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Preliminary Investigation of the Effect of Chemical Sanitizers and UV-C Light on *Listeria monocytogenes* Biofilm Survivability

ABSTRACT

The ability of *Listeria monocytogenes* to adapt under different environments and form biofilms is a challenge for food safety. Mature biofilms are difficult to disrupt. Chemical sanitizers combined with nonthermal technologies might be an effective way to control *L. monocytogenes* biofilms. This study was conducted to investigate *L. monocytogenes* biofilm survival after treatments with chemical sanitizers and UV-C light alone or in combination. A Centers for Diseases Control and Prevention biofilm reactor was used to grow 4-day-old multistrain *L. monocytogenes* biofilms on stainless steel. Biofilm survival was evaluated after 10 min of exposure to 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 (254 nm). The sequential treatment effect was also evaluated. Reductions of 2.6 to 3.6 log CFU/cm^2 were observed with chemical sanitizers, whereas a maximum of 1.8 log CFU/cm^2 reduction was recorded after UV-C treatment. Combined treatments had an enhanced effect, and the sequence of antimicrobial treatments was significant for lactic acid and peroxy acid ($P < 0.05$). The results obtained in this research offer an initial understanding of the response of *L. monocytogenes* biofilm to chemical sanitizers and contribute to development of effective strategies to control this pathogen in the food processing environment.

INTRODUCTION

Listeria monocytogenes can adapt and proliferate in a variety of environments by changing responses to external variations *(33)*, and its ability to form a biofilm represents a challenge for food safety. A biofilm is defined as a community of microorganisms that live in a sessile form attached to a substratum or interface *(13)*. *L. monocytogenes* in a processing facility can attach to various surfaces such as stainless steel, glass, plastic, or rubber and form biofilms *(17, 23)*. These biofilms are difficult to control and usually develop in hard-to-clean sites *(13, 23)*. Drains, floors, conveyor belts, scratches, joints, and porous or rough surfaces provide ideal niches for cell adhesion and biofilm formation and protection from mechanical and chemical disruption *(23)*. The process of forming a biofilm includes three main steps: (i)

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attachment to the surface; (ii) aggregation into microcolonies in a semipermanent association; and (iii) growth and maturation of macrocolonies. At the end of the development stage, cells are irreversibly attached to the surface. The microorganisms are in a semipermanent association with the surface because the embedded cells in the matrix have transitioned from a motile to a sessile lifestyle. The biofilm is now mature, and its cells cannot be removed by simply rinsing *(1, 10, 32)*. Biofilms offer greater protection to microorganisms against external challenges such as temperature, pH, and antimicrobial solutions through the secretion of extracellular polymeric substances *(10)*. Factors such as biofilm age and presence of other species or other *L. monocytogenes* strains allow biofilms to acquire higher resistance to sanitation strategies such as chemical sanitizers *(4, 21)*. Such resistance of mature biofilms is due to the protection provided by the extracellular polymeric substances and the multiple layers of bacterial cells in the biofilm *(38)*.

Food processing facilities follow sanitation standard operating procedures to ensure sanitary conditions in the processing plant *(24)*. The use of chemical sanitizers is one of the most common practices for combating contamination, bacterial adhesion, and biofilm formation *(29)*. However, novel technologies such as the use of UV light are being implemented to enhance the effectiveness of standard sanitation methods and ensure the safety of the products *(18)*. Among the most commonly used sanitizers are quaternary ammonium compounds (QACs), organic acids, and peroxyacetic acid, and their antimicrobial activity against biofilms have been previously investigated *(5, 8)*. When biofilms of *L. monocytogenes* were treated with chemical sanitizer, reductions of 1 to 3 log CFU/cm^2 were observed *(4, 21)*. However, results indicate that the use of chemical sanitizer as a single strategy is not always sufficient to control biofilms in food processing environments *(4, 21, 26)*. Therefore, some sanitation standard operating procedures include use of sanitizers in rotation or the implementation of hurdle technologies (i.e., combined intervention strategies) to achieve a greater antimicrobial effect. Among alternative germicidal technologies, nonionizing UV irradiation has been effective against bacterial biofilms *(15, 19, 31)*. The combination of UV light with chemical sanitizers also has been investigated. A UV-C light lamp was ceiling mounted in a fish smokehouse after daily cleaning and disinfection procedures, and a significant decrease in *Listeria*-positive environmental samples was reported after irradiation for 48 h *(7)*. The combination of sodium hypochlorite and UV light was used effectively against *L. monocytogenes* biofilms in industrial kitchens, facilities, and restaurants *(19)*. Combined physical and chemical techniques appear to represent a good strategy for overwhelming bacterial responses and achieving a greater reduction of *L. monocytogenes* biofilms.

