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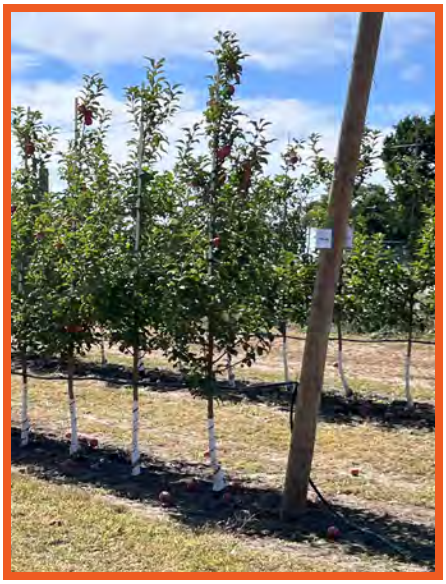
2900 100th Street, Suite 309, Des Moines, IA 50322-3855, USA

E. Manville,[1] L. Nwadike,[2, 3] and V. Trinetta[1*]

[1]Food Science Institute, Kansas State University, 216 Call Hall, Manhattan, KS 66503, USA
[2]Kansas State University, K-State Research and Extension, 22201 W. Innovation Drive, Olathe, KS 66061, USA
[3]University of Missouri, K-State Research and Extension, 22201 W. Innovation Drive, Olathe, KS 66061, USA



Combined Effects of Sanitizers and UV-C Light on *Listeria monocytogenes* Biofilm Growth and Survivability on Produce-Harvesting Materials Used in the Tree Fruit Production Industry

ABSTRACT

Listeria monocytogenes is an aggressive biofilm former that can establish and persist in food processing environments. Commonly associated with ready-to-eat and dairy products, this pathogenic bacterium has recently been increasingly linked to fresh produce outbreaks. Equipment used during harvesting and handling of produce can provide a niche environment for biofilm growth and persistence. Based on a survey conducted among stakeholders in the tree fruit production industry, three favored materials for storing and harvesting produce were identified: nylon, wood, and plastic. The purpose of this study was to investigate the application of the generally recognized as safe sanitizers lactic acid, thymol, and silver citric acid (SDC) and UV-C light alone or in combination for 2 or 5 min on different food-contact surfaces used during tree fruit harvesting and storing. Multistrain *L. monocytogenes* biofilms were grown in a Centers for Disease Control and Prevention biofilm reactor for 96 h on wood, nylon, and polycarbonate coupons at 20 ± 2°C. After each treatment, coupons were neutralized and the remaining cells were enumerated. Results showed that the most effective treatment was the simultaneous use of UV-C light and SDC (4-log reduction) and that the least effective treatment was UV-C light alone ($P < 0.05$). The type of material was found to play a significant role in the efficacy of the sanitizers ($P < 0.05$). This study demonstrates the ability of *L. monocytogenes* to grow and form biofilms on different surfaces and contributes to an understanding of the response of this food safety threat against antimicrobial intervention strategies.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen commonly associated with Ready-To-Eat (RTE) products, dairy products, and cold stored meat products *(34)*. This ubiquitous bacterial pathogen is the cause of listeriosis, an invasive infection resulting in high rates of mortality and morbidity *(15)*. *L. monocytogenes* is a saprophyte that is able to invade the cytosol of eukaryotic cells *(13)*. It can adapt and grow at refrigeration temperatures, low pH, and high salinity *(29)*. Its biofilm-forming capabilities enable

*Author for correspondence: Phone: +1 785.532.1667; Fax: +1 785.532.5861; Email: vtrinetta@ksu.edu

it to survive sanitation treatments, demonstrating concern for the safety of food products *(27)*. Researchers have been focusing efforts on understanding this pathogenic microorganism's ability to infect, survive, and persist in food processing environments and on equipment; however, the rising numbers of *L. monocytogenes* outbreaks linked to fresh produce have hastened the need to understand how to effectively control this pathogen on produce.

The prevalence, persistence, and diversity through which *L. monocytogenes* infects fresh produce is a growing concern, especially considering the zero-tolerance regulation put in place by the U.S. Food and Drug Administration for RTE products *(2)*. Equipment such as picking bags and storage bins used during the harvesting and handling of produce are known reservoirs of microbial biofilms *(18, 25, 30)*. Their regular food-contact surfaces provide a niche environment for biofilms to persist even after sanitizer application.

