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Evaluation of Single or Double Hurdle Sanitizer Applications in Simulated Field or Packing Shed Operations for Cantaloupes Contaminated with *Listeria monocytogenes*

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Abstract: *Listeria monocytogenes* contamination of cantaloupes has become a serious concern as contaminated cantaloupes led to a deadly outbreak in the United States in 2011. To reduce cross-contamination between cantaloupes and to reduce resident populations on contaminated melons, application of sanitizers in packing shed wash water is recommended. The sanitizing agent of 5% levulinic acid and 2% sodium dodecyl sulfate (SDS) applied as a single hurdle in either a simulated dump or dip treatment significantly reduced *L. monocytogenes* to lower levels at the stem scar compared to a simulated dump treatment employing 200 ppm chlorine; however pathogen reductions on the rind tissue were not significantly different. Double hurdle approaches employing two sequential packing plant treatments with different sanitizers revealed decreased reduction of *L. monocytogenes* at the stem scar. In contrast, application of sanitizers both in the field and at the packing plant led to greater *L. monocytogenes* population reductions than if sanitizers were only applied at the packing plant.

Keywords: levulinic acid; sodium dodecyl sulfate; cantaloupes; stem scar; *Listeria monocytogenes*

1. Introduction

Listeria monocytogenes has been identified as a pathogen of concern for the cantaloupe industry. In 2011, Colorado (USA) grown cantaloupes were determined to be contaminated with *L. monocytogenes* present on harvesting equipment and in the packing facility [1]. An ensuing outbreak resulted in 147 illnesses, 1 miscarriage, and 33 deaths [2]. Its widespread prevalence is another cause for concern. A study of five farms in New York State revealed the prevalence of *L. monocytogenes* in produce fields was 15% compared to 4.6% and 2.7% for *Salmonella* and Shiga toxin-producing *Escherichia coli*, respectively [3]. *L. monocytogenes* has been detected in sewage, water, soil, vegetation, silage, in domestic and wild animals, and in food processing plants [4].

Given the potential for *L. monocytogenes* contamination in cantaloupe packing facilities, the application of sanitizers in such environments is critical. Currently, many processing facilities utilize sanitizers (primarily chlorine or chlorine dioxide) in dump tanks that may contain more than several thousand liters of water. The water in these tanks serves both as a vehicle for unloading and moving the melons onto conveyor belts as well as to wash debris from the product's surface. However, to prevent cross-contamination of contaminated to uncontaminated melons during this stage of processing, it is recommended that the water be chlorinated at a rate of 150 ppm of free chlorine [5]. Reduction of pathogens on cantaloupe surfaces may occur during exposure of the melons to chlorine for nearly 10 min in these tanks, but it is minimal (1 to 2 log). Hence, alternative sanitizers have been evaluated. Rodgers *et al.* [6] reported greater than 5-log colony forming units (CFU)/g reductions of *L. monocytogenes* on inoculated cantaloupes treated for 5 min with peracetic acid (80 ppm), chlorinated trisodium phosphate (100 and 200 ppm), chlorine dioxide (3 and 5 ppm), and ozone (3 ppm). In another study, a chlorine dioxide gas (5 ppm) treatment for 10 min reduced *L. monocytogenes* levels by 4.3 log CFU/5 cm² [7]. Unfortunately, sanitizers such as chlorine dioxide and ozone must be generated on site and used in concentrations less than 3 ppm to ensure worker safety in processing facilities [8]. Hence, plant-derived antimicrobials, such as carvacrol, thymol, β -resorcylic acid, and caprylic acid, that would not jeopardize worker safety have been studied for their potential as a sanitizing agent in the presence or absence of hydrogen peroxide and have been shown to reduce *L. monocytogenes* levels from 2.5 to 6 log CFU/cm² on cantaloupe rinds [9]. Unfortunately, application in a commercial dump tank could require large quantities of the antimicrobial. Insertion of a smaller volume dip tank (<1000 liters) into the packing line where the melons would be exposed to the antimicrobial for a short period of time prior to their being sorted and packed could be a long-term, cost-effective alternative and one that would not reduce the packing line speed. However, additional up-front costs would be required for purchasing and incorporating these dip tanks into packing lines and an alternative mode for unloading melons in the absence of a dump tank would need to be devised and implemented.

Levulinic acid and sodium dodecyl sulfate (SDS) are generally recognized as safe food additives by the United States Food and Drug Administration (FDA) for specific purposes and have the potential to be produced in large quantities at low cost [10,11]. Previous studies have revealed that these combined chemicals can reduce pathogenic bacteria on lettuce, alfalfa seed, and tomatoes [12–15]. Based on the potential low cost and demonstrated efficacy of these chemicals, they have recently been investigated as a sanitizing agent in cantaloupe wash water. In that investigation, 2% levulinic acid in combination with 0.2% SDS in cantaloupe wash water reduced *Salmonella* Poona populations by 3.4 and 4.5 log CFU/g

on netted rind tissue after a 6 min simulated dump tank or dump tank with brushing treatment, respectively, compared to reductions of 1.5 and 2.6 log CFU/g when chlorine (120 ppm) was used as the sanitizing agent under those same treatment conditions [16].

