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Appl. Environ. Microbiol. 2012, 78(19):6789. DOI:
10.1128/AEM.01094-12.
Published Ahead of Print 29 June 2012.

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Enhanced Inactivation of *Salmonella* and *Pseudomonas* Biofilms on Stainless Steel by Use of T-128, a Fresh-Produce Washing Aid, in Chlorinated Wash Solutions

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The effect of the washing aid T-128 (generally recognized as safe [GRAS] formulation, composed mainly of phosphoric acid and propylene glycol) on inactivation of *Salmonella* and *Pseudomonas* populations in biofilms on stainless steel was evaluated under conditions of increasing organic matter loads in chlorinated wash solutions dominated by hypochlorous acid. Biofilms were formed statically on stainless steel coupons suspended in 2% lettuce extract after inoculation with *Salmonella enterica* serovar Thompson or Newport or with *Pseudomonas fluorescens*. Coupons with biofilms were washed in chlorine solutions (0, 0.5, 1, 2, 5, 10, or 20 mg/liter at pH 6.5, 5.0 and 2.9), with or without T-128, and with increasing loads of organic matter (0, 0.25, 0.5, 0.75, or 1.0% lettuce extract). Cell populations on coupons were dispersed using intermittent, pulsed ultrasonication and vortexing and enumerated by colony counts on XLT-4 or *Pseudomonas* agars. Cell responses to fluorescent viability staining of biofilm treatment washing solutions were examined using confocal laser scanning microscopy. Results showed that 0.1% T-128 (without chlorine) reduced *P. fluorescens* biofilm populations by 2.5 log₁₀ units but did not reduce *Salmonella* populations. For both *Salmonella* and *Pseudomonas*, the sanitizing effect of free chlorine (1.0 to 5.0 mg/liter) was enhanced ($P < 0.05$) when it was combined with T-128. Application of T-128 decreased the free chlorine depletion rate caused by increasing organic matter in wash waters and significantly ($P < 0.05$) augmented inactivation of bacteria in biofilms compared to treatments without T-128. Image analysis of surfaces stained with SYTO and propidium iodide corroborate the cultural assay results showing that T-128 can aid in reducing pathogen viability in biofilms and thus can aid in sanitizing stainless steel contact surfaces during processing of fresh-cut produce.

Biofilm formation by food-borne pathogens or spoilage microorganisms on food processing equipment and contact surfaces is a major concern in fresh-cut produce safety (1, 3). *Salmonella* spp. biofilms on plastic, cement, glass, and stainless steel surfaces (4, 11, 13) can be persistent sources of contamination in the fresh-food processing environment. Spoilage bacteria, such as *Pseudomonas fluorescens*, form biofilms that provide a protective matrix for surviving pathogenic cells and may cross-contaminate various stages of food production operations (16).

Typical cleaning methods, e.g., physical scrubbing followed by sanitizer treatment, that are used to remove bacteria from food processing equipment and contact surfaces (8) do not always inactivate bacteria in biofilms as these are more resistant to chemical sanitizers than planktonic cells. For example, 200 mg/liter free chlorine (FC) did not inactivate *Escherichia coli* O157:H7 in biofilms (24), and FC at 10 mg/liter for 10 min resulted in less than a 10-fold reduction in *Salmonella* biofilm-associated cells (13). Studies of the limitations of chlorine on viability of biofilm populations suggest that high concentrations of FC are effective in removing food spoilage bacterial biofilms on certain surfaces. Free chlorine at 200 mg/liter reduced *Bacillus cereus* and *Pseudomonas* biofilm populations more than 4 and 6 log CFU per stainless steel coupon, respectively (14), and FC at 100 mg/liter for 2 min reduced *P. fluorescens* biofilm populations by approximately 3 log CFU per stainless steel coupon (5).

Sodium hypochlorite solution at pH 6.5 is currently the most common sanitizer used in the fresh-cut produce industry. At pH 5.0 to 6.5, the free chlorine in solution is dominated by hypochlo-

rous acid (HOCl), the most effective disinfectant of the forms of chlorine including hypochlorite ion, OCl⁻, and gaseous chlorine, which off-gases from solutions in substantial amounts at pH < 4.0 (26). In addition to the capacity of hypochlorous acid to reduce the viable microbial bioburden and to prevent cross-contamination, it is also used as a sanitizer during equipment cleaning. Its overall minimal impact on nutritional and esthetic qualities of the produce, its well-known capability to inactivate pathogens in suspensions, and its low cost contribute to its common usage (19). Maintaining free chlorine in washing solutions with fresh-cut produce is challenging because fresh-cut products release copious amounts of juice into the wash water. The combined high solids and plant sap loads in the wash solution exert a high demand for the oxidation potential of FC (i.e., free available chlorine [FAC], comprising primarily HOCl, with minor amounts of Cl₂ and OCl⁻), and with very high processing rates and intermittent addition of sanitizer, FC concentrations rapidly decline to less than 0.5 mg/liter (9, 19). Replenishing chlorine periodically, either mechanically or manually, is common practice in fresh-produce processing; however, repeated additions of sodium hypochlorite to

Received 3 April 2012 Accepted 25 June 2012

Published ahead of print 29 June 2012

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doi:10.1128/AEM.01094-12

high-organic-load wash water can cause formation of toxic chlorine by-products, accumulation of chloramines, and generation of chlorine off-gas in the processing environment (23).

Recently, a formulation (T-128; New Leaf Food Safety Solutions, LLC, Salinas, CA) containing generally recognized as safe (GRAS) chemicals was reported to stabilize free chlorine concentrations in wash solutions receiving high organic loads (K. E. Lemons, U.S. patent application 20090192231). The main components of T-128 are phosphoric acid and propylene glycol; thus, the pH of pure T-128 is <1.0 (Lemons, U.S. patent application 20090192231). By pulsed addition of T-128 into sodium hypochlorite solution, controlled acidic solutions at pH 6.5 and 5.0 can be achieved. Our recent studies show that using T-128 in fresh-cut lettuce processing reduced pathogen survival in wash solutions containing high organic loads and prevented cross-contamination when inoculated and uninoculated lettuce were washed together (23). We hypothesized that T-128, developed as a novel fresh-cut washing aid, may also enhance the effects of chlorine sanitizer in reducing pathogen survival in biofilms. Thus, the objectives of this study were to (i) determine the effect of T-128 on the efficacy of FC in wash water in reducing viability of bacteria in biofilms and (ii) evaluate the effect of FC in T-128-treated wash water on the survival of pure or cocultured biofilms of *Salmonella* and *Pseudomonas* in solutions of different organic loads.

MATERIALS AND METHODS

Bacterial strains and culture preparation. *Salmonella enterica* serovar Thompson strain RM1987 (cilantro outbreak isolate; referred to as S. Thompson), *S. enterica* serovar Newport strain F3307 (mango isolate; referred to as S. Newport), and *Pseudomonas fluorescens* (ATCC 17400) were used in this study. Strains were maintained in brain heart infusion broth with 20% glycerol and stored at -80°C . Prior to experiments, the stock cultures were streaked on XLT-4 agar (BD, Sparks, MD) (*Salmonella* strains) or *Pseudomonas* agar F (Fluka/Sigma-Aldrich, St. Louis, MO) (*P. fluorescens*) and incubated at 37°C (*Salmonella* strains) or 28°C (*P. fluorescens*) for 24 to 48 h.

Media and material preparation. Lettuce juice extract (LJE) was prepared from cored, whole fresh iceberg lettuce that was processed in a commercial household juice maker (model BJE200XL Juice Fountain; Breville, Shanghai, China). The extracted juice was filtered through a double layer of cheesecloth and centrifuged ($4,629 \times g$ for 10 min at 4°C) twice to remove coarse particles. Supernatants were membrane filter sterilized (0.22- μm pore size) and then diluted in sterile distilled water to make 2% LJE, pH 6.3. Stainless steel coupons (type 302; 0.9-mm thickness, 50 by 20 mm; 21.26 cm^2) were cleaned by being soaked in methanol for 30 min to remove oil/grease and were rinsed in distilled water prior to being soaked in an alkaline detergent for 1 h at 60°C ; they were then ultrasonicated for 5 min to remove attached debris. Coupons were rinsed in distilled water and separated between paper tissues before sterilization by autoclaving at 121°C for 15 min.