The use of chemical sanitizers is a common practice within the produce industry for controlling *Listeria* spp. Most studies have analyzed the effect of commercially available sanitizers such as peroxyacetic acid (PAA), ozone, halogen-based compounds, hydrogen peroxide, acid anionic compounds, and quaternary ammonium compounds on food processing surfaces *(3, 12, 16)*. The implementation of technologies using UV-C light has provided a method for disinfecting surfaces that may eliminate the need for mechanical scrubbing *(1)*. Today, food safety is moving toward synergistic processes of simultaneous or sequential germicidal applications to obtain greater pathogen reduction *(7)*. Studies analyzing the combined effects of UV light and chemical treatment have found that such combined applications have higher antimicrobial effects than single-application treatments. Ding et al. *(11)* found that when treating leafy greens, the simultaneous application of UV-A light and benzoic acid was most effective. The combined use of chemical and physical means of sanitation has been presented as a positive strategy to overcome contamination in food processing environments. Based on a survey conducted among stakeholders in the tree fruit production industry (in the Midwest), three favored materials for storing and harvesting produce were identified: nylon (for picking bags), wood, and plastic (for bins).

The objective of this study was to investigate the survival of *L. monocytogenes* biofilms on wood, nylon, and polycarbonate produce-harvesting materials after treatment with chemical sanitizers alone and/or concurrent with UV-C light. The chemical sanitizers lactic acid, SDC and thymol were selected to represent nontoxic or generally recognized as safe chemicals. All of these products are commercially available and certified organic (GRAS). Their market price places them in a slightly higher category than bleach; nevertheless, these products are accessible and commonly used in the organic farming sector.

MATERIALS AND METHODS

Bacterial strains

Three strains of *L. monocytogenes* were used in this study to form biofilms: L2624 (serotype 1/2b) and L2626 (serotype 1/2a), isolated from the 2001 U.S. cantaloupe outbreak, and J2230 (serotype 4b) from a clinical sample *(21)*. All strains were kept on CyroCare Bacteria Preserver beads (Key Scientific, Stamford, TX, USA) and stored at −80°C until used in experiments. An *L. monocytogenes* cocktail, as described by Mendez et al. *(21)*, was created by combining individual strains in equal ratio to reach an initial population of ca. 1×10^8 CFU/ml.

Substrate materials

The materials selected for this study included wood, nylon, and polycarbonate, all of which are representative of substrates used during the harvesting and handling of tree fruit production. Coupons made of polycarbonate (1.27 cm in diameter) were purchased from BioSurface Technologies (Bozeman, MT, USA). Wood coupons were made of plywood of unfinished basswood (*Tilia americana*) purchased from a local store and cut into coupons of 1.27 cm in diameter. Nylon fabric was obtained from a local store and was cut into 2- by 2-cm squares to fit within the rods of the reactor.

Biofilm formation

Based on a protocol developed in our laboratory *(21)*, with slight modifications, biofilms were grown in a Centers for Disease Control and Prevention (CDC) biofilm reactor (BioSurface Technologies) for up to 96 h. To begin the 24-h batch phase, 1 ml of *L. monocytogenes* cocktail was used to inoculate the reactor. This phase was followed by an additional 72 h of continuous nutrient flow at a rate of 8 ml/min by using tryptic soy broth (Difco, BD, Sparks, MD, USA). The reactor was maintained at 20 ± 2°C for 96 h of incubation.

Chemical sanitizer and UV-C light

The following chemical sanitizers were used to evaluate efficacy against *L. monocytogenes* biofilms: 4% lactic acid solution (Purac, Corbion, Blair, NE, USA), confirmed using a lactic acid test kit (ChemWorld, Kennesaw, GA, USA); 5% SDC-based sanitizer, as per label (PURE Bioscience, Inc., El Cajon, CA, USA); and 0.23% thymol solution, as per label (Bioesque Solutions, Lighthouse Point, FL, USA). Because microbial DNA absorbs UV-C light between 200 and 300 nm, with optimal absorption at ca. 260 nm, the lamp (Lumalier, Memphis, TN, USA) used as the emission source of the UV-C light was set at 254 nm for germicidal and disinfection applications. The power output was monitored using a radiometer (Sper Scientific Ltd., Scottsdale, AZ, USA) during all of the experiments to ensure constant UV-C light irradiance.