Although single hurdle interventions would be desirable from the standpoint of reduced cost and processing time, adoption of double hurdle interventions could be potentially advantageous if their implementation were to generate additive or synergistic reductions to pathogen populations on contaminated cantaloupes. Therefore, the purpose of this study was to determine the fate of *L. monocytogenes* on stem scar and netted rind tissues when cantaloupes were exposed to sanitizers in single or double hurdle applications. Single hurdle approaches included a simulated dump tank or 1-min dip treatment, whereas the double hurdle approach included either: (1) a simulated dump tank treatment followed by a simulated dip treatment; or (2) a field-applied injection and spray treatment followed by the simulated dump tank treatment. During these treatments, several sanitizers (*i.e.*, chlorine, chlorine dioxide, and levulinic acid and SDS) were compared for their efficacy.

2. Results and Discussion

2.1. Effectiveness of Sanitizers for Reduction of *L. monocytogenes* from Cantaloupe Stem Scar and Rind Tissue

Sanitizers (chlorine and chlorine dioxide) commonly used in dump tanks by cantaloupe growers and processors were compared with 5% levulinic acid/2% SDS for their efficacy in reducing *L. monocytogenes* populations on cantaloupe surface tissue. *L. monocytogenes* populations in stem scar and rind tissue of cantaloupes exposed for 8 min to chlorine (200 ppm) or chlorine dioxide (3 ppm) in a simulated dump tank were not statistically different from non-treated (control) melons (Tables 1 and 2). In contrast, *L. monocytogenes* populations on both stem scar and netted rind tissues decreased significantly by 2.4 log CFU/sample when cantaloupes were treated in a simulated dump tank containing 5% levulinic acid/2% SDS compared to the non-treated control melons (Tables 1 and 2). In a previous study, greater reductions had been observed with levulinic acid and SDS compared to chlorine for netted rind tissue contaminated with *Salmonella* Poona, but in that case, the effectiveness of both chemicals in reducing *Salmonella* populations was much greater than seen here with *L. monocytogenes* [16].

2.2. Fate of Surviving *L. monocytogenes* during Storage at 4 °C Following Sanitizer Treatment

Storage at 4 °C slows or inhibits the growth of most bacteria; however, the psychrotrophic characteristics of *L. monocytogenes* allow it to grow in cold temperatures [17,18]. Hence, cantaloupes treated with chlorine, chlorine dioxide, and levulinic acid/SDS were analyzed for *L. monocytogenes* after short-term (3-day period representing transport time to market) and long-term (15-day period representing melons held refrigerated for duration of shelf life) storage. Growth of *L. monocytogenes* did not occur in any of the treated or non-treated cantaloupes during storage. In fact, populations on treated and non-treated Day 3 stem scar samples were statistically less than found on Day 0 samples and treated Day 3 rind samples were statistically less than Day 0 samples (Tables 1 and 2). With continued storage to 15 days, further decreases in populations were statistically significant for treated rind samples but not for stem scar samples. As population decreases during storage were greater when the cantaloupes

had been treated with either chlorine or levulinic acid/SDS, it may be presumed that for many of the cells not inactivated by these chemicals, the tolerance of the pathogen to stresses encountered during storage was reduced and contributed to further inactivation of *L. monocytogenes*.

Table 1. *Listeria monocytogenes* populations on cantaloupe stem scar surface tissue after sanitizer treatment and storage at 4 °C for 0, 3 or 15 days.

Sanitizer ^b	log CFU/sample ^a		
	(log reduction compared to Day 0 control)		
	Day 0	Day 3	Day 15
None (control) ^c	6.5 ± 0.6 A	4.6 ± 0.7 CD (1.9)	5.6 ± 0.3 A–C (0.9)
Chlorine, 200 ppm	5.6 ± 1.0 A–C (0.9)	3.5 ± 1.0 E (3.0)	4.2 ± 0.4 DE (2.3)
Chlorine dioxide, 3 ppm	6.3 ± 0.4 AB (0.2)	4.8 ± 0.2 CD (1.7)	5.4 ± 0.4 BC (1.1)
5% Levulinic acid/2% SDS	4.1 ± 1.5 DE (2.4)	2.3 ± 0.8 F (4.2)	2.3 ± 1.8 F (4.2)

^a Mean ± SD per 2.5 cm² sample. Each value was derived from two replicate trials and in each trial $n = 3$. Mean ± SD not followed by the same letter are statistically different ($p < 0.05$); ^b Cantaloupes exposed to sanitizer for 8 min in simulated dump tank; ^c Control, non-treated.

