Effect of Chemical Sanitizers with and without Ultrasonication on *Listeria monocytogenes* as a Biofilm within Polyvinyl Chloride Drain Pipes†

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ABSTRACT

As part of a biofilm in a floor drain, *Listeria monocytogenes* is exceedingly difficult to eradicate with standard sanitizing protocols. The objective of these studies was to test the use of ultrasonication to break up biofilm architecture and allow chemical sanitizers to contact cells directly. *L. monocytogenes* biofilms were created in model polyvinyl chloride drain pipes. Chemical sanitizers (quaternary ammonium, peroxide, or chlorine) were applied to the drain pipes with and without a 30-s ultrasonication treatment. Controls using sterile water were included for comparison. *L. monocytogenes* cells were enumerated from the liquid in the drain and the inside wall surface of the pipe. All chemicals lowered numbers of planktonic cells from approximately 6.0 log CFU/cm² of the inner wall surface was detected in water control pipes, and ultrasonication did not lower these numbers. With or without ultrasonication, the peroxide-based sanitizer was effective for reducing the numbers of attached *L. monocytogenes* cells, resulting in approximately 2.0 log CFU/cm². Both the chlorine- and quaternary ammonium–based sanitizers reduced the number of attached *L. monocytogenes* cells to a lesser degree, resulting in 4.2 to 4.4 log CFU/cm². However, addition of ultrasonication improved the performance of both these sanitizers, causing a further reduction to 3.1 and 2.9 CFU/cm² for quaternary ammonium– and chlorine-based chemicals, respectively. These results indicate that a peroxide-based sanitizer alone can be very effective against biofilm *L. monocytogenes* in drain pipes, and the addition of ultrasonication can improve the effectiveness of chlorine or quaternary ammonium sanitizers.

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*Listeria monocytogenes* is a foodborne pathogen that has been found in fully cooked poultry products (10). Unlike *Campylobacter* or *Salmonella,* *L. monocytogenes* is not thought to enter broiler slaughter plants in large numbers with live birds, but its numbers can be amplified during processing (8). *L. monocytogenes* can accompany raw poultry meat into a commercial poultry cooking plant (4). Once in a poultry cooking facility, *L. monocytogenes* can become a long-time resident and can be detected in a wide variety of environmental sites (4, 5, 11). When further processing plants become colonized with *L. monocytogenes,* the potential exists for cross-contamination of fully cooked ready-to-eat product after the thermal lethality step.

*L. monocytogenes* can form a biofilm on surfaces commonly found in food processing plants (3, 6). Persistent food processing plant strains of *L. monocytogenes* show improved ability to adhere to these surfaces (13). *L. monocytogenes* cells in a biofilm are more resistant to chemical sanitizers than are free-living planktonic cells (1, 9). Although the exact mechanism is not understood, a biofilm can develop resistance to common food plant sanitizing chemicals. Such resistance is lost when attached cells are resuspended in a liquid, suggesting that the biofilm itself may include extracellular substances that contribute to sanitizer resistance (14). Resistance to sanitizers and the physical structure of the attached bacterial community make it difficult to eradicate *L. monocytogenes* from sites where it is found as part of a biofilm.

Bacteria that are present in a food plant are likely to be washed into floor drains at some point during processing. *L. monocytogenes* can colonize floor drains in poultry cooking plants (4, 5, 11). Once a drain has been contaminated with *L. monocytogenes,* this pathogen can become a member of a biofilm community and as such can be very difficult to remove. During wash down, water is commonly sprayed across the floor or into a drain, and an aerosol with the potential to disseminate *L. monocytogenes* may be created. An effective method for cleaning and decontaminating the upper area of drain pipes may help to minimize the spread of *L. monocytogenes* through the environment of a poultry cooking plant.

Ultrasonic treatment at 20 to 100 kHz can be used to kill bacteria (15). When applied in a liquid medium, sound waves at this frequency lead to cavitation and changes in pressure (15). In food processing, the effectiveness of a mild heat treatment can be enhanced by ultrasonic treatment (2, 7). Ultrasonic treatment can remove cells from fresh produce and is especially effective when used with chlorine...
treatment (17). Increasing the time of ultrasound treatment and adding solids to a liquid can also increase the bactericidal effect (12, 16). The cavitation created in a liquid by ultrasonic waves may have the mechanical effect of breaking up the biofilm architecture, thereby releasing attached cells into a planktonic state where they would be more susceptible to chemical sanitizers.

The objective of the current study was to test the use of three different types of chemical sanitizers with and without ultrasound treatment to kill planktonic and attached L. monocytogenes in a polyvinyl chloride drain pipe. Treatments were applied in a model drain pipe system with an established L. monocytogenes biofilm on the inner surface. Numbers of cells were measured after treatment with a hydrogen peroxide–peroxyacetic acid, quaternary ammonium or chlorine sanitizer with and without ultrasound.