Biofilm formation. A single colony of each *Salmonella* strain on XLT-4 agar or *P. fluorescens* on *Pseudomonas* agar F was cultured in 25 ml of 1/4 strength Bacto M9 minimal salt broth (0.4% glucose, pH 7.0; Difco Laboratories, Detroit, MI) and incubated at 22°C for 24 h. Cells were washed by centrifugation at $4,629 \times g$ for 10 min at 4°C three times with 25 ml of phosphate-buffered saline (PBS; pH 7.4). Cell pellets were resuspended and diluted in PBS to 5 to 6 $\log_{10}$ CFU/ml. Pure cultures or coculture cell suspensions (0.1 ml) of *Salmonella* strains and *P. fluorescens* were inoculated into 30 ml of LJE in a 50-ml centrifuge tube containing a sterile stainless steel coupon which was fully submerged in the solution to allow biofilm formation on the coupon surface. Tubes were incubated statically at 22°C for 24 h to allow formation of biofilms.

Preparation of chlorine solutions and T-128 treatments. The chlorine wash solution (CWS) was prepared by diluting 6% NaOCl (Clorox, Oakland, CA) into cold distilled water (5°C). The washing aid T-128 (New Leaf Food Safety Solutions, LLC, Salinas, CA) was directly added to the CWS to reach pH 5.0, which corresponds to approximately 0.075% (referred to as pH 5.0 with T-128) or pH 2.9, corresponding to approximately 0.1% (referred to as pH 2.9 with T-128). When T-128 was not used, pH was adjusted to 6.5, 5.0, or 2.9 using citric acid. The FC concentration of each treatment was measured with a commercial test system including a DPD (*N,N*-diethyl-*p*-phenylenediamine) powder dispenser and chlorine photometer (CP-15; HF Scientific Inc., Ft. Myers, FL).

Water quality characteristics of treatments, including pH, turbidity, total dissolved solids (TDS), and chemical oxygen demand (COD), were determined using a digital pH meter (Oakton Instruments, Vernon Hills, IL), a turbidity meter (Aquafast, Thermo Orion Research Inc., Beverly City, MA), a conductivity meter (model 135A; Orion Research Inc., Beverly City, MA), and a reactor digestion method (COD2 Mercury-free COD reagent; Hach Co., Loveland, CO) (12, 18), respectively.

The different organic loads in solutions were prepared by adding fresh iceberg LJE to chlorine solutions, with or without T-128, to the desired concentration; solutions were mixed for 30 s prior to determining the residual FC concentrations and used immediately. All chlorine solutions were freshly prepared and used within 2 min.

The possible interference by manganese and monochloramine with the DPD free-chlorine test with wash waters also was evaluated to ensure that free chlorine was not overestimated by any of the unknown components in the wash solutions. Manganese and chloramine concentrations in distilled water, 0.5 or 1.0% LJE, and CWS (FC of 1 to 10 mg/liter), with or without T-128 (pH 5.0), were tested according to the indophenol method (10). For manganese tests, samples (25 ml; pH adjusted to 6 to 7) were either pretreated or not by mixing with three 5- μl drops of KI (30 g/liter) and NaAsO_2 (5 g/liter); measurements were made with a chlorine photometer (CP-15; HF Scientific Inc., Ft. Myers, FL) after the addition of powdered DPD reagent. Manganese content was calculated by subtracting the pretreated concentration from the untreated one. For the chloramine test, samples were mixed with Monochlor-F pillows (Hach, Loveland, CO), followed by the addition of one drop of free ammonia reagent solution, and the optical density 655 nm (OD_{655}) was recorded in a UV-1601 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). Chloramine-T standard solutions (0.5 to 10 mg/liter, reagent grade; Fisher Scientific, Fair Lawn, NJ) were tested likewise and measured to generate a standard curve. The chloramine content of samples was determined from the standard curve regression line.

Treatment of biofilms. Coupons with biofilms were removed from the LJE broth and rinsed with 10 ml of PBS to remove unattached cells. Two experiments were conducted in this study. In the first experiment, coupons were immersed for 2 min in 30 ml of sterile distilled water (control) and 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 mg/liter FC in CWS, with or without T-128 at pH 2.9 or 5.0. In the second experiment, coupons were placed into 10 to 12 mg/liter of CWS containing 0, 0.25, 0.5, 0.75, or 1.0% LJE, with or without T-128 at pH 2.9 or 5.0. The pH adjustment with citric acid was included to determine the effect of pH on bacterial population survival due to the pH achieved with T-128. During the 2-min exposure to CWS, the test tubes containing the coupons were agitated on an orbital shaker (VWR International, LLC; West Chester, PA) at 118 rpm. After treatment, each coupon was transferred to a 50-ml centrifuge tube containing 30 ml of Dey-Engley (DE) neutralizing both (pH 7.4) (BBL/Difco, Detroit, MI); thereafter tubes were ultrasonicated for 2 min (eight 15-s pulses with 10-s static intervals) and vortexed at 3,000 rpm for 2 min to disperse biofilm cells from the coupons. Cell suspensions were diluted in PBS and spiral plated using a spiral plater (WASP2; Microbiology International, Frederick, MD) on XLT-4 agar (*Salmonella*) or *Pseudomonas* F agar (*P. fluorescens*). Petri dishes were incubated at 28°C for 48 h (*P. fluorescens*) or at 37°C for 24 h (*Salmonella*) before automated colony counting (ProtoCol; Microbiology International, Frederick, MD).

Crystal violet staining of biofilms on stainless steel. Cocultured *S. Thompson* and *P. fluorescens* formed biofilms on stainless steel coupons as described above. Coupons were then statically immersed or shaken (118 rpm) for 2 min in 30 ml of sterile distilled water and 3.0 mg/liter FC in CWS, pH 5.0, adjusted with citric acid or T-128. After treatment, each coupon was transferred to 30 ml of PBS containing dechlorinating agent (Hach, Loveland, CO); the tubes were either kept stationary or ultrasonicated and vortexed (2 min). Each coupon was stained with 10% crystal violet for 15 min, gently rinsed with sterile distilled water, and examined and photographed using an Olympus SZX12 microscope (Melville, NY) and Nikon charge-coupled device (CCD) camera (DS-2 M series) with ACT-2U, version 1.52, software (Nikon Instruments Inc., Melville, NY). Both wash water (mixed with dechlorination agent) and cell suspension solutions were spiral plated onto tryptic soy agar (BBL/Difco, Detroit, MI) and then incubated at room temperature for 72 h before colonies were counted.

CLSM. Samples for confocal laser scanning microscopy (CLSM) were prepared in 50-mm glass-bottom culture dishes (part number P50G-1.5-14-F; MatTek Co., Ashland, MA) and on stainless steel coupons (type 302; 0.9-mm thickness, 17 by 17 mm) by inoculation of 5 ml of sterile 2% LJE with, respectively, 0.1 ml of cocultured *S. Thompson* and *S. Newport* or cocultured *S. Thompson* and *P. fluorescens* incubated at 25°C for 24 h. The 24-h culture liquid was then removed gently by pipetting and replenished with fresh LJE; then the samples were reinoculated with the same amount of the bacterial strains and incubated at 25°C for another 24 h. For glass-bottom culture dishes, the culture liquid was removed, and 5 ml of 2 mg/liter FC in CWS (pH 5.0) solution, adjusted with citric acid or T-128, was added to the culture dishes and left for 2 min under static conditions. Stainless steel coupons with biofilms were shaken (118 rpm) for 2 min in 5 ml of sterile distilled water and 2.0 mg/liter FC in CWS, adjusted with citric acid or T-128 to pH 5.0. Finally, the culture dishes or coupons were rinsed gently with PBS containing dechlorinating reagent.