Application of treatments

To simulate industrial conditions, a UV-C lab bench apparatus was built to demonstrate the effectiveness of dual antimicrobial processes *(Fig. 1)*. The apparatus was designed to deliver uniform and quantified irradiation and an even distribution of sanitizer to ensure coverage of all coupons. Sanitizers were applied with a steady flow rate of 266.67 ml/min delivered through a diaphragm pump feeding into a food-grade nozzle.

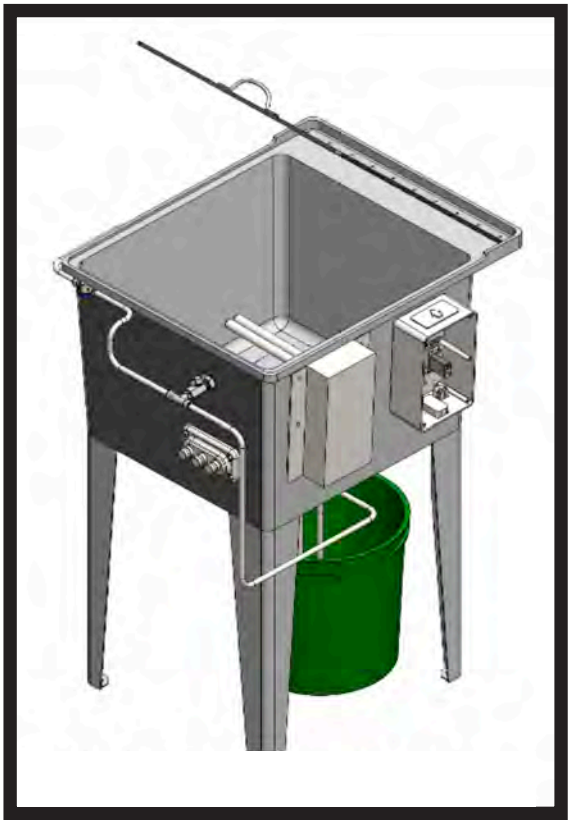


Figure 1. Schematic of the scaled lab device equipped with a UV-C lamp and commercial spray system.

The electrical energy per order (UV-C efficiency) was evaluated and defined as the number of kilowatt-hours of electrical energy required to reduce the concentration of a microbial cell by 1 order of magnitude. Because the intensity of the light is a function of the distance between the light source and the sample, the UV-C lamp was 16 cm away from the coupon, with an average output intensity of 850 μW/cm^2 emitting light at 254 nm (based on our preliminary experiments). At two application times, 2 and 5 min, the UV dose was 102,000 and 255,000 μWs/cm^2, respectively, based on the average intensity multiplied by the exposure time (in seconds) *(8)*. UV dose is defined as UV intensity (in milliwatts per square centimeter) × exposure time (in seconds).

After 4 days of biofilm development, the rods containing coupons (wood, nylon, and polycarbonate) were removed from the reactor and placed in the sanitizing apparatus. The preferred sanitizer treatment was applied at 20 ± 2°C. Halfway through the application time, the rod was rotated to ensure treatment exposure to both sides of the coupon.

Recovery and enumeration of microbial cells

After treatment exposure, coupons treated with a chemical sanitizer were individually placed in 10 ml of Dey and Engley neutralizing broth (Hardy Diagnostics, Santa Maria, CA, USA). Control treatments were evaluated by placing a coupon with untreated biofilm in 10 ml of phosphate-buffered saline (VWR, Solon, OH, USA). A 100-ppm PAA (Synergex, Ecolab, St. Paul, MN, USA) control was also performed, because PAA is approved in organic farming and commonly used by producers.

