterile cow manure slurry and inoculated by submerging Valencia oranges for 5 min, followed by drying for 1 h at ambient temperature. Pao and Davis (1999a) used a cocktail of *E. coli* ATCC 2599, ATCC 35218, ATCC 11229, and SPI1-97 (an orange surface isolate); and inoculate Valencia oranges by submersion into nutrient broth containing isolates. Pao et al. (1999b), used *E. coli* ATCC 25922 and *E. coli* 1–97 (possibly the same orange surface isolate from Pao and Davis (1999a)), and prepared it by scraping overnight cultures off of TSA plates, combining with 0.1% peptone water in a stomacher, then immersing Hamlin oranges for 15 min, with 25 min drying at 35 °C.

The survival of *Salmonella* and *E. coli* inoculated onto the equator surface of stored grapefruit over 14 days at 20 °C is shown in Fig. 1. Both organisms behaved similarly, and at no point were there significant differences ($P \geq 0.05$) between the populations. While not significantly different, *E. coli* population reductions at 14 days were 4.04 ± 0.4 log CFU/grapefruit, while *Salmonella* populations had decreased by 4.73 ± 0.5 log CFU/grapefruit over the same period, implying that *E. coli* is a suitable surrogate for *Salmonella* under storage conditions and significant reductions of both organisms occur during storage at ambient temperatures. The use of these strains of *E. coli*, as a surrogate for *Salmonella* when using a time interval (in days) between harvest and the product reaching a retail location, where microbial reductions over time are known as a Corrective Measure (US FDA, 2015a) should be further explored.

Subsequent laboratory studies were performed to compare the reductions of *E. coli* and *Salmonella* populations on the surface of grapefruit using various sanitizer treatments on a lab-scale brush wash system. Treatments included wetting (2 min: ground water and 200 ppm free chlorine), washing (1 min wash, 5 s rinse with ground water, PAA, PAA + XA-15 additive, alkaline detergent, or alkaline detergent plus 2% SOPP), and controls. Microbial reductions under different treatments at two inoculation sites can be seen in Fig. 2. On equator surfaces (Fig. 2A), average log reductions of *E. coli* ranged from 2.70 (2 min, water wetting) to 4.87 (2 min, chlorine 200 ppm wetting) log CFU/grapefruit, and average *Salmonella* reductions ranged from 2.83 (1 min water wash, or 2 min water wetting) to 4.87 (2 min 200 ppm free chlorine wetting, and 1 min alkaline detergent plus SOPP wash) log CFU/grapefruit. On stem scar surfaces (Fig. 2B), average *E. coli* reductions ranged from 2.29 (1 min water wash), to 4.93 (2 min, 200 ppm free chlorine wetting, or 1 min, alkaline detergent plus SOPP