MATERIALS AND METHODS

Experimental design. A monoculture L. monocytogenes biofilm of approximately 10⁶ CFU/cm² was established on the inner surface of model floor drains made from polyvinyl chloride pipe. Two sets of experiments were conducted to determine the effect of chemical sanitizers (quaternary ammonium, hydrogen peroxide–peroxyacetic acid, or chlorine) with and without the use of ultrasonication to break up biofilm architecture. In the first experiment, a 30-s ultrasonication treatment was applied to water-filled drain pipes that had an L. monocytogenes biofilm in place. After sonication, the water was replaced with one of three chemical sanitizers (or water as a control), which was left in place for 5 min. In the second set of experiments, the chemical and sonication treatments were concurrent. Pipes with an established biofilm were filled with one of the three chemical sanitizers, the 30-s ultrasonication treatment was applied with the chemical sanitizer in place, and the chemical remained in the pipe for an additional 5 min. In both sets of experiments, after all treatments were completed, planktonic L. monocytogenes cells in the liquid and attached cells on the inner wall surface of the drain pipe were enumerated. Five replications, each with two duplicate pipes, were conducted for each set of experiments.

Biofilm development. Model drain pipes were constructed from polyvinyl chloride pipe. Schedule 40 polyvinyl chloride pipe with a 5-cm inside diameter was cut to 10-cm lengths, and an end cap was glued onto one end of each 10-cm pipe. A field isolate of L. monocytogenes previously found to effectively form biofilms (data not shown) was grown as a lawn on multiple modified Oxford plates, which was then poured off, and the inner surface of each pipe was rinsed three times with sterile water in a squeeze spray bottle. Each pipe was then refilled with 103 ml of sterile brain heart infusion (BHI) broth and incubated at 25°C for an additional 24 h. After incubation, BHI broth was poured off, and the inner surface of each pipe was again rinsed by spraying sterile water over the entire surface three times to remove nonadherent cells.

Ultrasonication treatment. Ultrasonication at a frequency of 20 kHz was applied using a 750-W, 120-V ultrasonic processor outfitted with a high-intensity probe, which had a tip diameter of 13 mm (Daigger, Vernon Hills, Ill.). The probe was placed 0.5 cm below the liquid surface in the model drain pipe. Ultrasonication was conducted at 100% amplitude for 30 s, which in preliminary experiments caused a 4°C increase in liquid temperature (data not shown). Ultrasonication was conducted once per pipe at a time, and the probe was sanitized with 70% ethanol between samples. Unsonicated controls were conducted simultaneously for comparison.

Chemical sanitizers. Three chemical sanitizers were tested, and sterilized water was used as a control. The three chemical sanitizers (Zep Manufacturing Co., Atlanta, Ga.) were Perosan, a blend of hydrogen peroxide and per oxyacetic acid; FS Amine-Z, a blend of quaternary ammonium compounds; and Zep FS formula 4665, a chlorine-based sanitizer. Perosan was applied at the concentration recommended on the label for killing L. monocytogenes (1.2 oz/5 gal [1.9 ml/liter]; 111 ppm per oxyacetic acid and 473.6 ppm hydrogen peroxide). FS-Amine-Z was applied at the highest concentration recommended on the label for decontamination of food plants (0.5 oz/gal [4.0 ml/liter]; 400 ppm active ingredients). Zep FS Formula 4665 was applied at the concentration recommended on the label for sanitizing floors (6 oz/10 gal [4.8 ml/liter]; 600 ppm sodium hypochlorite).

L. monocytogenes culture. Planktonic L. monocytogenes cells were recovered from the liquid by removing 1 ml immediately following 5 min of chemical treatment and placing in 9 ml of sterile Dey-Engley (DE) neutralizing broth (Becton Dickinson, Sparks, Md.) to halt sanitizer activity. Further serial dilutions were made in sterile PBS, and 1 ml was plated on the surface of duplicate modified Oxford agar plates. All plates were incubated at 35°C for 24 h.

After removal of the aliquot for sampling, the liquid was poured out of the drain pipe. L. monocytogenes was recovered from the inner wall surface of model drain pipes with a sterile sponge (18-oz [511-g] Speci-Sponge, Nasco, Fort Atkinson, Wis.) premoistened with 10 ml of DE neutralizing broth (Becton Dickinson, Sparks, Md.) to halt sanitizer activity. Further serial dilutions were made in sterile PBS, and 0.1 ml was plated on the surface of duplicate modified Oxford agar plates. All plates were incubated at 35°C for 24 h.

Statistical analysis. The L. monocytogenes CFU per milliliter or per square centimeter were calculated and log transformed. A general linear model (GLM) was conducted to test for the effect of replication, sonication, and chemical on the log CFU recovered per milliliter or per square centimeter. When the GLM indicated a significant effect, Fisher least significant difference tests were used to separate means. Significance was assigned at P < 0.01.