The objectives of this study were to (i) investigate *L. monocytogenes* biofilm survival after treatments with chemical sanitizers (i.e., quaternary ammonium, lactic acid, and peroxy acid) and UV-C light (254 nm) alone or in combination and (ii) understand the effect of changing the sequence of treatments to identify possible enhancement of overall antimicrobial activity.

MATERIALS AND METHODS

Bacterial strains

The same strains of *L. monocytogenes* investigated in previous research *(25)* were used for this study. FSL B2-323 (serotype 4b; Texas Tech University) was isolated from a dairy processing environment *(6)*, ATCC 7644 (serotype 1/2c) was from a clinical case of human meningitis, NRRL B-33043 (serotype 1/2a; California) and NRRL B-33260 (serotype 1/2c; U.S. Department of Agriculture, Agricultural Research Service) were obtained from a meat slaughter facility *(36)*. Each strain was kept in a CryoCare organism preservative system (Key Scientific, Stamford, TX) and stored at −80°C until needed for the experiments.

Biofilm formation

A biofilm-growing device developed by the Centers for Disease Control and Prevention (https://biofilms.biz/products/biofilm-reactors/cdc-biofilm-reactor/) was used to develop 4-day-old biofilms on circular stainless steel coupons 1.27 cm in diameter. A protocol developed in our laboratory was used *(25)*, and coupons were treated and cleaned following the manufacturer's instructions. Each *L. monocytogenes* strain was grown overnight at 37 ± 2°C in 10 ml of tryptic soy broth (Difco, BD, Sparks, MD) with 0.6% yeast extract (Hardy Diagnostics, Santa Monica, CA) (TSBYE). Equal amounts of bacteria were combined to create a cocktail, and 1 ml was used to inoculate the reactor containing 350 ml of TSBYE. The initial cocktail population was verified by spread plating on tryptic soy agar (TSA; Difco, BD) and enumerating after 24 h of incubation at 37 ± 2°C. Biofilms were grown at 30 ± 2°C following a 24-h batch phase and a 72-h continuous flow stirring phase (11 ml/min flow rate and 60 rpm stirring force).

Chemical sanitizers exposure

Coupons with mature biofilms were removed from the reactor after 4 days. Three chemical sanitizers were used at room temperature for an exposure time of 10 min: 4% lactic acid solution, pH 3 (Purac, Corbion, Blair, NE); a peroxy acid-based sanitizer diluted to 100 ppm, pH 4.5 (SYNERGEX, Ecolab, St. Paul, MN); and a quaternary ammonium-based sanitizer (STER-BAC, Ecolab) diluted to 400 ppm, pH 6, following the manufacturer's recommendations. Immediately after treatment, solutions were neutralized by transferring the coupons into 10 ml of D/E neutralizer broth (Difco, BD).

UV-C light treatments

Experiments to evaluate the effect of UV-C light on biofilms were conducted in a small chamber in which UV irradiance could be measured and controlled. UV-C light (254 nm) was applied for 15 min (0.2 J/cm^2) or 30 min (0.45 J/cm^2) at room

temperature. A germicidal UV lamp (Lumalier, Memphis, TN) was the emission source, and a research radiometer (ILT1700, International Light Technologies, Peabody, MA) was used to monitor the intensity (W/cm^2) of the UV irradiation. Coupons with 4-day-old mature biofilms were placed 20 cm from the emission source and treated for 0, 15, or 30 min. Because biofilms developed on both sides of the coupons, coupons were flipped halfway through the total exposure time. Exposed coupons were then aseptically transferred to 10 ml of phosphate buffered saline (PBS; VWR, Radnor, PA).

Combined treatment application

The combined application of chemical sanitizers and UV-C light was evaluated to determine any increase in antimicrobial activity. Surfaces with mature biofilms were exposed for 10 min to chemical sanitizers and then for 0, 15, and 30 min to UV-C light treatment. Because no differences among holding times without UV-C light treatment were observed, this parameter was not added to the experimental design. Treatment sequence effect was also evaluated based on differences in antimicrobial effectiveness when UV-C was applied before or after the sanitizer step. After each treatment, coupons were aseptically placed in 10 ml of D/E neutralizer broth. Control treatments consisted of coupons with untreated biofilm in 10 ml of PBS or D/E neutralizer broth.