The biofilms on the glass portion of the petri dishes or on stainless steel coupons were stained using a Live/Dead BacLight Bacterial Viability Kit (Invitrogen, Eugene, OR) according to the manufacturer's recommendations. The BacLight Bacterial Viability Kit contains both SYTO9 stain and propidium iodide; when used alone, SYTO9 stain labels both live and dead bacteria. No interference by T-128-adjusted solutions was found in pretests conducted on biofilms stained with the Live/Dead kit and observed with a fluorescence microscope (Nikon Instruments, Inc., Melville, NY). In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. A Zeiss 710 confocal laser scanning microscopy (CLSM) system with a Zeiss Axio Observer inverted microscope equipped with a 100× (1.4 numerical aperture [NA]) oil immersion Plan-Apochromat objective was used to observe and digitally capture images of the stained bacteria in the biofilms. A 488-nm argon laser with a pinhole of 32 μ m passing through an MBS 488/561/633 beam splitter filter with limits set between 490 and 560 nm was used for detection of green fluorescence for live cells stained with SYTO9. A 561-nm diode-pumped solid-state laser with a pinhole of 32 μ m passing through a Main Beam Splitter (MBS) 488/561/633 beam splitter filter with limits set between 651 and 720 nm was used for detection of red fluorescence, indicative of dead cells, in which propidium iodide overstates the SYTO9. Dual lasers were used simultaneously to observe the differentially stained bacteria. At least 20 fields were examined to ascertain the consistency of the staining reaction for each different treatment, and images were captured of fields with large numbers of cells aligned within a clearly resolved focal plane. Zeiss Zen 2009 software (Carl Zeiss Microscopy, Germany) was used to capture the images, and Axiophot, version 4.6 (Carl Zeiss Microscopy, Germany), and Photoshop, version 7.0 (Adobe Systems, San Jose, CA), were utilized to design the figures.

Data analysis. Each test series was repeated twice, and in each replicate, three samples were analyzed per treatment. Microbiological data

TABLE 1 Water quality characteristics of chlorine wash water containing increasing amounts of organic matter as lettuce juice extract^a

LJE concn (%) ^b	Turbidity (NTU) ^c	TDS (mg/liter)	COD (mg/liter)
0	0.02 $\pm$ 0.01	38.67 $\pm$ 1.15	192.00 $\pm$ 10.00
0.25	4.59 $\pm$ 0.26	75.33 $\pm$ 0.58	323.67 $\pm$ 24.28
0.50	7.07 $\pm$ 0.15	94.67 $\pm$ 0.58	392.83 $\pm$ 20.82
0.75	10.60 $\pm$ 0.96	113.67 $\pm$ 1.53	486.17 $\pm$ 5.20
1.0	15.27 $\pm$ 1.22	124.67 $\pm$ 1.53	540.33 $\pm$ 34.49

^a Means and standard deviations of water quality parameters measured as described in Materials and Methods.

^b Volume of lettuce juice relative to the volume of chlorine wash water, expressed as a percentage.

^c NTU, nephelometric turbidity units.

(survivors or reductions, converted to log₁₀ CFU/coupon) were analyzed using the "mixed procedure" of the SAS program, with independent variables including the chlorine or LJE concentrations, pH of wash solutions (adjusted by citric acid or T-128), and the interaction between them. Means and standard deviations for microbiological data were calculated, and mean significant differences ($\alpha = 0.05$) among interactions were separated with the least significant difference procedure.

RESULTS

Water quality and FC concentration in CWS. CWS pH was not affected by increasing levels of LJE, as evidenced by the less than 0.1-pH unit fluctuation (data not shown). As expected, turbidity, TDS, and COD of CWS increased proportionally with addition of LJE (Table 1), which agreed with our previous results (23).

The FC concentration decreased significantly as increasing concentrations of LJE were added to chlorine wash solutions, regardless of T-128 application (Fig. 1). CWS with T-128 (pH 2.9 or 5.0) resulted in higher residual FC levels than the wash solutions of the same pH level adjusted by citric acid. In the presence of 1.0% LJE, the FC concentration dropped to 0.26 and 0.48 mg/liter for pH 5.0 and 2.9 for CWS adjusted by citric acid. In contrast, in the presence of the same level of LJE, FC levels decreased to 0.71 and 1.08 mg/liter in pH 5.0 and 2.9 in chlorine solutions containing T-128.

The manganese and monochloramine concentrations in wash water samples, including distilled water, 0.5 and 1.0% LJE, and CWS with FC (10 mg/liter) mixed with 1.0% LJE, with or without 0.1% T-128, ranged from 0.0 to 0.02 mg/liter for manganese and from 0.001 to 0.004 mg/liter for monochloramine. Thus, the trace levels of manganese and monochloramine in the CWS of this study did not interfere with the free chlorine DPD test results or thereby lead to an overestimate of the FC in CWS.

Effect of chlorine water with/without T-128 on *Salmonella* or *Pseudomonas* biofilm cells. After overnight incubation at 22°C for 24 h, *S. Thompson*, *S. Newport*, or *P. fluorescens* biofilm populations reached 7.6, 7.7, and 8.0 log₁₀ CFU/coupon, respectively. Compared to chlorine water without T-128 (pH 2.9 or 5.0 adjusted with citric acid), chlorine water with T-128 (at both pH 2.9 and 5.0) significantly ($P < 0.05$) enhanced the inactivation of bacteria in biofilms (Fig. 2). Notably, treatment with T-128 at neither pH 2.9 nor 5.0 without chlorine (0 at x axis) produced no reduction in *Salmonella* biofilm populations (Fig. 2).

For *Salmonella* cells, chlorine water (FC of 0.5 to 5.0 mg/liter, pH 6.5) reduced biofilm cell populations by 1.6 to 4.6 (*S. Thompson*) and 0.8 to 4.4 (*S. Newport*) log₁₀ CFU/coupon compared to

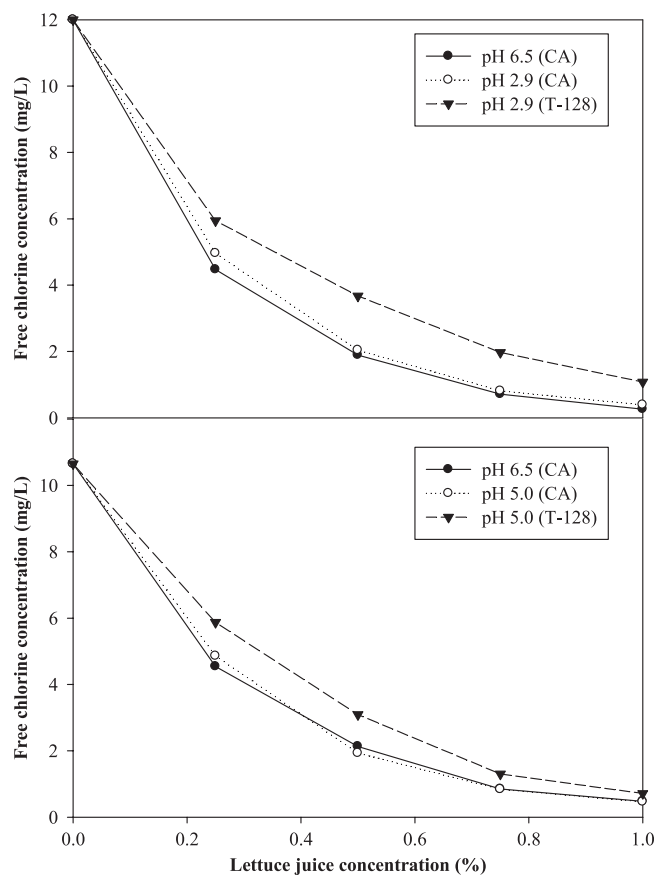


FIG 1 Effect of T-128 on chlorine retention in wash solutions containing various amounts of organic materials. CA, citric acid.

the populations of the untreated coupons (Fig. 2A and B). Pathogen population reductions in biofilms were not different in CWSs adjusted with citric acid to pH 6.5 and pH 2.9 (Fig. 2). In CWS at pH 2.9 adjusted by T-128, 1 and 2 mg/liter FAC significantly ($P < 0.05$) reduced viability of biofilm populations by an additional 1.6 to 1.7 $\log_{10}$ CFU/coupon (*S. Thompson*) (Fig. 2A) and 1.7 to 2.8 $\log_{10}$ CFU/coupon (*S. Newport*) (Fig. 2B) compared to populations in pH 2.9 chlorine solutions without T-128. At 5 mg/liter, CWS adjusted to pH 2.9 with T-128 reduced viability of pathogens in biofilms to below the detection limit, whereas without T-128 (pH 2.9), pathogens survived in biofilms at 3.0 $\log_{10}$ CFU/coupon (Fig. 2A and B). A similar trend in enhancing the sanitizing effect on biofilm populations was observed in chlorine solutions adjusted to pH 5.0 by T-128, compared to the same pH level adjusted by citric acid (Fig. 2). In *S. Thompson* and *S. Newport* biofilm cells, treatments (0.5 to 2.0 mg/liter FAC, pH 5.0) containing T-128 resulted in additional ($P < 0.05$) reductions of 0.7 to 1.5 $\log_{10}$ CFU/coupon compared to those of the citric acid-adjusted pH 5.0 (Fig. 2).