RESULTS

The numbers of planktonic and attached L. monocytogenes cells detected in model drain pipes following ultrasonic and chemical treatments are shown in Tables 1 and
TABLE 1. Mean L. monocytogenes populations detected in model drain pipes when treated with ultrasonication in water followed by a 5-min sanitizer rinse

<table>
<thead>
<tr>
<th>Ultrasonication</th>
<th>Sanitizer</th>
<th>Planktonic (log CFU/ml)</th>
<th>Attached (log CFU/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Water</td>
<td>6.63 ± 0.39 A</td>
<td>6.23 ± 0.26 A</td>
</tr>
<tr>
<td>30 s</td>
<td>Water</td>
<td>6.35 ± 0.53 A</td>
<td>6.16 ± 0.33 A</td>
</tr>
<tr>
<td>None</td>
<td>Quat</td>
<td>0 ± 0 B</td>
<td>3.57 ± 0.35 B</td>
</tr>
<tr>
<td>30 s</td>
<td>Quat</td>
<td>0 ± 0 B</td>
<td>3.65 ± 0.37 B</td>
</tr>
<tr>
<td>None</td>
<td>Peroxide</td>
<td>0.17 ± 0.38 B</td>
<td>2.12 ± 0.95 C</td>
</tr>
<tr>
<td>30 s</td>
<td>Peroxide</td>
<td>0 ± 0 B</td>
<td>1.80 ± 1.09 C</td>
</tr>
<tr>
<td>None</td>
<td>Chlorine</td>
<td>0.17 ± 0.38 B</td>
<td>3.82 ± 0.40 B</td>
</tr>
<tr>
<td>30 s</td>
<td>Chlorine</td>
<td>0.17 ± 0.38 B</td>
<td>3.59 ± 0.67 B</td>
</tr>
</tbody>
</table>

a Ultrasonication was conducted at 20 kHz and 750 W.

b Quat, a blend of quaternary ammonium compounds, 0.5 oz/gal (4.0 ml/liter) FS Amine-Z (Zep Manufacturing Co., Atlanta, Ga.); peroxide, hydrogen peroxide and peroxyacetic acid, 1.2 oz/5 gal (1.9 ml/liter) PeroSan (Zep Manufacturing Co.); chlorine, chlorine-based sanitizer, 6 oz/10 gal (4.8 ml/liter) Zep FS Formula 4665 (Zep Manufacturing Co.).

c Means within columns followed by different letters are significantly different (Fisher least significant difference test, P < 0.01).

TABLE 2. Mean L. monocytogenes populations detected in model drain pipes when treated with ultrasonication in sanitizer chemical followed by an additional 5-min chemical rinse

<table>
<thead>
<tr>
<th>Ultrasonication</th>
<th>Sanitizer</th>
<th>Planktonic (log CFU/ml)</th>
<th>Attached (log CFU/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Water</td>
<td>6.61 ± 0.39 A</td>
<td>6.63 ± 0.10 A</td>
</tr>
<tr>
<td>30 s</td>
<td>Water</td>
<td>6.63 ± 0.47 A</td>
<td>6.43 ± 0.24 A</td>
</tr>
<tr>
<td>None</td>
<td>Quat</td>
<td>0 ± 0 B</td>
<td>4.22 ± 0.28 B</td>
</tr>
<tr>
<td>30 s</td>
<td>Quat</td>
<td>0 ± 0 B</td>
<td>3.13 ± 0.41 C</td>
</tr>
<tr>
<td>None</td>
<td>Peroxide</td>
<td>0 ± 0 B</td>
<td>2.36 ± 0.80 Cd</td>
</tr>
<tr>
<td>30 s</td>
<td>Peroxide</td>
<td>0 ± 0 B</td>
<td>2.00 ± 1.10 C</td>
</tr>
<tr>
<td>None</td>
<td>Chlorine</td>
<td>0 ± 0 B</td>
<td>4.44 ± 0.22 B</td>
</tr>
<tr>
<td>30 s</td>
<td>Chlorine</td>
<td>0 ± 0 B</td>
<td>2.86 ± 0.84 C</td>
</tr>
</tbody>
</table>

a Ultrasonication was conducted at 20 kHz and 750 W.

b Quat, a blend of quaternary ammonium compounds, 0.5 oz/gal (4.0 ml/liter) FS Amine-Z (Zep Manufacturing Co., Atlanta, Ga.); peroxide, hydrogen peroxide and peroxyacetic acid, 1.2 oz/5 gal (1.9 ml/liter) PeroSan (Zep Manufacturing Co.); chlorine, chlorine-based sanitizer, 6 oz/10 gal (4.8 ml/liter) Zep FS Formula 4665 (Zep Manufacturing Co.).

c Means within columns followed by different letters are significantly different (Fisher least significant difference test, P < 0.01).