Table 2. *Listeria monocytogenes* populations on cantaloupe rind surface tissue after sanitizer treatment and storage at 4 °C for 0, 3 or 15 days.

Sanitizer ^b	log CFU/sample ^a		
	(log reduction compared to Day 0 control)		
	Day 0	Day 3	Day 15
None (control) ^c	4.4 ± 1.9 AB	3.4 ± 1.7 BC (1.0)	2.1 ± 1.7 C–E (2.3)
Chlorine, 200 ppm	3.6 ± 1.2 B (0.8)	2.2 ± 1.3 CE (2.2)	0.1 ± 0.2 G (4.3)
Chlorine dioxide, 3 ppm	5.2 ± 0.4 A (–0.8)	3.3 ± 0.8 B–D (1.1)	1.6 ± 1.0 EF (2.8)
5% Levulinic acid/2% SDS	2.0 ± 1.4 DE (2.4)	0.1 ± 0.3 G (4.3)	0.4 ± 0.6 FG (4.0)

^a Mean ± SD per 2.5 cm² sample. Each value was derived from two replicate trials and in each trial $n = 3$. Mean ± SD not followed by the same letter are statistically different ($p < 0.05$); ^b Cantaloupes exposed to sanitizer for 8 min in simulated dump tank; ^c Control, non-treated.

2.3. Detection of *L. monocytogenes* in Cantaloupe Flesh after Simulated Dump Tank Treatment

The immersion of cantaloupes into dump tanks after harvest has been described by Richards and Beuchat [19] as a potential route for infiltration of pathogens into internal tissues. Consequently, the grower practice of sanitizing cantaloupes in tanks using ground water (20 to 22 °C) plus sanitizer was simulated to determine if surface-inoculated *L. monocytogenes* could infiltrate stem scar and netted rind tissue. *L. monocytogenes* was isolated in both positive controls (inoculated but non-immersed) as well as cantaloupes immersed in sanitizer solutions with more *Listeria* found in internal stem scar tissue (17%–28%) than in internal tissue beneath the rind (6%–11%, Table 3). Only 3 of 144 surface samples (2.0%) tested *L. monocytogenes*-positive by enrichment culture, but in all those cases, the corresponding flesh sample tested *L. monocytogenes*-negative (Table 3). Hence, steaming was considered an effective method for inactivating pathogens on the surface and preventing cross-contamination during sampling of internal flesh samples. In terms of the mode by which the pathogen reaches the internal tissues, the similar frequency of internalized contamination observed between non-immersed and immersed melons

for each type of tissue argues against immersion in dump tanks as being a likely scenario in this study (Table 3). Hence, contamination of internal tissue likely occurred during inoculation through passive diffusion of the liquid inoculum. Moreover, the increased prevalence of infiltration of *Listeria* into stem scar tissue compared to rind tissue could likely be attributed to the greater porosity of the stem scar tissue compared to the rind tissue. Following harvest of cantaloupes, physiological changes by the cantaloupe tissue may occur as the fruit encounters different temperatures. Thus during the commercial practice of holding melons in shaded staging areas, the pore size may shrink, in which case its subsequent transfer to the dump tank could lead to a decreased possibility of infiltration of pathogens through the stem scar.

Table 3. Presence of *Listeria monocytogenes* in cantaloupe flesh after simulated dump tank treatment and storage for 0, 3, and 15 days at 4 °C ^{a,b}.

Sanitizer ^c	# <i>L. monocytogenes</i> Positive by Enrichment Culture/# Samples Analyzed			
	Stem scar tissue w/o surface layer ^d	Stem scar surface layer ^d	Rind tissue w/o surface layer ^d	Rind surface layer ^d
None (control)	5/18	0/18	2/18	0/18
Chlorine, 200 ppm	3/18	2/18 ^e	2/18	0/18
Chlorine dioxide, 3 ppm	5/18	1/18 ^e	1/18	0/18
5% Levulinic acid/2% SDS	5/18	0/18	1/18	0/18

^a Two replicate trials were performed with $n = 18$. Day 0, 3, and 15 day storage data combined; ^b No positive values were obtained for non-inoculated, non-treated controls, data not shown; ^c Cantaloupes treated for 8 min in sanitizer solution, then held for 5 min at room temperature before either storing at 4 °C or analyzing the sample; ^d Cantaloupes were subjected to a 6-min steam treatment to inactivate pathogens on the surface. Stem scar and rind pieces were then immediately removed using a sterile knife and divided into flesh and surface samples of 3.13 cm³ each; ^e *L. monocytogenes*-positive surface samples in this column corresponded to *L. monocytogenes*-negative flesh samples, indicating no cross contamination during sample preparation.