For *P. fluorescens*, 5 mg/liter of CWS (pH 6.5) reduced biofilm populations by 2.9 $\log_{10}$ CFU/coupon (Fig. 2C). CWS (FAC of 0 to 5 mg/liter) at pH 2.9 (adjusted by citric acid) reduced the *P. fluorescens* biofilm cell population by 3.2 to 3.7 $\log_{10}$ CFU/coupon (Fig. 2C), and these reductions were greater ($P < 0.05$) than those with pH 6.5 or 5.0 CWS at the same chlorine concentrations, indicating that *P. fluorescens* cells are sensitive to the low pH. The

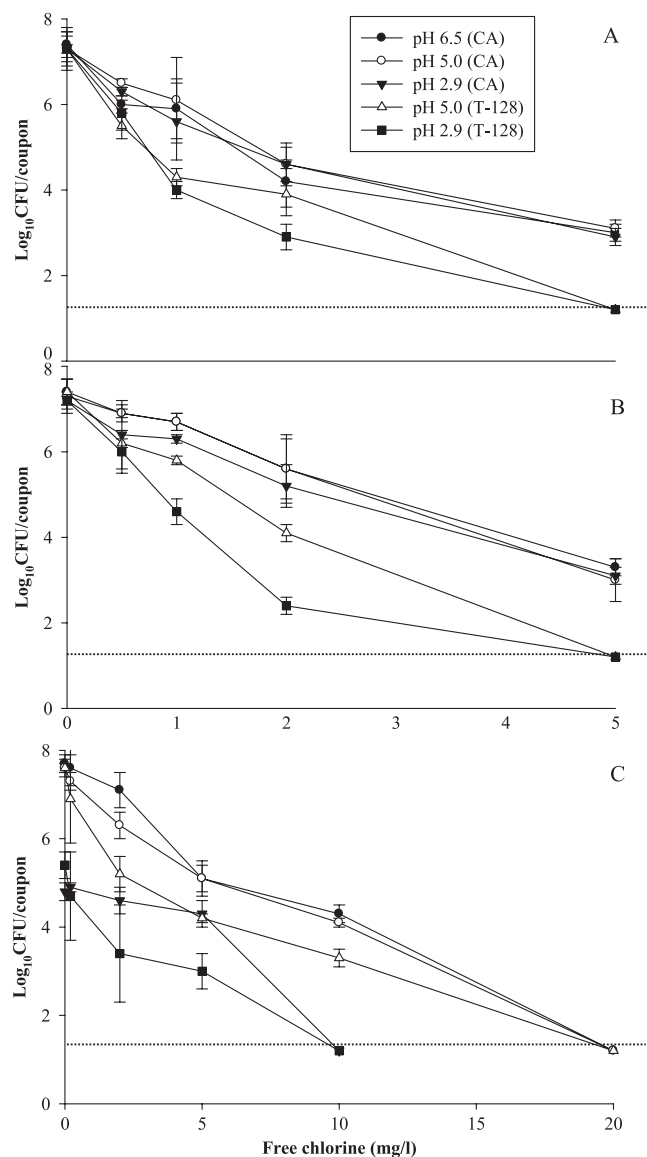


FIG 2 Effects of free chlorine (0 to 20 mg/liter) and T-128 (0.075% at pH 5.0 and 0.1% at pH 2.9), including T-128 without chlorine, in wash solution on the survival of *S. Thompson* (A), *S. Newport* (B), and *P. fluorescens* (C) in biofilms formed on stainless steel surfaces. The detection limit (dashed line) is 1.3 $\log_{10}$ CFU/coupon. The unwashed-biofilm populations of *S. Thompson* or *S. Newport* and *P. fluorescens* were 7.5 and 8.0 $\log_{10}$ CFU/coupon, respectively.

pH 2.9, nonchlorine, T-128 treatment reduced *P. fluorescens* populations by 2.6 $\log_{10}$ CFU/coupon (Fig. 2C), whereas the pH 5.0, nonchlorine, T-128 treatment did not show similar reductions (Fig. 2C). CWS at pH 2.9 or 5.0 with T-128 and with 2 to 10 mg/liter FAC showed an additional ($P < 0.05$) approximately 1.0 $\log_{10}$ CFU/coupon reduction of the biofilm population compared to CWS at the same citric acid-adjusted pH.

Effect of T-128 and organic loads on CWS efficacy against pure-culture *Salmonella* or *Pseudomonas* biofilms. The sanitizing effect of CWS on bacterial biofilms decreased with increasing concentrations of LJE in wash solutions, regardless of T-128 application. Generally, in comparison with the pH 6.5 CWS, adjustment of CWS to pH 2.9 with T-128 significantly ($P < 0.05$)

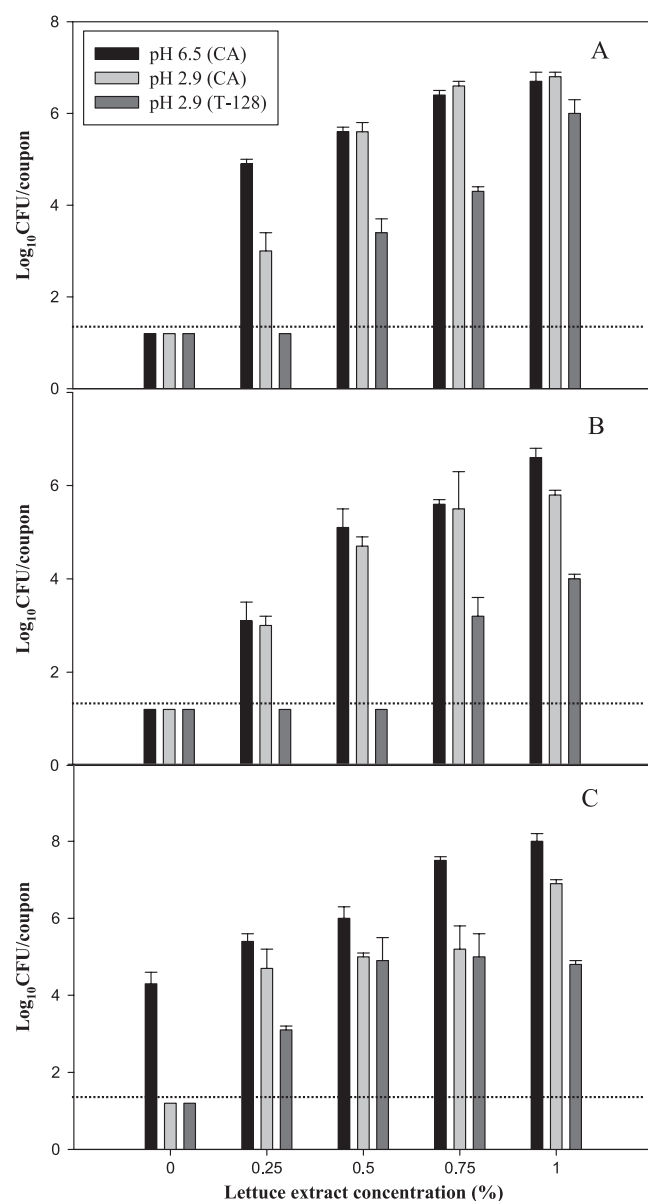


FIG 3 Recovery of *S. Thompson* (A), *S. Newport* (B), and *P. fluorescens* (C) biofilms formed on stainless steel coupons exposed to 10 to 12 mg/liter initial free-chlorine solutions at pH 2.9, with or without T-128, in the presence of different amounts of lettuce juice. The detection limit (dashed line) is 1.3 log₁₀ CFU/coupon. The unwashed-biofilm populations of *S. Thompson* or *S. Newport* and *P. fluorescens* were 7.5 and 8.0 log₁₀ CFU/coupon, respectively.

enhanced the killing of bacterial biofilms when different concentrations of LJE were added as a source of increasing organic loads. For instance, adjustment of chlorine solutions containing 0.75% LJE to pH 2.9 with T-128 resulted in an additional ($P < 0.05$) 2.3 log₁₀ CFU/coupon reduction of *S. Thompson* compared to the corresponding solution without T-128 (Fig. 3A). Similarly, CWS adjusted to pH 2.9 with T-128 and 1.0% LJE resulted in an additional ($P < 0.05$) 2.1 to 2.6 log₁₀ CFU/coupon reduction of *Pseudomonas* compared to CWS at pH 6.5 or 2.9 adjusted by citric acid instead of with T-128 (Fig. 3C).