To recover cells, coupons underwent 30 s of sonication at 40 kHz followed by 30 s of vortexing; this procedure was repeated three times to ensure full recovery of remaining cells *(4)*. Serial dilutions were then performed in 0.1% peptone water (Bacto, Sparks, MD, USA) and spread plated on tryptic soy agar (Difco, BD) in duplicate. After 24 h of incubation at 37 ± 2°C, colonies were counted and the results reported as log CFU per square centimeter.

Experimental design and statistical analysis

Seven different treatment combinations, including the application of chemical sanitizers and UV-C light alone and simultaneously, were analyzed. Each treatment was randomized across the eight rods residing within the CDC reactor, and all experiments were completed four times. Statistical significance was defined by a P-value < 0.05. A mixed model was used for the analysis of log reduction among treatments, materials, and application times. All data were analyzed using SAS 9.4TS Level 1M5 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

The fresh produce industry is constantly battling *L. monocytogenes* on surfaces that are difficult to clean and sanitize. With more recent outbreaks being associated with pre- and postharvest practices, evaluating the effectiveness of interventions that are efficient and practical for small produce growers is becoming more prudent to food safety. In our previous study *(22)*, we evaluated the efficacy of lactic acid (4%), peroxy acid (100 ppm), and quaternary ammonium (400 ppm) alone or in combination with 15 or 30 min of exposure to UV-C light on stainless steel surfaces, whereas in our current study, different materials and organic sanitizers were evaluated. The volume of organic farming practices in the United States has grown rapidly in the past decades; therefore, there is a need to study and understand effective sanitation practices for the organic market. Control coupons

TABLE 1. Number of recovered cells on wood, nylon, and polycarbonate after exposure to treatments lactic acid, SDC, UV-C light, and the simultaneous use of UV-C light and a chemical sanitizer

Material	Intervention[a]	Log CFU/cm² after 2-min exposure	Log CFU/cm² after 5-min exposure
Wood	PAA control	7.31 ± 0.64^{AB}	7.20 ± 0.52^{A}
	Lactic acid	8.09 ± 0.09^{A}	7.84 ± 0.18^{A}
	Thymol	7.87 ± 0.10^{A}	7.94 ± 0.06^{A}
	SDC	7.86 ± 0.16^{A}	7.61 ± 0.18^{A}
	UV-C	8.70 ± 0.14^{A}	8.52 ± 0.13^{A}
	UV + lactic acid	7.94 ± 0.12^{A}	7.49 ± 0.41^{A}
	UV + thymol	8.18 ± 0.51^{A}	7.65 ± 0.10^{A}
	UV + SDC	7.91 ± 0.05^{A}	7.31 ± 0.58^{A}
Nylon	PAA control	4.83 ± 0.28^{A}	4.51 ± 0.22^{A}
	Lactic acid	7.05 ± 0.90^{B}	6.96 ± 0.55^{B}
	Thymol	7.84 ± 0.32^{B}	5.85 ± 1.56^{B}
	SDC	6.83 ± 0.21^{B}	6.80 ± 1.25^{B}
	UV-C	9.45 ± 0.30^{C}	8.85 ± 1.03^{C}
	UV + lactic acid	6.94 ± 0.57^{B}	6.71 ± 0.44^{B}
	UV + thymol	6.89 ± 0.14^{B}	7.12 ± 0.77^{B}
	UV + SDC	6.83 ± 0.54^{D}	5.60 ± 0.53^{D}
Polycarbonate	PAA control	3.98 ± 0.60^{A}	3.27 ± 0.87^{A}
	Lactic acid	5.62 ± 0.22^{B}	5.20 ± 0.35^{B}
	Thymol	4.10 ± 0.4^{A}	4.06 ± 1.08^{A}
	SDC	4.55 ± 0.52^{A}	4.04 ± 0.42^{A}
	UV-C	6.75 ± 0.28^{C}	6.35 ± 0.77^{C}
	UV + lactic acid	5.64 ± 0.33^{B}	4.43 ± 0.40^{B}
	UV + thymol	4.57 ± 0.47^{A}	3.64 ± 0.24^{A}
	UV + SDC	3.81 ± 1.72^{A}	3.35 ± 1.53^{A}

[A]Two controls were included in this experiment: controls with untreated biofilms and PAA controls (see text for details). The following counts were recovered on untreated coupons with mature biofilms after 96 h: 8.87 ± 0.13, 9.60 ± 0.32, and 7.68 ± 0.06 log CFU/cm² on wood, nylon, and polycarbonate, respectively.