2.4. Comparison of Single Hurdle to Double Hurdle Approaches Incorporating Sanitizer Treatments

Effectiveness of both single and double hurdle sanitizer treatments was greater on rind tissue than stem scar tissue in that *L. monocytogenes* populations in treated stem scar samples were still large enough to be detected by plate count enumeration but the pathogen could only be detected by enrichment culture in treated rind samples (Table 4). Similar levels of prevalence of the pathogen was found in treated rind samples implying that double and single hurdle treatments were equally effective. In the case of stem scar samples, the chlorine tank treatment (200 ppm) resulted in only a 1-log CFU/sample reduction of *L. monocytogenes* compared to non-treated cantaloupes. However, in comparison to non-treated cantaloupes, treatments incorporating 5% levulinic acid/2.5% SDS reduced *L. monocytogenes* in stem scar tissue by 3.4 and 1.4 log CFU/sample in the single and double hurdle approaches, respectively (Table 4). The decreased effectiveness of the double hurdle approach suggests that chlorine altered the pathogen or tissue in some manner as to diminish the effectiveness of the levulinic acid and SDS dip treatment. One possibility could include activation of the pathogen's defenses by chlorine. Alternatively, chlorine may react with the stem scar tissue to make it less porous and accessible for levulinic acid and SDS to penetrate and reach the pathogen. In any event, incorporation of a levulinic acid/SDS dip treatment as a second hurdle would likely have only a minimal impact on reducing *Listeria* on cantaloupes in commercial operations employing chlorine dump tanks. As was the case with stem scar tissue, there was

no advantage to employing a double hurdle rather than a single hurdle approach to eliminating the pathogen from rind tissue.

Table 4. *Listeria monocytogenes* recovered from cantaloupe stem scar and rind tissue ^a following treatment with sanitizers in single hurdle versus double hurdle approach ^b.

Hurdle	Sanitizer ^d	Log <i>L. monocytogenes</i> CFU/ sample ^e or # positive by enrichment culture/# samples (log reduction compared to control)	
		Stem Scar ^e	Netted Rind ^f
None	None (control)	4.4 ± 1.0 C	4.4 ± 1.4
Single	200 ppm chlorine in dump tank	3.3 ± 1.5 BC (1.1)	38/41
	5% levulinic acid/2.5% SDS in dip tank	1.0 ± 1.4 A (3.4)	34/41
Double	200 ppm chlorine in dump tank followed by 5% levulinic acid/2.5% SDS in dip tank	3.0 ± 0.8 B (1.4)	35/41

^a Stem scar and netted rind samples are 3.13 cm³, two replicate trials performed; ^b No positive values were obtained for non-inoculated, non-treated controls, data not shown; ^c Mean ± SD, stem scar means not followed by the same letter are significantly different ($p < 0.05$); ^d Dump tank treatments were for 10 min, whereas dip treatments were for 1 min; ^e The number of samples analyzed was $n = 6$ (control) and $n = 8$ (single/double hurdle treatments); ^f The number of samples analyzed was $n = 24$ (control) and $n = 41$ (single/double hurdle treatments).

2.5. Stem Scar Injection and Spray Treatment of Cantaloupes Prior to Inoculation and Simulated Dump Tank Treatment

An injection treatment of cantaloupe stem scar tissue with 200 µL 7.5% levulinic acid/0.5% SDS combined with a spray sanitizer treatment of 30 mL 7.5% levulinic acid/0.5% SDS administered to the entire cantaloupe surface immediately after harvest was evaluated as a means to prevent subsequent cross-contamination of freshly harvested cantaloupes during transport to the packing shed. Therefore, after application of the field sanitizer treatments, cantaloupes were spot- or soil-inoculated to simulate contact with contaminated liquids or surfaces on transport trailers. Moreover, in this set of experiments, cultures were prepared from solid media compared to liquid media as studies conducted by Uesegi *et al.* and Theofel and Harris [20,21] and preliminary studies conducted in our laboratory have demonstrated that cultures prepared from solid media were more resistant to desiccation than liquid cultures. Once inoculated, the cantaloupes were held for a short period before subjecting them to the dump tank washing hurdle, typically applied by growers in the southeastern United States, using either 200 ppm chlorine or 5% levulinic acid/2% SDS.