In CWS adjusted to pH 5.0 with T-128, the number of surviving *Salmonella* bacteria in biofilms was approximately 0.5 to 1.5

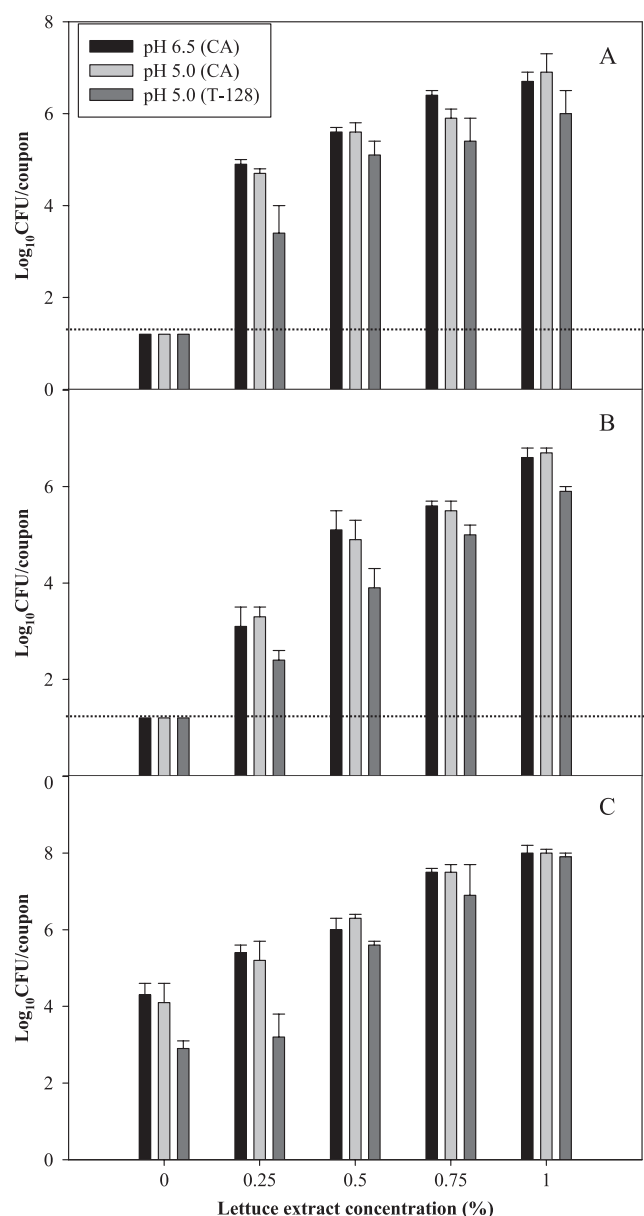


FIG 4 Recovery of *S. Thompson* (A), *S. Newport* (B), and *P. fluorescens* (C) biofilm populations formed at pH 5.0, with or without T-128, in the presence of different amounts of lettuce juice. The detection limit (dashed line) is 1.3 log₁₀ CFU/coupon. The unwashed-biofilm populations of *S. Thompson*, *S. Newport*, and *P. fluorescens* were 7.5, 7.2, and 8.0 log₁₀ CFU/coupon, respectively.

log₁₀ CFU/coupon less than in similar solutions at the same pH without T-128 (Fig. 4A and B). For *P. fluorescens* biofilms, an additional reduction of 0.6 to 1.4 log₁₀ CFU/coupon was observed in wash solutions containing T-128 compared to the citric acid-adjusted pH when 0.75% LJE was added to wash solutions (Fig. 4C). However, no reduction of *P. fluorescens* populations was observed in pH 5.0 washing solutions containing 1.0% LJE compared to populations in unwashed coupons (Fig. 4C).

Effect of T-128 and organic loads on CWS efficacy against cocultured *Salmonella* and *Pseudomonas* biofilms. In cocultures, after incubation at 22°C for 24 h, *S. Thompson* biofilm

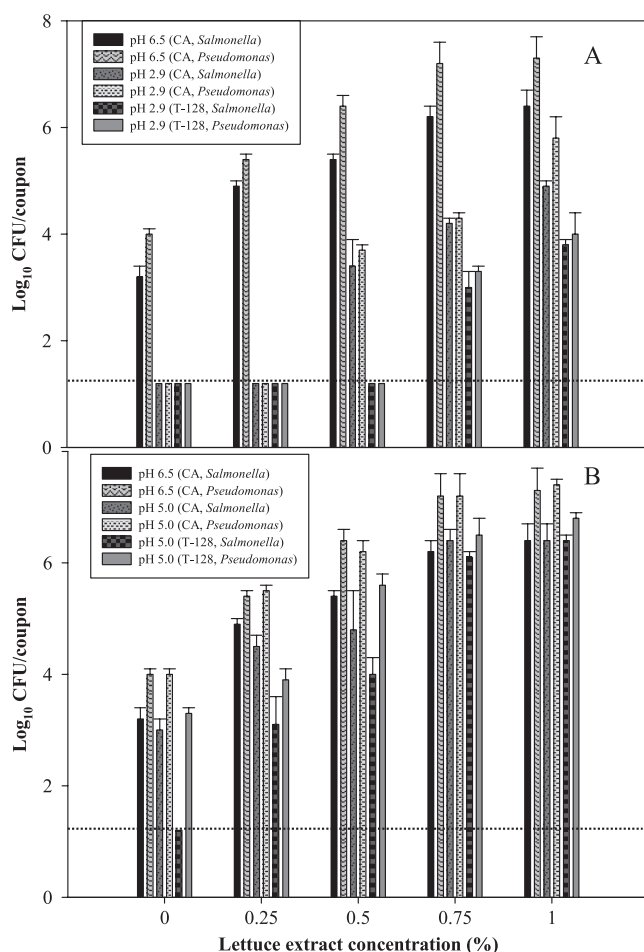


FIG 5 Recovery of cocultures of *S. Thompson* and *P. fluorescens* biofilm populations formed on stainless steel coupons exposed to free chlorine solutions at pH 2.9 (A) or 5.0 (B), with or without T-128, in the presence of different amounts of lettuce juice. The detection limit (dashed line) is $1.3 \log_{10}$ CFU/coupon. Unwashed-biofilm populations of *Salmonella Thompson* and *P. fluorescens* were 6.5 and $8.2 \log_{10}$ CFU/coupon, respectively.

populations ($6.5 \log_{10}$ CFU/coupon) were $1.0 \log_{10}$ CFU/coupon less than those of the pure-culture biofilms ($7.5 \log_{10}$ CFU/coupon) (Fig. 3 to 5). Coculture *P. fluorescens* populations were similar to those of the pure culture.

Compared to the pure cultures, there was a distinct difference in the responses of cocultured biofilm cells of *S. Thompson* and *P. fluorescens* to CWS at different pH levels. In the pH 6.5 or 5.0 CWS, pure-culture and coculture *P. fluorescens* biofilm cells were similar in their sensitivities to CWS, whereas *S. Thompson* coculture biofilms were less susceptible to CWS than the pure culture (Fig. 3 to 5). For example, no significant reductions of cocultured *S. Thompson* populations were found in wash solutions with 0.75% LJE (Fig. 5), while the reduction of its respective pure-culture cells ranged from 1.1 to 2.1 log CFU/coupon among all treatments (Fig. 4A). In contrast, in the pH 2.9 CWS, cocultured cells of both bacterial species were more sensitive to the chlorine treatment than their respective pure-culture cells (Fig. 3 to 5).

Coculture biofilm studies showed, in general, that CWS adjusted to pH 2.9 or 5.0 with T-128 again showed higher ($P < 0.05$)

inactivation on bacterial biofilms than CWS of the same pH adjusted with citric acid. In the pH 2.9 CWS with T-128, no survival of *S. Thompson* or *P. fluorescens* in biofilm was observed even when 0.50% LJE was added to the CWS (Fig. 5). In the high-organic-load (0.75 to 1.0% LJE) wash solutions, bacterial survival in biofilm was less ($P < 0.05$) in pH 2.9 CWS adjusted by T-128 than in the citric acid-adjusted CWS (Fig. 5A). At pH 5.0, CWS with T-128 reduced survival ($P < 0.05$) of *S. Thompson* and *P. fluorescens* until 0.50% and 1.0% LJE was added to the wash solutions, respectively (Fig. 5B).