[B]Uppercase letters compare individual and simultaneous treatments within the same material.

of wood, nylon, and polycarbonate were enumerated after 96 h, resulting in counts of 8.87 ± 0.13, 9.60 ± 0.32, and 7.68 ± 0.06 log CFU/cm², respectively. *Table 1* shows the effects of single and simultaneous sanitizer applications of lactic acid, SDC, thymol, and UV-C light on experimentally inoculated wood, nylon, and polycarbonate coupons.

Materials and sanitizers were the only interaction found to be significant ($P < 0.05$; *Table 2*). This highlights that the effectiveness of the sanitizer on mature biofilms is influenced by the substrate material. Biofilms developed on wood were found to be the most resistant to treatments compared with those on nylon and polycarbonate, resulting in ≤ 1-log CFU/cm² reduction for all selected sanitizers at each application time. As expected, the use of PAA was the most effective among the single-treatment applications. Nevertheless, a significant difference was only observed for nylon coupons ($P < 0.05$). Even if in wood and polycarbonate the highest log reduction was achieved with PAA, no statistical difference was observed compared with the other treatments.

The sanitizers applied were highly effective on biofilms grown on polycarbonate coupons, with reductions nearing 3.6 log CFU/cm². In Bang et al. *(6)*, similar results were observed wherein *Escherichia coli* O157:H7 biofilms were most persistent on wood surfaces after treatments with NaOCl and

TABLE 2. Statistical output of type 3 tests of fixed effects comparing experimental factors

Effect	Pr > F
Sanitizer	<0.0001
Time	0.0007
Sanitizer × time	0.2391
Material	<0.0001
Sanitizer × material	<0.0001
Time × material	0.9267
Sanitizer × time × material	0.0973

ClO_2. Yang et al. *(31)* reconfirmed this finding when studying *L. monocytogenes* biofilms on smooth and rough high-density polyethylene (HDPE): sanitizers were found to be less effective on rough HDPE. Both studies imply that surface texture provides protection to cells and limits sanitizer penetration. In our study, UV-C light exposure was found to be the least effective strategy for all materials, regardless of application time ($P < 0.05$). Textural discrepancies could explain the low success of the application of UV-C light alone. UV-C light shows germicidal properties when UV light is absorbed by DNA, creating cyclobutane pyrimidine dimers that stop cellular transcription and replication, and UV-C's effectiveness as a sanitizer is dependent on its ability to reach genetic material *(14, 23, 24)*.

As shown in *Table 2*, the individual factors, material, sanitizer, and time were all significant against *L. monocytogenes* biofilms ($P < 0.05$). Nevertheless, there was high variability among bacterial reductions. Lactic acid (4%) alone resulted in up to a 2.5-log CFU/cm^2 reduction of *L. monocytogenes*, depending on the substrate: after 2 min of exposure on nylon, there was a 2.5-log CFU/cm^2 reduction, and after 2-min exposure on polycarbonate, there was a 2-log CFU/cm^2 reduction. Lactic acid is an organic compound that permeates the cell membrane, leading to extreme pH distortion and causing loss of cell contents, lysis, and eventual cell death *(32, 33)*. The effectiveness of lactic acid to control *L. monocytogenes* biofilms was previously studied by Ban et al. *(5)* on 6-day-old biofilms developed on polyvinyl chloride and stainless steel. Using 0.5–2.0% lactic acid, these researchers were able to achieve a 0.19- to 0.94-log reduction after 5–30 s of immersion in sanitizer. In our study, a greater reduction was observed due to the longer exposure times (2 and 5 min).