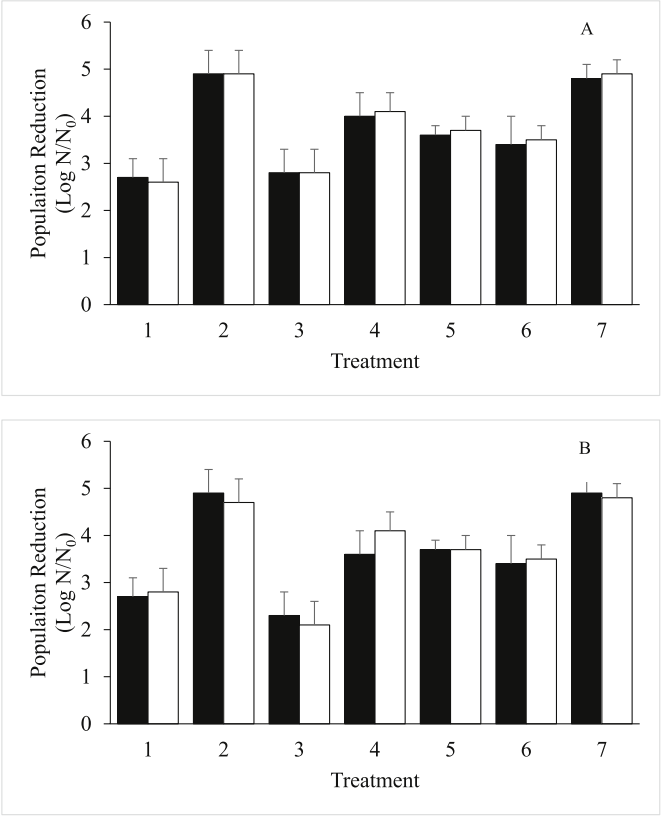

Fig. 2. Average log reductions and standard deviation (vertical bars) of *E. coli* (black) and *Salmonella* spp (white) populations inoculated onto the equator (A) or stem scar (B) of grapefruit and treated with various commercial packingline treatments (defined in Table 1) on a lab-scale brush wash system (n = 12 grapefruit).

wash) log CFU/grapefruit, and *Salmonella* reductions ranged from 2.06 (1 min, water wash) to 4.84 (1 min alkaline detergent + SOPP wash) log CFU/grapefruit. An ideal surrogate for *Salmonella* is a non-pathogenic microorganism with similar or higher resistance when exposed to same conditions (Busta et al., 2003). In all treatments, population reductions of *E. coli* were not significantly different ($P \geq 0.05$) from *Salmonella*, indicating *E. coli* is a suitable surrogate for *Salmonella* under these treatment scenarios. Significant differences ($P \leq 0.05$) were not seen between populations inoculated onto the equator or the stem scar of the grapefruit for either *E. coli* or *Salmonella* (Fig. 2); therefore, all pilot plant scale experiments were carried out using only equator inoculations.

Packingline studies, five each at each the CREC in Lake Alfred and IRREC in Ft. Pierce, over two harvest seasons to evaluate thirteen different treatments using *E. coli* (as a surrogate for *Salmonella*) inoculated onto fruit surfaces; control fruit were inoculated but not treated or treated but not inoculated. The mean, standard deviation, median, maximum, and minimum log CFU/grapefruit reductions of *E. coli* populations at each packingline location are shown in Table 2; the bolded number is the minimum log reduction obtained for the treatment on either the CREC or the IRREC packingline. Average individual packingline processes at CREC and IRREC reduced *E. coli* populations from 3.59 to > 5.11 and 3.30 to > 5.12 log CFU/grapefruit, respectively, depending on the detection media, individual processes, treatments, and packingline system. Mean log reductions seen following wetting with ground or municipal water (3.59 CREC, 3.30 IRREC), were higher than those reported by Pao and Brown (1998), who observed a 2.4 log reduction using overhead water on a brush roller, however, the treatment time was not reported. The addition of 200 ppm free chlorine (pH 6.5) significantly increased the log reductions seen at both CREC and

Table 2
Mean log reduction of *E. coli* populations (log CFU/grapefruit) from the surface of grapefruit during different pilot packingline treatments (n = 150 fruit total per treatment at each CREC and IRREC from 5 trials per packingline, 3 replicates per trial, 10 fruit per replicate).