Crystal violet staining of biofilm cells on stainless steel. As stained with 10% crystal violet, the purple matrix shown in Fig. 6AA indicates that cocultured bacterial cells of *S. Thompson* and *P. fluorescens* formed biofilm on stainless steel after 24 h of cultivation in LJE. Purple-stained bacterial biofilms were present on stainless steel coupons that were stationary or shaken in distilled or chlorinated water, regardless of T-128 presence (Fig. 6B, D, F, and H). The washed coupons that were ultrasonicated and vortexed showed reduced purple staining, as demonstrated in Fig. 6C, E, G, and I, indicating that this treatment actually removed some of the biofilm matrix. The viability of biofilm cells associated with these various treatments was verified by ultrasonication and vortexing and then plating cells recovered from a parallel set of stainless steel coupons not stained with crystal violet. Results in Table 2 show that ultrasonication and vortexing caused approximately a 1.2 to 1.3 $\log_{10}$ CFU/coupon increase in bacterial cell recovery in suspensions compared to those from stationary coupons. The number of surviving bacteria in distilled-water solutions was as great as $5.3 \log_{10}$ CFU/ml regardless of stationary or shaking treatment. Again, it is observed that T-128-adjusted CWS led to a 1.0- to 1.4- $\log_{10}$ reduction of bacterial survival in wash water and cell suspensions compared to samples without T-128.

Imaging viability of biofilm cells with CLSM. Biofilm cells and their response to sanitizers were evaluated using specialized glass-bottom petri dishes and stainless steel coupons to facilitate imaging. The clear matrix of green cells in Fig. 7A and B and 8A and B shows that *Salmonella* and *Pseudomonas* biofilms were formed on the glass-bottom dishes and stainless steel coupons after 48 h of cultivation with LJE. The two-channel laser confocal images of the biofilm cells in Fig. 7A, B, and C or Fig. 8A, B, C, and D correspond to the colocalization scatter graphs in Fig. 7D, E, and F or Fig. 8E, F, G, and H, respectively. The graphs illustrate the relative prevalence of live and dead cells in each corresponding frame based on the distribution and intensity of pixels detected, which are displayed as a color spectrum using an absolute intensity frequency scale across the x - and y -axis space. This array of the intensity distribution corresponds to detection of live (x axis) and dead (y axis) cells as well as the biofilm matrix. The SYTO9/propidium iodide stain produced a range of intermediate fluorescent colors (yellow, orange, rust, and brown) between green and red in the T-128 treatment, indicating loss of membrane integrity (25). As recommended (22), all green cells were considered live, whereas the other colors are considered to represent dead cells. A few red (dead) cells appear in the 2 mg/liter FC in CWS-treated samples on glass-bottom dishes (Fig. 7B) and stainless steel coupons (Fig. 8C), but a significantly greater number of orange-red-stained cells was present in samples treated with 2 mg/liter FC in CWS with T-128 at pH 5.0 (Fig. 7C and 8D).

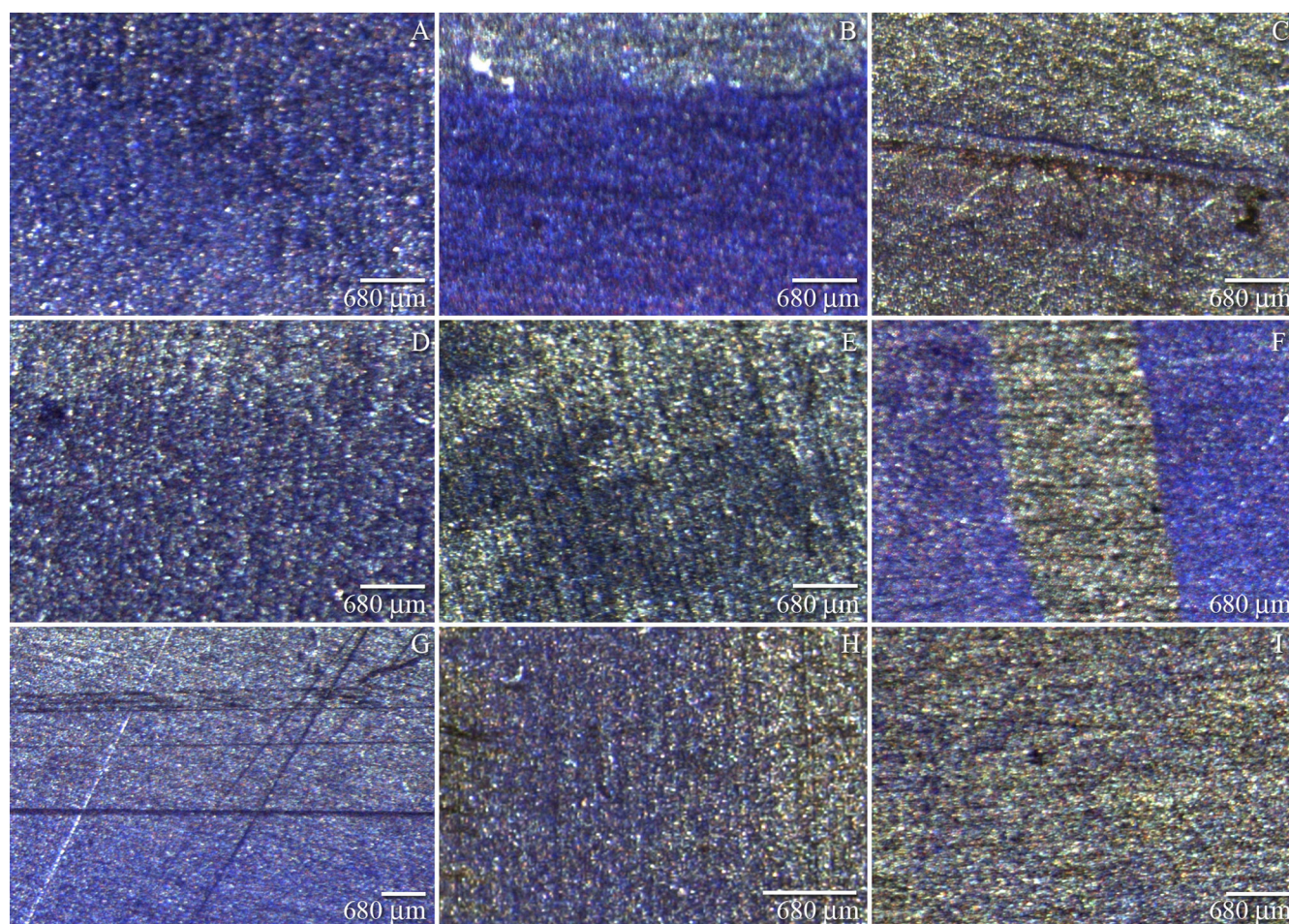


FIG 6 Crystal violet-stained microscopic images of cocultures of *S. Thompson* and *P. fluorescens* biofilms on stainless steel coupons exposed to chlorine wash solutions (CWS) at pH 5.0, with and without T-128. Treatments were as follows: control biofilm, untreated (A); distilled water, static treatment, without (B) or with (C) ultrasonication (U) and vortexing (V); distilled water, shaken treatment, without (D) or with (E) ultrasonication and vortexing; CWS (pH 5.0, citric acid), shaken treatment, without (F) or with (G) ultrasonication and vortexing; CWS (pH 5.0, T-128), shaken treatment, without (H) or with (I) ultrasonication and vortexing.

DISCUSSION

In the commercial fresh produce industry, the degradation of sanitizing concentrations of free chlorine results from accumulation of organic materials in wash water as freshly cut produce is introduced into processing wash solutions. The typical approach to maintaining sanitizer concentration involves periodic replenishment of chlorine. However, repeated addition of chlorine into

high-organic-load wash solutions will increase chlorine off-gas and, as some have suggested, possibly lead to formation of chlorine by-products, such as trihalomethanes and haloacetic acids (19). Furthermore, bacterial cells can accumulate on the surface, niches of washing machines, or tanks; biofilm formation can ensue, and this can be exceptionally resistant to physical and chemical removal.

To test the effect of T-128 on FC in different organic-load wash waters, LJE (0.25 to 1.0%) was added to 10 to 12 mg/liter of CWS in the presence or absence of T-128. The increases in turbidity, COD, and TDS in wash waters were mainly attributed to release of organic material from LJE into wash solutions (19). CWS in which pH was adjusted with T-128 resulted in higher residual FC than solutions with citric acid controls. This indicates that T-128 decreased the free chlorine depletion rate when high organic loads existed in wash waters. These results are in agreement with our previous studies (23) showing that 0.1% T-128 significantly decreased the free chlorine depletion rate in the presence of soil with organic matter. The mechanism by which T-128 tends to stabilize free chlorine in high-organic-load solutions is still unclear.