Cells recovery and microbial counts

After exposure to single or combined treatments, coupons were sonicated for 30 s at 45 kHz and then vortexed for 30 s, following ASTM standard E2871-19 *(2)*. This process was repeated three times to assure complete detachment of biofilm cells. Serial dilutions were made in 0.1% peptone water (Difco, BD) and spread plated in duplicate on TSA. Plates were incubated at 37 ± 2°C for 24 h. Colonies were manually counted, and results were recorded as log CFU per square centimeter.

Statistical analysis

Treatments were randomized across coupons, and experiments were run six times to reduce experimental error. Statistical significance was defined at $P < 0.05$. An analysis of variance and a multiple comparison of means test were used to evaluate differences in the treatment results. Data were analyzed using the GLM procedure of SAS 9.4 (SAS Institute, Cary, NC).

RESULTS

Effect of single treatment application on the reduction of *L. monocytogenes* biofilms

The effects of UV-C light, lactic acid, peroxy acid, and quaternary ammonium treatments alone on *L. monocytogenes* biofilms are shown in *Table 1*. After 4 days at 30°C in TSBYE, control biofilms reached a population of 6.04 ± 0.49 log CFU/cm^2. When mature biofilms were exposed to UV-C light for 15 or 30 min, significant reductions ($P < 0.05$) of 1.73 ± 0.79 and 1.68 ± 0.97 log CFU/cm^2, respectively, were observed. However, no significant differences were found between the 15- and 30-min exposures to UV light. After 10 min of exposure to 4% lactic acid, a reduction of 3.06 ± 0.85 log CFU/cm^2 was obtained, exposure to 400 ppm of quaternary ammonium resulted in 2.61 ± 0.91 log CFU/cm^2 reduction, and exposure to 100 ppm of peroxy acid achieved a 3.66 ± 0.90 log CFU/cm^2 reduction. No significant differences were observed between lactic acid and quaternary ammonium treatments. Peroxy acid was the most effective of the single treatment applications ($P < 0.05$). Overall, all the chemical sanitizers investigated in this study significantly reduced *L. monocytogenes* biofilms compared with the untreated controls ($P < 0.05$).

Effect of combined treatment application on the reduction of *L. monocytogenes* biofilms

Table 1 shows *L. monocytogenes* biofilm reductions obtained after exposure to the combination treatments of chemical sanitizers and UV-C light.

Lactic acid

Evaluation of the order of treatment application revealed a significant effect ($P < 0.05$) for the combination of lactic acid and UV-C light. When lactic acid was followed by 15 or 30 min of UV-C light, biofilms were reduced by 5.11 ± 0.66 and 4.78 ± 1.02 log CFU/cm^2, respectively, compared with the control. Conversely, when lactic acid was preceded by 15 min of UV-C light, no significant significance ($P > 0.05$) in biofilm reduction (3.26 ± 0.62 log CFU/cm^2) was observed compared with the use of lactic acid alone (3.06 ± 0.85 log CFU/cm^2). However, a significant effect ($P < 0.05$) was observed when UV-C exposure time was extended to 30 min: a reduction of 4.02 ± 0.67 log CFU/cm^2.

Quaternary ammonium

Evaluation of the order of treatment application revealed no significant effect ($P > 0.05$) for the combination of quaternary ammonium and UV-C light. Nevertheless, an enhanced log reduction was observed when the antimicrobial treatments were used together compared with their use alone. When quaternary ammonium was applied before UV-C light, a significant difference was found ($P < 0.05$) between the 15- and 30-min treatments, with 3.28 ± 1.32 and 4.02 ± 1.19 log CFU/cm^2 reductions, respectively. However, when UV-C light was used first, no difference ($P > 0.05$) was found between the 15- and 30-min treatments, with 3.45 ± 0.93 and 3.85 ± 0.84 log CFU/cm^2 reductions, respectively.