L. monocytogenes populations on rind samples of soil-inoculated cantaloupes were not significantly different for melons receiving either the chlorine or levulinic acid/SDS treatment as their second hurdle (Table 5). In contrast, both rind samples from spot-inoculated cantaloupes and stem scar samples from either spot- or soil-inoculated cantaloupes were significantly less when the second hurdle employed a levulinic acid/SDS treatment as opposed to a chlorine treatment ($p < 0.05$). To determine whether any antimicrobial activity had occurred by the first hurdle employing the levulinic acid/SDS injection and spraying, comparisons of the net reductions in this experiment to the previous experiments employing a levulinic acid/SDS (Table 1) or a chlorine (Tables 1 and 4) dump tank were made. Stem scar and rind samples averaged net reductions of more than 1.5 and 2.0 fold greater, respectively, for the double hurdle

approach compared to the single hurdle. These comparisons would therefore suggest that both stem scar injection and a field spray treatment with levulinic acid/SDS did exert some antimicrobial activity. These results are in agreement where *Salmonella* inactivation was observed following vacuum diffusion of sanitizers into tomato stem scar tissue [22].

Table 5. *Listeria monocytogenes* populations on stem scar and rind tissues of non-treated cantaloupes and cantaloupes treated by injection of stem scar and spraying of cantaloupes ^a prior to inoculation ^b and then exposing the cantaloupes to a sanitizer as a second hurdle ^c.

2nd Hurdle Sanitizer ^e	Log CFU/sample ^d			
	(log reduction compared to control)			
	Spot Inoculation		Soil Inoculation	
	Stem Scar	Rind	Stem Scar	Rind
None (control) ^f	6.7 ± 0.2 A	7.2 ± 0.3 A	5.8 ± 0.4 A	5.5 ± 0.5 A
200 ppm Chlorine	5.3 ± 0.2 B (1.4)	2.6 ± 1.4 B (4.6)	3.9 ± 1.0 B (1.9)	0.4 ± 0.9 B (5.1)
5% levulinic acid/2% SDS	3.9 ± 1.2 C (2.8)	1.4 ± 1.5 C (5.8)	2.2 ± 1.6 C (3.6)	0.3 ± 0.7 B (5.2)

^a Stem scars were injected with 200 µL of 7.5% levulinic acid/1.0% SDS after which the entire cantaloupe was sprayed with ca. 30 mL of 7.5% levulinic acid/0.5% SDS; ^b Solid media preparation used for inoculation; ^c No positive values were obtained for non-inoculated, non-treated controls; ^d Mean ± S.D per 3.13 cm³ sample. Within each column, means not followed by the same letter are significantly different ($p < 0.05$). Four replicate trials occurred with $n = 5$ for each trial; ^e Cantaloupes were treated in simulated dump tanks for 10 min; ^f Positive controls were inoculated but did not receive a first or second hurdle treatment.

3. Experimental Section

3.1. Cantaloupes

Freshly harvested Eastern variety cantaloupes (*Cucumis melo* L. var. *reticulatus* cv. Athena) were obtained from a commercial grower in Tifton, GA. The melons were chosen from the transport trailers prior to washing and packing, to be of similar maturity, size, degree of netting, and free of any visible blemishes.

3.2. Bacterial Strains

Five strains of *Listeria monocytogenes* (2011L-2624, 2011L-2625, 2011L-2626, 2011L-2663, and 2011L-2676) isolated from patients who had consumed cantaloupe involved in a 2011 outbreak, were obtained from the Centers for Disease Control and Prevention. Each strain was transformed with plasmid pNF8 [23] containing genes to produce green fluorescence and erythromycin resistance [24]. The resulting *L. monocytogenes* pNF8 strains LD22 (2011L-2625), LD23 (2011L-2626), LD24 (2011L-2663), and LD25 (2011L-2676) produced bright green colonies under a Dark Reader trans-illuminator at ~500 nm (Clare Chemical, Dolores, CO, USA). *L. monocytogenes* wild type and pNF8 strains were stored at -80 °C in brain heart infusion broth (BHIB) (Neogen, Lansing, MI, USA) with 25% glycerol. Strains from frozen stock were struck on to brain heart infusion agar (BHIA) (Neogen) or BHIA supplemented with 8 µg/mL erythromycin (MP Biomedicals, LLC, Santa Ana, CA, USA), BHIAE, and incubated at

37 °C for 18–21 h. *L. monocytogenes* wild-type or brightly glowing green colonies were re-streaked on BHIA or BHIAE, respectively, and incubated at 37 °C for 18–21 h.

3.3. Preparation of Liquid- and Soil-based Inoculum

Wild-type *L. monocytogenes* strains were used for the simulated dump tank storage studies due to the potential instability of the pNF8 plasmid during storage where potential proliferation could occur [23], whereas *L. monocytogenes* pNF8-containing strains were used in all non-storage studies for ease of detection and limited plasmid segregation pressure after short-term exposure to cantaloupe surfaces.