TABLE 2 Recovery of bacterial biofilm cells in wash waters and suspension solutions with or without ultrasonication and vortexing

Treatment(s) ^a	Bacterial recovery (log ₁₀ CFU/coupon)		
	Before ultrasonication and vortexing	After ultrasonication and vortexing	In washing liquid
DW, static	4.21 ± 0.05	5.42 ± 0.35	5.28 ± 0.12
DW, shaken	4.29 ± 0.12	5.52 ± 0.10	5.33 ± 0.24
HOCl, shaken	3.14 ± 0.14	4.51 ± 0.12	1.33 ± 0.16
HOCl + T-128, shaken	2.03 ± 0.25	3.44 ± 0.21	0.40 ± 0.05

^a DW, distilled water.

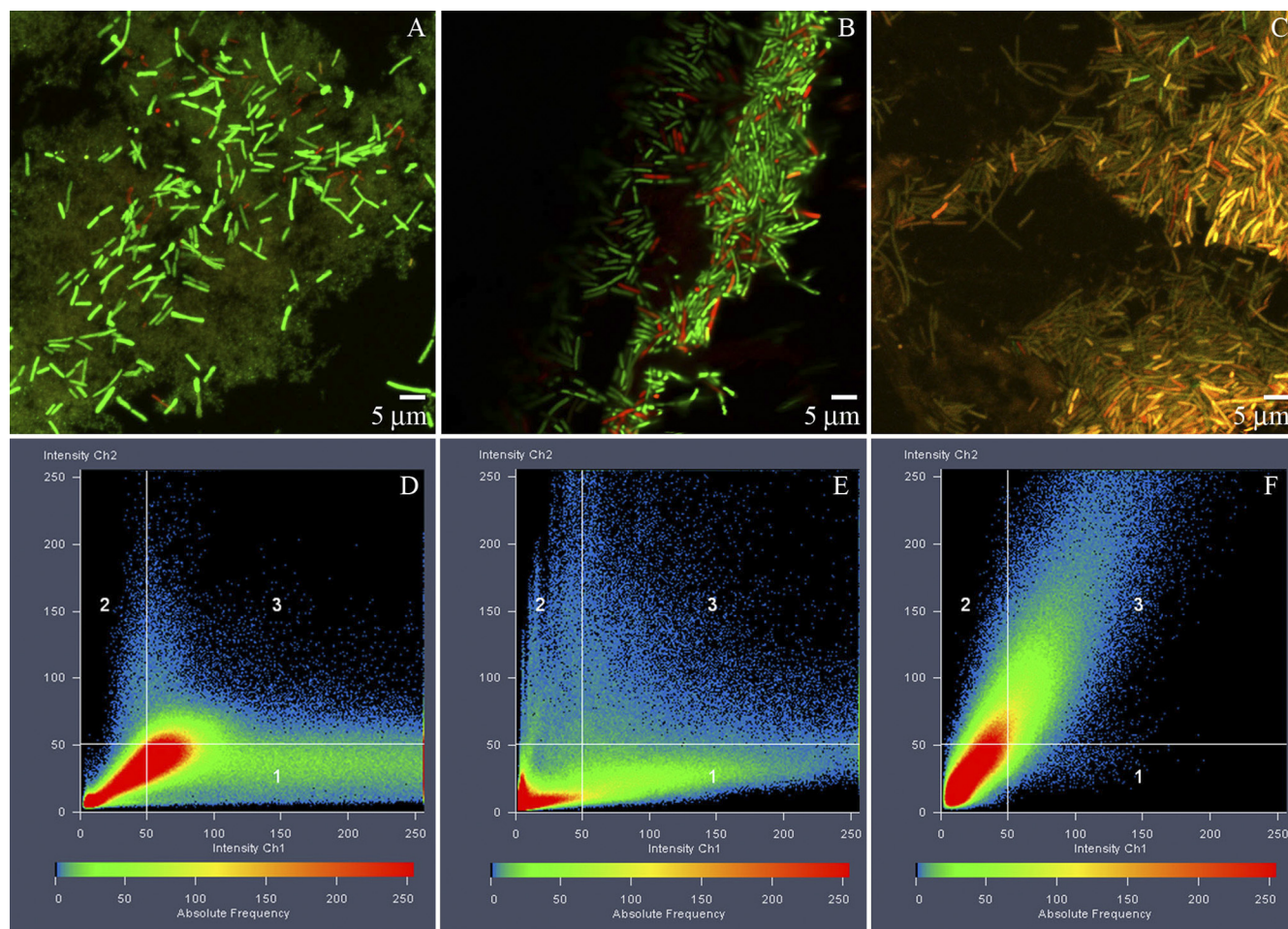


FIG 7 CLSM images of cocultured *S. Thompson* and *S. Newport* biofilms on glass coverslips exposed to chlorine wash solutions (CWS) at pH 5.0, with and without T-128. (A) Control biofilm, untreated. Green cells are live. (B) CWS (pH 5.0, citric acid) treatment of biofilm. Red cells are dead. (C) CWS containing 0.075% T-128 at pH 5.0. Orange, yellow, and red cells are membrane damaged and considered dead. Colocalization intensity graphs of green fluorescent, live (x axis) versus red, dead (y axis) cells in the untreated control biofilm from panel A (D) and in the CWS-treated biofilm from panel B (E) are shown. (F) Colocalization intensity graph of green fluorescent, live (x axis) versus red-orange, dead and dying (y axis) cells in the biofilm treated with CWS and T-128 shown in panel C. For all colocalization graphs, the regions designated 1, 2, and 3 correspond to regions where 50% or more of the pixels detected were green fluorescent (live cells), red fluorescent (dead cells), or intermediate colors (membrane-damaged cells), respectively.

The effect of T-128 on CWS inactivation of bacterial biofilm populations was determined using coupons inoculated with pure cultures of *Salmonella* or *P. fluorescens* followed by direct plating and colony enumeration on appropriate media. Our direct-plating results, which show significant reductions compared to the control treatments, are consistent with a similar trend reported for *Salmonella* detection from lettuce and in CWS by enrichment after 1-min exposures to 0 to 1% LJE and FC of 0.5 to 16 mg/liter in CWS (23). This suggests that our direct-plating approach did not substantially underestimate the survival of *Salmonella*.

Our results suggest that there is a synergistic effect between T-128 and FC in CWS relative to reduction of *Salmonella* biofilms on stainless steel coupons. This effect does not rely solely on low pH because a similar enhancing effect was not observed in pH 2.9 CWS adjusted with citric acid instead of T-128; and the enhanced reductions by T-128 in CWS on biofilm populations were also observed at pH 5.0, which is the target pH currently used by many fresh-cut produce processors that have implemented T-128 use. The mechanism for the synergistic effect between T-128 and CWS

in the inactivation of *Salmonella* biofilms remains to be determined. However, T-128 alone, without chlorine, did not show any reductions in *Salmonella* biofilm cell counts. This result agrees with a previous study (23), in which T-128 alone was found to have only weak bactericidal activity against *Escherichia coli* O157:H7 on lettuce.

Although *P. fluorescens* is not a food-borne pathogen, it can contribute to formation of biofilms by food-borne pathogens on contact surfaces. In this study, *P. fluorescens* biofilm cells were less sensitive to CWS than the *Salmonella* biofilm cells; however, they were sensitive to low-pH wash solutions. The low pH of wash water achieved by adding 0.1% T-128 was due to the phosphoric acid, one of its major components (Lemons, U.S. patent application 20090192231). Furthermore, the synergistic effect between T-128 and CWS against bacterial biofilms also occurred with *P. fluorescens*. Thus, T-128, as a washing aid, enhances the sanitizer efficacy of chlorine solutions against *P. fluorescens* biofilm populations, and this effect is likely due in part to low pH and the surfactant action of polyethylene glycol.