Peroxy acid

Evaluation of the order of treatment application revealed a significant effect ($P < 0.05$) for peroxy acid and UV-C light. Greater reduction was observed when peroxy acid was applied before the UV-C light. However, no significant difference ($P > 0.05$) was observed between the reduction achieved by

TABLE 1. Effect of treatment application on the reduction (mean ± standard deviation) of *L. monocytogenes* biofilms

1st Treatment[a]	2nd Treatment[a]	Biofilm cell reduction (log CFU/cm^2)[b]
Lac		3.06 ± 0.85[a]
Lac	15-UV	5.11 ± 0.66[c]
Lac	30-UV	4.78 ± 1.02[c]
15-UV	Lac	3.26 ± 0.62[a]
30-UV	Lac	4.02 ± 0.67[b]
Qua		2.61 ± 0.91[a]
Qua	15-UV	3.28 ± 1.32[ab]
Qua	30-UV	4.02 ± 1.19[c]
15-UV	Qua	3.45 ± 0.93[bc]
30-UV	Qua	3.85 ± 0.84[bc]
Poa		3.66 ± 0.90[a]
Poa	15-UV	4.66 ± 0.66[c]
Poa	30-UV	4.38 ± 0.91[bc]
15-UV	Poa	4.00 ± 0.81[ab]
30-UV	Poa	4.51 ± 0.92[bc]

[a]15-UV, 15-min exposure to UV-C light; 30-UV, 30-min exposure to UV-C light; Lac, 4% lactic acid sanitizer; Qua, 400 ppm quaternary ammonium–based sanitizer; Poa, 100 ppm peroxy acid-based sanitizer.

[b]The population in control biofilms was 6.04 ± 0.49 log CFU/cm^2. Within treatment groups, means with different letters are significantly different ($P < 0.05$).

the 15- and 30-min UV-C light exposures, with 4.66 ± 0.66 and 4.38 ± 0.91 log CFU/cm^2 reductions, respectively. Likewise, no difference ($P > 0.05$) was found between 15 and 30 min of UV-C exposure when UV-C light treatments preceded the sanitizer (4.00 ± 0.81 and 4.51 ± 0.92 log CFU/cm^2 reductions, respectively).

DISCUSSION

In the food industry, *L. monocytogenes* is widely known for forming biofilms in difficult-to-clean sites such as floors, waste water pipes, conveyor belts, and stainless steel surfaces *(12)*. In the present study, we found that 4-day-old multistrain biofilms of *L. monocytogenes* were reduced by 3.06, 2.61, and 3.66 log CFU/cm^2 by lactic acid (4%), quaternary ammonium (400 ppm), and peroxy acid (100 ppm), respectively.

The use of lactic acid to control microbial biofilms has been previously studied. Yang et al. *(38)* observed a reduction of 4.21 log CFU/cm^2 in a 7-day-old *L. monocytogenes* biofilm on polyethylene surfaces treated with 0.18% lactic acid, a greater log reduction than that found in our study. This discrepancy in results may be attributed to key factors such as biofilm age, attachment surface, and conditions under which the biofilms were grown (e.g., temperature and shear force). Wang et al. *(35)* found that lactic acid can cause a leakage of proteins through the cell membrane, especially in the first 2 h of sanitizer exposure, and can inhibit the synthesis of bacterial cell soluble proteins. These authors found that *L. monocytogenes* in the planktonic state was reduced below detectable limits after exposure to 0.5% lactic acid for 2 h. However, because biofilms provide bacteria with increased protection from stressors, a higher concentration of antimicrobial solutions or the combination with other intervention strategies might offer a more effective way to control biofilm formation. Ban et al. *(4)* achieved a 0.92 log CFU per coupon reduction in *L. monocytogenes* biofilms on stainless steel after exposure to 2% lactic acid for 30 s. However, a 4.5-log reduction was obtained when the sanitizer was combined with steam for 20 s. In our study, when 4% lactic acid was combined with UV-C light, a 5.11-log reduction was obtained.

QAC sanitizers have been evaluated by many researchers for control of microbial biofilms on various surfaces *(5, 21, 26, 27)*. Concentrations of 200 ppm of QACs used to treat 7-day-old *L. monocytogenes* biofilms resulted in a 1.35 log CFU per well reduction on polystyrene *(21)* and a 3.4 log CFU/cm^2 reduction on stainless steel *(26)*. In our experiment with 4-day-old *L. monocytogenes* biofilms, a reduction of 2.61 log