Preliminary experiments revealed that *L. monocytogenes* inoculum prepared from solid media plates was more resistant to sanitizer treatment after a 2-h incubation prior to sampling, therefore solid media-prepared cultures were used for spot and soil inoculation in the field spray treatment study (Table 5). Liquid-media prepared cultures were used for all other studies because preliminary experiments revealed no difference in the two culture preparations inoculated on cantaloupes and incubated for 16 to 18 h prior to treatment and sampling (data not shown).

Inoculum preparation was initiated by taking either one or two colonies of each wild-type strain to individually inoculate 50 mL of BHIB (liquid media) or one or two *L. monocytogenes* pNF8 colonies to inoculate either 50 mL of BHIB supplemented with 8 µg/mL erythromycin, BHIBE (liquid media) or 3 BHIAE plates (solid media). The liquid media cultures were incubated at 37 °C with agitation (150 rpm) for 21–24 h, and the solid media plates were incubated for 24 h at 37 °C. After incubation, 4 mL of 0.1% peptone water was added to each solid media plate, the colonies were gently removed with a glass spreader, collected in a 50-mL centrifuge tube, and the process was repeated to dislodge remaining cells from the plate. Both liquid media cultures and suspended solid media cultures were sedimented by centrifugation (4193× *g* for 15 min at 4 °C) and washed two times in sterile 0.1% peptone water. *L. monocytogenes* wild-type strains were resuspended in 45 mL of sterile water, combined in equal proportions, and an additional 50 mL of sterile water was added to make a 5-strain mixture of *ca.* 9 log CFU/mL. *L. monocytogenes* pNF8 strains were resuspended in 3 mL of 0.1% peptone water and combined in equal proportions to make a 5-strain mixture of *ca.* 10 log CFU/mL.

Soil was collected from the cantaloupe farm and sifted to remove rocks and other large debris. A portion (200 g) was placed in a 3.07 L Glad container (The Clorox Company, Oakland, CA, USA) and 4 mL of 10 log CFU/mL of the *L. monocytogenes* pNF8 (solid media culture) mixture was applied in a fine mist spray. The inoculated soil was mixed for 1 min with a spoon, covered, and held 18 h in the dark at room temperature for pathogen acclimation. *L. monocytogenes* concentration in the inoculated soil ranged from 7.11 to 8.46 log CFU/g.

3.4. Preparation of Sanitizing Solutions

Chlorine (200 ppm) was prepared by adding 40 mL of sodium hypochlorite solution containing 5% available chlorine (Ricca Chemical Company, Arlington, TX, USA) with 10 L of sterile deionized water. The chlorine solution was adjusted to pH 7.0 with sulfuric acid (Sigma-Aldrich, St. Louis, MO, USA). Free chlorine was determined with the Hach digital titrator using the DPD (*N,N*-diethyl-*p*-phenylenediamine)-ferrous ethylenediammonium sulfate titration cartridge (Hach Co., Loveland, CO, USA).

A chlorine dioxide stock solution was prepared by dissolving 4 g of Aqua-Tab (Beckart Environmental, Inc., Kenosha, WI, USA) in 1L of sterile deionized water. The chlorine dioxide stock was further diluted in sterile deionized water to prepare three 10-L batches of a 3 ppm solution, pH 4.36, per trial. Chlorine dioxide concentrations were measured using a Chlorine Dioxide Pocket Colorimeter™ II (Hach, Co. Loveland, CO, USA).

Levulinic acid (98%, Acros Organics, Fair Lawn, NJ, USA) and sodium dodecyl sulfate (SDS, 20%, Acros Organics) were combined with sterile deionized water to make 10 liters of 5% levulinic acid/2.0% SDS, pH 2.84, for dump tank treatments; 4 liters of 5.0% levulinic acid/2.5% SDS, pH 2.85, for dip treatments; 2 liters of 7.5% levulinic acid/0.5% SDS, pH 2.70, for spray treatments; and 200 mL of 7.5% levulinic acid/0.5% SDS, pH 2.71, for injection treatments.

All sanitizer solutions were prepared daily for each type of treatment and for each replicate trial. The temperature of all sanitizer solutions was 20 to 22 °C at the time of treatment.