2. Table 1 lists data from the first set of experiments, in which ultrasonication was tested as a treatment in water before sanitizer application. In water control drains, more than 6 log CFU/ml (planktonic cells) and more than 6 log CFU/cm² (attached cells) were detected. Each experimental chemical lowered the numbers of planktonic L. monocytogenes to the same extent resulting in counts at or close to the limit of detection of 10 cells per ml. Although not as dramatic as the effect noted for planktonic cells, chemical treatment also lowered numbers of attached cells detected on the inner wall surface of drain pipes. Under the conditions of this study, the peroxide–peroxyacetic acid sanitizer was significantly more effective (P < 0.01) than the quaternary ammonium or chlorine-based chemicals for killing attached L. monocytogenes cells. A 30-s ultrasound treatment before application of sanitizer did not significantly affect the numbers of attached or planktonic L. monocytogenes cells detected in any of the drain pipes, including those in the water control.

Data from the second set of experiments in which ultrasonication and chemical sanitizers were applied concurrently are listed in Table 2. Ultrasonication did not cause any change in the numbers of planktonic or attached L. monocytogenes cells detected in the water control drain pipes. All chemical sanitizers (regardless of ultrasonication treatment) significantly lowered numbers of planktonic L. monocytogenes cells below the limit of detection, representing a 6-log decrease compared with the water control. All sanitizers were effective for significantly lowering numbers of L. monocytogenes cells attached to the inner wall surface of model drain pipes in comparisons with the water control. When used without sonication, the peroxide–peroxyacetic acid sanitizer was again the most effective treatment tested for lowering numbers of attached L. monocytogenes cells. Ultrasonication did not improve the performance of the peroxide–peroxyacetic acid sanitizer but it did improve the effectiveness of the quaternary ammonium and chlorine-based sanitizers. When ultrasonication was used in concert with the chlorine-based sanitizer, its ability to lower numbers of attached L. monocytogenes cells was equivalent to that of the peroxide–peroxyacetic acid sanitizer.

DISCUSSION

The finding that attached L. monocytogenes cells are more resistant to sanitizers than planktonic cells is consistent with other published data (1, 9, 14). All the sanitizers tested in the current study were very effective for reducing numbers of viable L. monocytogenes cells in drain liquid. Although ultrasonication has been reported to kill bacteria in liquid (16), under the conditions of this study we did not see a significant effect of ultrasonication when applied to a water-filled drain pipe. The effectiveness of ultrasound for killing bacteria is dependent on the type of organism being tested. L. monocytogenes has been reported to be susceptible to ultrasonic treatment (15). However, the effectiveness of such treatment against L. monocytogenes increases when combined with heat treatment (2, 15) and increases against other bacteria when used in combination with chemical sanitizers (12, 17).

As expected, chemical treatment alone did not eliminate attached L. monocytogenes from the inner surface of model drain pipes. Ultrasonic treatment alone also did not make a difference in the numbers of attached bacteria in a water-filled pipe. The cavitation produced by ultrasonic waves has been reported to help remove cells from the surface of fresh produce (17). We hypothesized that the ultra-
sonic waves would disrupt biofilm structure, releasing cells into a planktonic state where the chemical sanitizers would be more likely to kill them. The current data, however, do not suggest that ultrasonication as tested here had a large effect on biofilm architecture. There was no indication that ultrasonication released more cells into the diluent when the pipes were filled with water. If such a transfer had occurred, it would presumably have resulted in an increased number of planktonic cells detected in the water control drain pipes, but no such increase was noted.

Ultrasound treatment has been more effective when used in concert with chemical sanitizers (12, 17). In the current study, ultrasound treatment did improve the effectiveness of quaternary ammonium and chlorine-based sanitizers. However, even with the addition of ultrasonication, these chemicals did not outperform an oxidizing sanitizer.

The effectiveness of ultrasound treatment for killing bacteria is related to treatment time; extended treatments are generally more effective (16). It is possible that treatment times of longer than 30 s would be more effective than the 30-s treatments used in the current study. However, assuming all other engineering hurdles could be cleared (e.g., portable probe design and control of aerosol), it seems unlikely that a treatment time of more than 30 s per drain pipe would be practical in a food plant environment. Other possibilities for improving the performance of ultrasound treatment include the addition of heat (2, 7) or specific solids (12).

Floor drains in food plants will continue to be sites of L. monocytogenes harborage and a difficult area to clean effectively. More research is needed to address this problem. More information on ultrasonication intensity, treatment time, and chemical or heat application would be useful to determine what role, if any, ultrasonication could play in the ongoing effort to prevent dissemination of L. monocytogenes in food plants.

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REFERENCES