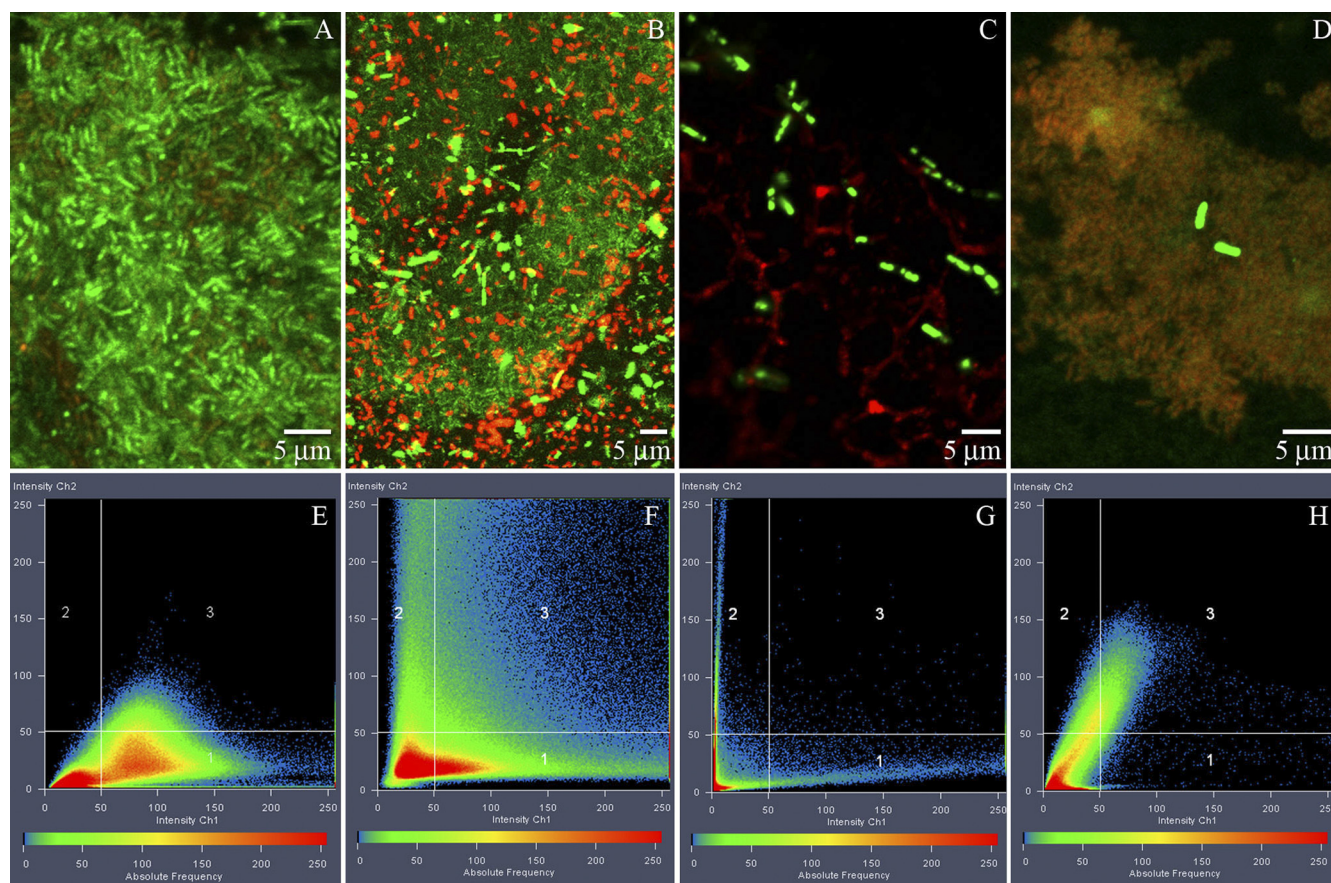


FIG 8 CLSM images of cocultured *S. Thompson* and *P. fluorescens* biofilms on stainless steel coupons exposed to chlorine wash solutions (CWS) at pH 5.0, with and without T-128. (A) Control biofilm, untreated. Green cells are live. (B) Distilled-water treatment of biofilm. Green cells are live, and red cells are dead. (C) CWS (pH 5.0, citric acid) treatment of biofilm. Red cells are dead. (D) CWS containing 0.075% T-128 at pH 5.0. Orange, yellow, and red cells are membrane damaged and considered dead. Colocalization intensity graphs of green fluorescent, live (x axis) versus red, dead (y axis) cells are shown for the untreated control biofilm from panel A (E) the distilled-water-treated biofilm from panel B (F), and the CWS-treated biofilm from panel C (G). (H) Colocalization intensity graph of green fluorescent, live (x axis) versus red-orange, dead and dying (y axis) cells in the biofilm treated with CWS and T-128 shown in panel D. For all colocalization graphs, the regions designated 1, 2, and 3 correspond to regions where 50% or more of the pixels detected were green fluorescent (live cells), red fluorescent (dead cells), or intermediate colors (membrane-damaged cells), respectively.

Evaluation of the effect of T-128 in CWS on biofilm inactivation in solutions of different organic loads was conducted using biofilms grown on stainless steel coupons with pure cultures of *S. Thompson*, *S. Newport*, or *P. fluorescens* treated with 10 to 12 mg/liter of FC in CWS with or without T-128. The enhanced reduction of bacterial biofilm cells observed in CWS adjusted to pH 2.9 with T-128 was partly due to its ability to maintain higher residual chlorine levels than citric acid.

Crystal violet staining as a method to verify the formation of bacterial biofilms on certain surfaces has been well documented (2, 7, 20). The purple matrix retained on stainless steel coupons that had been washed in distilled water and in CWS solutions, with or without T-128, shows that the surfactant component in T-128 did not simply dislodge the bacterial biofilm cells from the stainless steel during washing. The purple-stained matrix was present on coupons following ultrasonication and vortexing, indicating that biofilms were still present on coupons and available for further microbial analysis. The bacterial recovery results (Table 2) correspond to results from other experiments in this study and verify that T-128 assists CWS in reducing viability of bacterial biofilm cells in wash solutions and on stainless steel coupons.

The morphology and structure of bacterial biofilms can be effectively analyzed by CLSM combined with fluorescent staining techniques (15). The differential, live-dead staining of a slightly greater number of red cells present in the control than in the 2 mg/liter CWS-treated samples showed that 2 mg/liter FC killed some cells; however, most cells were still viable and thus fluoresced green. Viability was corroborated in the cultural assays. In contrast, most cells in samples treated with chlorine and T-128 stained yellow, orange, red, or rust, indicating loss of membrane integrity; these cells were deemed dead accordingly (21). The images of T-128 chlorine treatment corroborate the cultural assay data that show significantly reduced viability of *Salmonella* biofilm cells exposed to T-128 CWS.

In commercial fresh produce processing facilities, biofilms are most likely formed by a consortium of microorganisms. Thus, coculture of *Salmonella* and *Pseudomonas* was used in this biofilm study in addition to pure cultures of these bacteria. Results showed that in cocultures the *P. fluorescens* populations were similar to those of the pure culture, indicating the competitive advantage of *Pseudomonas* in coculture with *Salmonella* even when initial inoculum concentrations were equivalent. This result was

similar to that reported by Fatemi and Frank (6) and Lourenço et al. (17), in which *Pseudomonas* was the dominant species in the total coculture biofilm of *Listeria monocytogenes* and *Pseudomonas* sp. At pH 5.0, cells of *S. Thompson* in coculture biofilms were more resistant to CWS than those in pure-culture biofilms. This finding agrees with other reports (6, 17, 22), which state that pathogenic bacteria were protected by *Pseudomonas* sp. when mixed-culture biofilms were exposed to the sanitizers. However, at pH 2.9, the opposite was observed. This can be explained by the sensitivity of *P. fluorescens* cells in the biofilm matrix to low pH. When *S. Thompson* lost the protection of the *P. fluorescens* biofilm matrix, it became more sensitive to the sanitizer.

The present study has demonstrated that washing in CWS does not eliminate viable *Salmonella* or *P. fluorescens* from biofilms formed on stainless steel. In contrast, T-128 at pH 2.9 or pH 5.0 with CWS enhanced *Salmonella* and *P. fluorescens* population reductions in biofilms on stainless steel. Thus, use of T-128 in fresh-cut produce processing environments shows promise as a sanitization enhancer on stainless steel and possibly also other types of surfaces commonly present on food-processing equipment and for increased inactivation of pathogenic or spoilage bacteria in biofilms.

ACKNOWLEDGMENTS

We are grateful for the support provided by grant number 2009-74 from the Center for Produce Safety, University of California, Davis, CA, for this research and for the supply of T-128 provided by New Leaf Food Safety Solutions, LLC, Salinas, CA.

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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