3.5. Simulated Dump Tank Sanitizer Treatment of Cantaloupes and Subsequent Storage

Cantaloupes were spot inoculated, using a wild-type *L. monocytogenes* liquid medium cultured mixture by applying 100 µL of either an 8-log CFU/mL stock inoculum within a 2-cm diameter circle drawn on the netted rind, or a 7-log CFU/mL stock inoculum on the stem scar. After inoculation, the melons were held for 16 to 18 h at 22 °C. Inoculated melons for treatment were floated in 10 liters of 200 ppm chlorine, 3 ppm chlorine dioxide or 5% levulinic acid/2.0% SDS in a 53-L (51 × 35 × 30 cm) storage box (Roughneck, Rubbermaid Home Products, Fairlawn, OH, USA). The melons were held for a total of 8 min with the inoculation zones on stem scar and rind surface submerged the entire time, placed in 354-mL foam bowls (Walmart, Bentonville, AR, USA), and held for 5 min at 22 °C before analysis or storage. Positive control samples were not submerged in a sanitizer solution but were inoculated under conditions similar to treated cantaloupes. Two subsets of treated and non-treated melons were stored for 3 and 15 days at 4 °C and then analyzed. Two replicate trials were performed for each treatment. No positive values were obtained for non-inoculated, non-treated controls.

3.6. Sanitizer Treatment of Cantaloupes for the Single Hurdle or Double Hurdle Approach

Cantaloupes held at 4 °C for 48 and 72 h after harvest (replicate 1 and 2, respectively) were spot inoculated, using a *L. monocytogenes* pNF8 liquid medium cultured mixture, by applying 10 µL of either a 9-log CFU/mL stock inoculum within a 2-cm diameter circle drawn on the netted rind or an 8-log CFU/mL stock inoculum on the stem scar tissue. Inoculated cantaloupes were held for 16 h at 22 °C before treatment. Dip-treated cantaloupes were submerged for 1 min in 4 L of 5.0% levulinic acid/2.5% SDS held in 11.35-L pails and then placed in a foam bowl. Dump tank-treated melons were floated in 10 L of 200 ppm chlorine, as described in section 3.5, but with a 10-min treatment time. Cantaloupes designated for sequential dump tank and dip treatments first received the 10-min tank treatment in 200 ppm chlorine immediately followed by a 1 min dip treatment in 5.0% levulinic acid/2.5% SDS. All treated cantaloupes were held for 1 h prior to sample analysis. Non-treated melons (positive controls) were also inoculated and analyzed after holding for 16 h. Two replicate trials were performed for each treatment.

3.7. Stem Scar Injection and Spray Treatment of Cantaloupes Prior to Inoculation and Subsequent Simulated Dump Tank Treatment

Cantaloupes held at 22 °C for 20–24 h after harvest were injected at the stem scar with 0.2 mL of 7.5% levulinic acid/0.5% SDS at 60 psi using a P50 Microdose needle free injector fitted with a 3-stream nozzle (Pulse NeedleFree Systems, Lenexa, KS, USA) and attached to a CO₂ compressed gas canister. The stem scar-treated melons were immediately sprayed for 10 s with *ca.* 30 mL of 7.5% levulinic acid/0.5% SDS using a hand-held commercial garden sprayer (Flo Master Yard and Garden Sprayer, model 1401, Root-Lowell Manufacturing Co., Lowell, MI, USA). Stem scar- and spray-treated cantaloupes were placed in foam bowls and held at 22 °C for 2 h before either spot inoculation of cantaloupes with 100 µL of a 9 log (netted rind) and an 8 log (stem scar) CFU/mL *L. monocytogenes* pNF8 solid medium cultured mixture, or inoculation of cantaloupes with *L. monocytogenes* pNF8 solid medium cultured inoculated soil (2.5 g) pressed onto the stem scar and netted rind tissue (2-cm diameter circle) using waxed weighing paper (Thermo Fisher Scientific Inc., Waltham, MA, USA). Inoculated cantaloupes were held for 2 h at 22 °C prior to dump tank treatment of either 200 ppm of chlorine or 5% levulinic acid/2.0% SDS using the protocols described in Sections 3.5 and 3.6. The treated cantaloupes were held for 2 h at 22 °C prior to sample analysis. Non-treated melons (positive controls) were also inoculated and analyzed. Four replicate trials were performed for each treatment.