CFU/cm^2 was obtained after treatment with 400 ppm of QAC. These differences could be attributed to differences in biofilm age and sanitizer concentration. Chavant et al. *(11)* observed a decrease in biofilm cell mortality from 98% after 6 h to 45% after 7 days of biofilm maturation when samples were exposed to 20 ppm of QACs. In another study, solutions of hydrogen peroxide and sodium hypochlorite were more effective than QACs for controlling *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms *(22)*. However, the use of QACs is one of the most common strategies in the food industry *(16)*. Very few studies have been conducted to investigate the combination of QACs with other control strategies against biofilms *(8, 9)*. Berrang et al. *(8)* found an enhanced bactericidal effect when quaternary ammonium (400 ppm) was combined with ultrasonication, with a 3.5-log reduction of *L. monocytogenes* biofilms. Similar log reductions were observed in our study when QACs were combined with UV-C light (3.28 to 4.02 log CFU/cm^2).

Effective control of biofilms has been achieved by use of compounds with strong oxidizing properties, such as peroxyacetic acid and peroxyoctanoic acid *(3, 29)*. Peroxyacetic acid at 80 and 160 ppm against *L. monocytogenes* biofilms on polystyrene achieved reductions of 3.29 and 4.34 log CFU per well, respectively *(21)*. Fatemi and Frank *(14)* compared hydrogen peroxide, peroctanoic acid, and peracetic acid sanitizers. Peracetic acid and peroctanoic acid (80 ppm) inactivated *L. monocytogenes* biofilms on stainless steel, with 3.2 and 3.9 log CFU/cm^2 reductions after 5 min of exposure. Hydrogen peroxide and peroxyacetic acid are strong oxidizing agents, whereas peroxyoctanoic acid is a surface-active component retaining active antimicrobial agents *(3)*. In our study, peroxy acid followed by UV-C light resulted in a high level of biofilm reduction (4.4 to 4.7 log CFU/cm^2), indicating enhancement of antimicrobial activity when treatments were combined. Conversely, Berrang et al. *(8)* found no enhancement in biofilm reduction with use of peroxyacetic acid followed by ultrasonication. The use of UV light at 0.3 and 0.6 J/cm^2 against biofilms of *L. monocytogenes* on stainless steel achieved reductions of 0.26 and 0.42 log CFU/cm^2. A synergistic effect was observed when UV light was combined with sodium hypochlorite (200 ppm), with reductions of 3.1 and 3.8 log CFU/cm^2 *(19)*.

When intervention strategies are combined (e.g., as in this study), bacteria sublethally injured by the first treatment (e.g., peroxy acid) do not have time to recover before the immediate application of the second treatment (e.g., UV light). The bacteria sublethally injured by the first treatment activate defense mechanisms to address the damage caused by that particular stress. However, with the immediate application of the second treatment, the bacterial defense mechanisms are overwhelmed and not promptly activated, leading to a much greater population reduction than achieved by the application of a single intervention *(37)*. The response mechanisms of *L. monocytogenes* to these interventions differ; chemical sanitizers cause oxidative or acid stress conditions, activating bacterial cell wall repair genes, whereas UV light creates abnormal chemical bonds in the DNA, triggering the photo reactivation repair system *(28, 30, 34, 39)*. When bacterial cells encounter several stresses simultaneously, the defense mechanisms are overwhelmed, resulting in a much greater population reduction than achieved with the application of a single intervention *(37)*. UV irradiation damages nucleic acids, and chemical sanitizers weaken the cell wall and membranes. This multiple damage mechanism was also observed by Koivunen and Heinonen-Tanski *(20)*; microbial repair mechanisms are overloaded and unable to repair the injuries, resulting in death of the bacterial cells. We hypothesized that when the chemical sanitizer is followed by UV light, the sanitizer disrupts the membrane protecting the biofilms, allowing deeper penetration of the UV radiation. In our experiments, this combination resulted in the highest reduction in biofilms; however, more research is needed to determine whether the interventions were additive or synergistic by evaluating the effect of the second intervention on injured versus uninjured cells.

In the present study, we evaluated the effect of three chemical sanitizers (lactic acid, quaternary ammonium, and peroxy acid) alone or in combination with UV-C light to control *L. monocytogenes* biofilms on stainless steel. When lactic acid treatment was followed by UV-C light, the greatest log reduction in biofilm was observed. In contrast, quaternary ammonium followed by UV-C light had the least effect on the biofilm. Further research is needed to benchmark the proposed treatments in food processing environments. Nevertheless, the results obtained in this investigation support the use of combined treatments as effective strategies to control biofilms of *L. monocytogenes* on stainless steel.

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