	Location									
	CREC					IRREC				
	Mean	SD	Median	Max	Min*	Mean	SD	Median	Max	Min*
Wetting (2 min)										
1. Ground or municipal water	3.59^{d**}	0.61	3.40	4.06	2.18	3.30^{d}	0.61	3.00	3.85	**1.62**
2. 200 ppm free chlorine, pH 6.5	> 5.04^{abc}	0.08	4.99	> 5.12	> 4.96	> 5.13^{a}	0.24	> 5.16	> 5.39	> **4.66**
Washing (1 min, 5 s water rinse)										
3. Ground or municipal water	3.68^{d}	0.66	3.76	4.01	**1.70**	3.83^{d}	0.52	3.76	4.46	2.39
4. 85 ppm peroxyacetic acid (PAA)	4.73^{cd}	0.46	4.66	> 5.18	**3.92**	5.05^{abc}	0.39	4.79	> 5.39	4.05
5. 85 ppm PAA + XA-15 additive	4.97^{abc}	0.48	4.99	> 5.18	**3.59**	> 5.12^{ab}	0.27	> 5.16	> 5.39	> 4.56
6. Alkaline detergent	4.85^{abcd}	0.60	4.96	> 5.17	**3.19**	5.01^{abc}	0.35	4.90	> 5.39	4.22
7. Alkaline detergent + 2% sodium-O- penylpenate (SOPP)	> 5.01^{abc}	0.23	4.99	> 5.17	**4.22**	> 5.12^{ab}	0.27	> 5.16	> 5.39	> 4.56
Pre-wax drying of fruit										
8. pre-dryer (1 min)	> 5.09^{abc}	0.13	5.06	> 5.29	4.86	4.92^{abc}	0.39	4.73	> 5.26	**3.88**
9. pre-dryer (2 min)	> 5.11^{ab}	0.11	5.07	> 5.29	4.94	> 4.97^{abc}	0.28	> 4.92	> 5.26	> **4.49**
Waxing and drying of fruit										
10. Coated with shellac based wax	5.04^{abc}	0.22	5.08	> 5.20	4.38	4.84^{abcd}	0.62	4.51	> 5.48	**3.53**
11. Coated with carnauba based wax	> 5.07^{abc}	0.11	5.08	> 5.20	> 4.84	4.79^{bcd}	0.67	4.40	> 5.48	**3.50**
12. Coated with carnauba based wax + morpholine	> 5.07^{abc}	0.11	5.08	> 5.20	> 4.84	4.96^{abc}	0.49	4.81	> 5.48	**3.43**
Complete configuration										
13. Treatments 2, 7, 9, and 12	> 5.06^{abc}	0.11	5.08	> 5.12	> 4.83	> 4.98^{abc}	0.32	> 4.81	> 5.48	> **4.40**

*Bolded number is the minimum log reduction obtained for the treatment on either the CREC or the IRREC packingline.
**Superscripted letters indicate mean separation. All means with the same letter in Table 2 are statistically the same ($P < 0.05$). All treatment means in Table 2 are compared to each other, regardless of facility location; no significant treatment difference was indicated due to facility location.

IRREC, decreasing populations to below the limit of detection. Pao and Brown (1998) did not report an increase in log reduction following addition of SOPP, or SOPP and chlorine; the pH of the chlorine solution and the treatment time were not reported.

During the washing step (1 min washing, and a 5 s water rinse), mean log reductions vary at IRREC and CREC, however trends are consistent (Table 2). The lowest mean log reductions are seen with ground or municipal water (3.68 and 3.83 log CFU/grapefruit reduction, at CREC and IRREC, respectively). At CREC, the 200 ppm free chlorine, alkaline detergent + 2% sodium-O-penylpenate (SOPP), 1 min and 2 min pre-wax drying treatments, carnauba wax, carnauba wax + morpholine, and the complete configuration treatment all resulted in *E. coli* reductions above the upper limits of detection (> 5.04 - > 5.11 log CFU/grapefruit reduction); at IRREC the 200 ppm free chlorine, 85 ppm PAA + XA 15 additive, alkaline detergent + 2% sodium-O-penylpenate, 2 min pre-wax drying, and the complete configuration treatments resulted in reductions exceeding the upper limits of detection (> 4.97 – > 5.12 log CFU/grapefruit reduction). Pao and Davis (1999a) evaluated similar chemicals, however rather than treating grapefruit (Valencia oranges in their work) on a brush roller bed as is common in the Florida citrus industry, they submerged the fruit in a solution for 8 min at 30 °C. Their reported average log reductions for 80 ppm PAA following submersion was ca. 2.5 log CFU/cm^2, lower than the 4.73–5.05 average log reductions found at CREC and IRREC.