3.8. Sample Collection

Treatment and inoculation procedures were staggered to maintain equal time intervals before analysis of each melon. Control and treated melons (storage study only, Table 3) in which flesh beneath inoculated stem scar and netted rind tissue was to be analyzed, were first steam treated for 6 min in a 12.06 L stainless steel food steamer (Secura Inc., Brookfield, WI, USA) to remove residual wild-type *Listeria monocytogenes* from cantaloupe surfaces. Steamed cantaloupes were then placed in a sterile 3.79- or 7.58-L- Ziploc® bag (SC Johnson, Racine, WI, USA) containing 225 mL of 0.1% peptone water for non-treated control melons or sanitizer neutralizing buffers (0.1% buffered peptone water (Neogen, Lansing, MI, USA) for levulinic acid/SDS-treated melons or 0.1% peptone water supplemented with 0.01 g/liter of sodium thiosulfate (Sigma-Aldrich, St. Louis, MO, USA) for chlorine or chlorine dioxide-treated melons). The melons were massaged in the sealed bags by hand for 1 min, then were removed for sampling and the remaining buffer was collected for enrichment culture. Sampling of stored and non-stored cantaloupes involved placing cantaloupes on a sterile cutting board and using a stainless steel coring knife to cut out cores from the rind and stem scar inoculation sites. In the case of samples from non-stored cantaloupes, both the rind and stem scar samples were trimmed with a sterile knife into 3.13 cm³ samples (2.5 × 2.5 × 0.5 cm) which included surface tissue and flesh beneath the inoculation site before placing in a Whirl-Pak bag (Nasco, Fort Atkinson, WI, USA). In the case of samples from stored cantaloupes, the cored pieces were first separated into surface (2.5 cm² squares) or flesh (3.13 cm³) tissue, before placing in sterile Whirl-Pak bags. The surface and flesh samples collected during the storage study were weighed, and either sterile 0.1% peptone water or sanitizer neutralizing buffer was added at a 1:9 (wt/vol) ratio. Rind and stem scar surface samples collected in non-storage studies were combined either with 25 mL of 0.1% peptone water or neutralizing buffer.

3.9. Processing and Analysis of Cantaloupe Rind and Flesh Samples

Each sample was macerated in a Stomacher 400C (Seward Laboratory Systems, Port Saint Lucie, FL, USA) for 1 min at 260 rpm. The sample was directly plated, or a portion of the sample was diluted (1:9 wt/vol portions) in 0.1% peptone water and either 100 or 250 μL was plated in duplicate for enumeration of *L. monocytogenes* on modified Oxford agar (MOX) supplemented with 100 $\mu\text{g}/\text{mL}$ sodium pyruvate (Fisher Bioreagents, Fair Lawn, NJ, (MOXP)) or MOXP supplemented with 8 $\mu\text{g}/\text{mL}$ erythromycin (MOXPE) for wild-type *L. monocytogenes* and *L. monocytogenes* pNF8 strains, respectively. The MOX agar consisted of Oxford Listeria base (Neogen) and modified Oxford antimicrobial supplement (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The plates were incubated at 37 °C for 48 h. The limit of detection for directly plated samples was 2 log CFU/sample.

Wild-type *L. monocytogenes* samples (storage studies) were enriched according to the FDA Bacterial Analytical Manual protocol [25] and 2 \times BHIBE was added to *L. monocytogenes* pNF8 samples (non-storage studies) and incubated 24 h at 37 °C. The enrichment cultures (20 μL) were plated on MOXP or MOXPE plates and incubated at 37 °C for 48 h. The limit of detection by enrichment was 1 CFU per sample. Representative presumptive colonies of wild-type *L. monocytogenes* were streaked on tryptic soy agar (Neogen) and analyzed with API *Listeria* test kits (bioMérieux, Inc., Marcy l'Etoile, France) for confirmation.

3.10. Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using StatGraphics Centurion XVI statistical software package (Statpoint, Inc., Herndon, VA, USA). When statistical differences were observed ($p < 0.05$) with ANOVA, differences among sample means were determined using the least significant difference test at $p < 0.05$.

4. Conclusions

Using a single hurdle approach, treatment of cantaloupes with 5% levulinic acid/2% SDS either in a dump or dip tank provided greater reductions of *L. monocytogenes* than 200 ppm chlorine for stem scar tissue. In contrast, no significant differences were observed with these sanitizers on rind tissue samples. Regardless of sanitizer, it was revealed that stem scar-attached *L. monocytogenes* was more difficult to eliminate than rind-attached *L. monocytogenes*. This response was attributed to greater infiltration of the pathogen into stem scar tissue than rind tissue. A double hurdle approach employing a 200-ppm chlorine dump tank treatment followed by a 5% levulinic acid/2.5% SDS dip treatment did not provide any additional inactivation of *L. monocytogenes* on contaminated rind samples and actually had reduced efficacy compared to the dip treatment alone on contaminated stem scar samples. In contrast, a double hurdle approach employing field injection or spraying of 7.5% levulinic acid/0.5% SDS into stem scar and rinds, respectively, followed by exposure to either chlorine or levulinic acid/SDS in the packing plant dump tank appeared to have provided additional reductions of *L. monocytogenes* at stem scars and rinds compared to treatment of the cantaloupe with the dump tank sanitizer only. Based on these results, application of levulinic acid and SDS in the field could provide an additional hurdle to ensure the safety of the final product.

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Author Contributions

C.C.W., M.C.E. and M.P.D., conceived and designed the experiments. C.C.W., M.C.E. and L.E.D., performed the experiments. C.C.W. and M.C.E., analysed the data. C.C.W. and L.E.D., contributed reagents, material, and analysis tools. C.C.W. and M.C.E., wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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