Thymol (0.23%) and SDC (5%) alone were found to cause cell reductions up to 3-log CFU/cm^2, depending on the substrate on which the biofilm was developed. For example, nylon exposed to thymol for 5 min exhibited a 3.7-log CFU/cm^2 microbial reduction and polycarbonate exposed for 5 min exhibited a 3.6-log CFU/cm^2 reduction. SDC caused a 3.6-log CFU/cm^2 reduction of cells after 5 min on polycarbonate and a 2.8-log CFU/cm^2 reduction on nylon after a 5-min exposure. The strong antibacterial properties of thymol are due to the high concentration of phenolic compounds that can disrupt the cytoplasmic membrane, thereby interrupting the proton motive force, flow of electrons, and active transport as well as causing the congealing of cell contents *(9)*. Desai et al. *(10)* found 4-day-old biofilms (7 log CFU per coupon) to be eradicated on stainless steel coupons by using 0.5% thymol after 24 h of exposure. Our study using 0.23% thymol was able to achieve up to a 3-log reduction after only 5 min of exposure, depending on the material. There are few studies on the efficacy of SDC against mature *L. monocytogenes* biofilms. Masuku et al. *(19)* applied SDC for 2 min against 4-h-old *L. monocytogenes* cells on stainless steel and aluminum and achieved a 5-log reduction. In our study, a >3-log reduction of *L. monocytogenes* biofilms after 5-min exposure was observed on polycarbonate. Many of the discrepancies between our results and those of Masuku et al. *(19)* are probably due to biofilm growing conditions, biofilm age, sanitizer concentrations, substrate material, and sanitizer contact time.

As previously stated, simultaneous applications of sanitizers have been predicted to improve efficacy against target microorganisms. In this study, no significant difference was found when comparing the effectiveness of single-use sanitizers with simultaneous applications ($P < 0.05$). Studies analyzing concurrent sanitizers use have found that two stressors on an organism result in higher bacterial lethality. For example, Ding et al. *(11)* reported that treatment with benzoic acid and UV-A light on *E. coli* resulted in an ca. 6-log CFU/cm^2 reduction of cells after 30 min. Silva-Espinoza et

al. *(26)* found that the simultaneous use of clove essential oil and UV-C light was highly effective against *Salmonella* biofilms. Nevertheless, when Tajik et al. *(28)* treated *L. monocytogenes* biofilms by using UV-C light and *Zataria multiflora* Boiss (Shirazi thyme) essential oil simultaneously for 15 and 45 min, no significant difference was observed compared with single-use treatments, aligning with the results of our study. These results could be linked to the fact that irregular liquid particles from the chemical sanitizers could scatter light-shielding cells from UV wavelengths *(20)*, or sanitizers could interfere with the free radicals that are being produced with UV light *(28)* and/or the biofilm extracellular polymeric substances act as a shield from UV light and limit the diffusion of disinfectants *(17, 31)*. Nevertheless, in our study the most effective treatment was the simultaneous application of UV-C light and SDC ($P < 0.05$). This simultaneous sanitizer application resulted in average reductions of 1.2 log CFU/cm^2 on wood, 3.4 log CFU/cm^2 on nylon, and 4 log CFU/cm^2 on polycarbonate. SDC is a poor reflector of UV light, which could help explain its increased effectiveness compared with that of the other applications *(23)*.

CONCLUSIONS

We evaluated the effectiveness of lactic acid, SDC, thymol, and UV-C light alone or simultaneously against 96-h-old *L. monocytogenes* biofilms grown on wood, nylon, and polycarbonate. These materials are commonly used for storing and harvesting produce in the tree fruit production industry. The treatments investigated could be used at the end of the workday when tools are stored. We found that the biofilm growth substrate is an important factor to consider for the efficacy of sanitizers and that the simultaneous applications of UV-C and chemical sanitizers does not guarantee greater potency compared with single-sanitizer applications. Also, the choice of easy-to-sanitize harvesting tools is important. Materials with numerous textural discrepancies may lead to biofilms that are more resistant to sanitizers, as observed with biofilms grown on wood. The results of this study could help produce growers make informed decisions on the type of materials that they should use for handling their fresh products.

This research explored organic treatment options such as lactic acid, thymol, and SDC used alone or simultaneously with UV-C light that could be used to control *L. monocytogenes* biofilms. The most effective mitigation strategy used against *L. monocytogenes* biofilms that resulted in high cellular reduction was the simultaneous applications of SDC and UV-C light. Future research that investigates the use of other certified organic sanitizers, the effects of higher UV dose levels, and the role of a cleaning step before sanitation might be useful to understand the role and interaction mechanisms of physical interventions with sanitizers.

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