Average log reductions during the pre-wax drying of fruit, not previously evaluated, always decreased populations to below the limit of detection following a 2 min treatment, and in some replicates following a 1 min treatment on both packinglines. Similar average log reductions were seen following the addition of different wax formulations, with average log reductions between 4.79 and > 5.07 log CFU/grapefruit. Pao et al. (1999b) evaluated the addition of shellac based wax at various pH, and drying times and temperatures. They determined similar log reductions on the mid-section surface, up to 5.9 average log CFU/cm^2, however much lower log reductions (up to 2.0 log CFU/cm^2) were reported on the stem scar area of their Valencia oranges. In all cases of the current study, the complete line configuration, typical of a Florida citrus packinghouse, reduce inoculated *E. coli* populations to below the limit of detection.

Results from this study should represent worst-case conditions in terms of microbial contamination load within commercial packinghouses as the two pilot packinglines (CREC and IRREC) simulate commercial equipment/practices, but with shorter runs of line for washing, drying, and waxing. When evaluating microbial reduction data for use as a validation study under the FSMA rules, it is critical to look at the minimum log reductions each process obtained, in addition to the mean log reduction. In Table 2, the minimum log reductions obtained during any replicate of the experiment on both the CREC and IRREC packinglines are identified as the bold number in each row. It is this bolded number that should be used as the corrective measure or minimum log reduction obtained in validation at each given step in their processing. Pre-wetting fruit with chlorine (200 ppm, pH 6.5 for 2 min) effectively reduced *E. coli* by a minimum of > 4.66 log CFU/grapefruit; a water alone pre-wetting achieved a 1.62 log reduction. Brush washing fruit with PAA (85 ppm) achieved a minimum 3.92 log CFU/grapefruit reduction, PAA + XA-15 additive a minimum 3.59 log CFU/grapefruit reduction, alkaline detergent (2%) a minimum 3.19 log CFU/grapefruit reduction and SOPP (2%) plus alkaline detergent (2%) achieved a minimum 4.22 log CFU/grapefruit reduction. The lowest minimum reduction during brush washing was a 1.62 log CFU/grapefruit reduction, when using water alone for 1 min. Pre-wax drying lead to minimum 3.88 and > 4.49 log CFU/grapefruit reductions after 1 and 2-min drying, respectively. Waxing and drying of fruit lead to minimum log reductions of 3.43–3.53 log CFU/grapefruit reductions, depending on wax type, additives, and drying temperature. Complete packingline processing reduced *E. coli* populations to levels below the detection limit (< 1 log CFU/grapefruit), resulting in a minimum > 4.40 log CFU/grapefruit reduction. These minimum log reductions can be used by industry in complying with the Produce Safety Rule if corrective measures are needed and establishing a Food Safety plan if they are required to comply with the PC for Human Foods Rule.

While significant reductions in inoculated *E. coli* populations are noted, the average reduction of naturally occurring background bacterial populations ranged from 1.31 to 2.74 log CFU/grapefruit (Table 3). Minimum log reductions range between 0.03 log CFU/grapefruit for waxing fruit with carnauba wax at CREC, to 1.73 log

Table 3
Mean log reduction of total aerobic plate count (background microflora; log CFU/grapefruit) from the surface of grapefruit during different pilot packingline treatments (n = 50 fruit total per treatment at each CREC and IRREC from 5 trials per packingline, 1 replicates per trial, 10 fruit per replicate).

	Location									
	CREC					IRREC				
	Mean	SD	Median	Max	Min*	Mean	SD	Median	Max	Min*
Wetting (2 min)										
1. Ground or municipal water	2.08^{abcdef**}	0.47	2.36	2.57	1.27	1.34^{ef}	0.75	1.13	3.27	**0.37**
2. 200 ppm free chlorine, pH 6.5	2.75^{a}	0.67	2.90	3.62	1.39	1.87^{cdef}	0.53	1.87	2.81	**1.15**
Washing (1 min, 5 s water rinse)										
3. Ground or municipal Water	2.13^{abcdef}	0.62	2.26	3.05	1.06	1.37^{ef}	0.63	1.46	2.67	**0.50**
4. 85 ppm peroxyacetic acid (PAA)	1.87^{cdef}	1.08	1.89	3.53	**0.11**	1.92^{abcdef}	0.49	1.81	2.79	1.14
5. 85 ppm PAA + XA-15 additive	1.87^{cdef}	0.99	1.89	3.18	**0.11**	2.11^{abcdef}	0.61	2.13	3.34	1.11
6. Alkaline detergent	1.97^{abcdef}	0.75	2.04	3.13	**0.66**	1.90^{bcdef}	0.54	1.99	2.67	1.05
7. Alkaline detergent + 2% sodium-O-penylpenate (SOPP)	2.41^{abcd}	0.99	2.95	3.69	**1.00**	2.37^{abcd}	0.60	2.46	3.68	1.43
Pre-wax drying of fruit										
8. pre- dryer (1 min)	2.28^{abcd}	0.85	2.33	3.46	0.85	2.14^{abcdef}	0.72	2.22	3.17	**0.81**
9. pre-dryer (2 min)	2.58^{abc}	0.77	2.77	3.54	**0.89**	2.51^{abc}	0.80	2.56	3.79	1.04
Waxing and drying of fruit										
10. Coated with shellac based wax	1.31^{f}	0.77	1.35	2.34	**0.03**	2.11^{abcdef}	0.49	2.22	2.98	1.33
11. Coated with carnauba based wax	1.84^{cdef}	0.79	2.02	2.66	**0.28**	2.18^{abcde}	0.62	2.23	3.45	1.24
12. Coated with carnauba based wax + morpholine	1.58^{def}	0.78	1.57	2.67	**0.23**	2.26^{abcd}	0.53	2.28	3.00	1.18
Complete configuration										
13. Treatments 2, 7, 9, and 12	2.10^{abcdef}	0.63	1.95	3.56	**1.36**	2.74^{ab}	0.80	2.38	4.20	1.73

*Bolded number is the minimum log reduction obtained for the treatment on either the CREC or the IRREC line.
**Superscripted letters indicate mean separation. All means with the same letter in Table 3 are statistically the same ($P < 0.05$) All treatment means in Table 3 were compared to all other treatments, regardless of facility location.

CFU/grapefruit for the complete configuration at IRREC. These results are similar to the total aerobic plate counts reported in Pao and Brown (1998), where reductions seen during a packinghouse survey ranged from ca. 0.2 log CFU/cm^2 following washing to ca. 1.75 log CFU/cm^2 on Valencia oranges after the complete packingline configuration. When submerged in the various cleaners evaluated by Pao and Davis (1999a), log reduction on total aerobic counts decreased by ca. 1 log CFU/cm^2 on Valencia oranges.

While this work was performed on grapefruit collected from commercial groves or CREC trial plots, similar results are expected for all citrus varieties. All citrus fruits are composed of two major morphologically distinct regions, the pericarp (commonly known as the peel or rind), and the endocarp or pulp (Spiegle-Roy and Goldschmidt, 1996). A further distinction is made within the peel, where the external colored portion is known as the flavedo, and the internal, white layer is known of the albedo. The flavedo of all citrus fruits is composed of a cuticle-covered epidermis and a few compactly arranged parenchyma cell layers (Spiegle-Roy and Goldschmidt, 1996). While peel surface morphology (e.g., texture, characteristics of wax platelet, etc.) can vary somewhat with citrus variety, growing location, nutrition, and even canopy position, most commercial citrus varieties share similar characteristics. The inclusion of numerous fruit samples from many locations within Florida and collected over two seasons mean the results are representative of a large range of citrus fruit surface conditions (including with different amounts of dirt and organic matter). Thus, it is anticipated that the same log reductions are be expected on other citrus varieties used for fresh market to support postharvest corrective measure as defined by the Produce Safety Rule, or as a validation as defined by the Preventive Controls for Human Food Rules of FSMA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.10.014.

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