

Elimination of *Listeria monocytogenes* Biofilms by Ozone, Chlorine, and Hydrogen Peroxide

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ABSTRACT

This study evaluated the efficacy of ozone, chlorine, and hydrogen peroxide to destroy *Listeria monocytogenes* planktonic cells and biofilms of two test strains, Scott A and 10403S. *L. monocytogenes* was sensitive to ozone (O₃), chlorine, and hydrogen peroxide (H₂O₂). Planktonic cells of strain Scott A were completely destroyed by exposure to 0.25 ppm O₃ (8.29-log reduction, CFU per milliliter). Ozone's destruction of Scott A increased when the concentration was increased, with complete elimination at 4.00 ppm O₃ (8.07-log reduction, CFU per chip). A 16-fold increase in sanitizer concentration was required to destroy biofilm cells of *L. monocytogenes* versus planktonic cells of strain Scott A. Strain 10403S required an ozone concentration of 1.00 ppm to eliminate planktonic cells (8.16-log reduction, CFU per milliliter). Attached cells of the same strain were eliminated at a concentration of 4.00 ppm O₃ (7.47-log reduction, CFU per chip). At 100 ppm chlorine at 20°C, the number of planktonic cells of *L. monocytogenes* 10403S was reduced by 5.77 log CFU/ml after 5 min of exposure and by 6.49 log CFU/ml after 10 min of exposure. Biofilm cells were reduced by 5.79 log CFU per chip following exposure to 100 ppm chlorine at 20°C for 5 min, with complete elimination (6.27 log CFU per chip) after exposure to 150 ppm at 20°C for 1 min. A 3% H₂O₂ solution reduced the initial concentration of *L. monocytogenes* Scott A planktonic cells by 6.0 log CFU/ml after 10 min of exposure at 20°C, and a 3.5% H₂O₂ solution reduced the planktonic population by 5.4 and 8.7 log CFU/ml (complete elimination) after 5 and 10 min of exposure at 20°C, respectively. Exposure of cells grown as biofilms to 5% H₂O₂ resulted in a 4.14-log CFU per chip reduction after 10 min of exposure at 20°C and in a 5.58-log CFU per chip reduction (complete elimination) after 15 min of exposure.

Listeria monocytogenes is a gram-positive, aerobic to facultative anaerobic bacterium that is a major foodborne pathogen. Owing to its ubiquitous nature, *L. monocytogenes* is frequently isolated from food processing plants. A factor promoting colonization within a processing environment is adhesion to a wide range of surfaces including stainless steel, rubber, glass, and polypropylene (13). *L. monocytogenes* has been found on cooler floors, freezers, processing rooms, cases, mats (14), and lubricants used in the dairy industry (20). It has been found in a variety of products including poultry, milk, cheese, coleslaw, lettuce, and meat products (14). Biofilms have been defined as “matrix enclosed bacterial populations adherent to each other and/or to surfaces or interfaces” (3). This includes cell aggregates that are attached to, or imbedded into, solid surfaces or that are suspended in, or on top of, liquids. Kumar and Anand (9) defined a biofilm as a metabolically active matrix of cells and extracellular compounds. Donlan and Costerton (5) further refined the definition of a biofilm as “a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface, or to each other, are embedded in a matrix of extracellular polymeric substance that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.”

Chlorine as hypochlorous acid is an active form of chlorine and is produced by the hydrolysis of chlorine gas at acidic pH values (17). Studies have shown that chlorine rapidly inactivates suspended *L. monocytogenes* cells (2, 6). Lee and Frank (10) found that *L. monocytogenes* biofilms grown on stainless steel surfaces for 8 days were nearly twice as resistant to chlorine than cells grown for 4 days and were significantly more heat resistant. Norwood and Gilmour (16) examined a multispecies biofilm of *L. monocytogenes*, *Pseudomonas fragi*, and *Staphylococcus xylosum* and found increased chlorine resistance in the multispecies biofilm compared with monospecies biofilms. Ozone (O₃) is one of the most powerful oxidizing agents known. It is a 52% stronger oxidant than chlorine and acts more rapidly against a wide spectrum of microorganisms. Ozone is a protoplasm oxidant, and its bactericidal action is extremely rapid. Restaino et al. (18) found more than 5-log unit reductions in *Salmonella* Typhimurium and *Escherichia coli* counts after exposure to 0.18 ppm O₃, while *L. monocytogenes* cells were found to be more resistant. Kim and Yousef (8) reported that exposure of *Pseudomonas fluorescens*, *E. coli*, and *L. monocytogenes* to ozone at 2.5 ppm for 40 s caused 5- to 6-log decreases in counts. Hydrogen peroxide (H₂O₂) is an effective disinfectant that is used in the food industry. It is a strong oxidizing agent that has been shown to damage bacterial proteins, DNA, and cellular membranes (1). Lettuce contaminated with *L. mono-*

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cytogenes, *E. coli* O157:H7, and *Salmonella enterica* and treated with lactic acid, a hydrogen peroxide-based sanitizer, and a mild heat treatment was found to significantly reduce microbial contamination (12). Dominguez et al. (4) reported the bactericidal effect of hydrogen peroxide was enhanced with mild heat. Attached cells of *L. monocytogenes* are more resistant to sanitizers than their planktonic (unattached) counterparts (16). The most problematic phenomenon associated with biofilms is enhanced resistance to sanitizers (7, 15, 16). A biofilm that has withstood the cleaning process can potentially shed bacteria. In the case of foodborne pathogens, these shedding cells can continue to contaminate product lines even after a sanitizer has been used (20).

The purpose of this study was to examine the effectiveness of ozone, chlorine, and hydrogen peroxide on the destruction of planktonic and biofilm cells of *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains. *L. monocytogenes* strain 10403S was obtained from Dr. Daniel A. Portnoy, University of California, Berkeley; *L. monocytogenes* strain Scott A was obtained from Dr. Larry Beuchat, University of Georgia, Griffin.

Growth conditions. Frozen stocks of the cultures were prepared by inoculating 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) with 0.1 ml of an overnight stationary-phase inoculum. These tubes were then vortexed, frozen, and stored at -20°C . As needed, stocks were thawed and inoculated into 250-ml Erlenmeyer flasks containing 90 ml of TSB (Difco, Becton Dickinson) and grown at 37°C in a gyratory shaking water bath (New Brunswick Scientific, Edison, N.J.) to stationary phase, which corresponded to an optical density at 600 nm (OD_{600}) of 1.0 to 1.1 for approximately 12 h of growth.

Stainless steel chip preparation. The wash procedure was modified from Lee and Frank (10). Stainless steel (4 grade) was fabricated into chips (2.54 by 2.54 cm) giving a total surface area of 6.45 cm^2 . Chips were vigorously washed in Fisherbrand Sparkleen for manual washing (Fisher Scientific, Pittsburgh, Pa). After washing, distilled rinses were carried out (3 \times) in 400 ml of distilled water. Four chips were placed in Pyrex glass petri dishes and autoclaved for 15 min at 121°C .

Attachment and biofilm development. The procedure was a modification of that described by Leriche and Carpentier (11). An overnight culture (early stationary phase; 0.1 ml) was placed on each stainless steel chip and spread evenly over the surface of the chip using a sterile inoculating loop. The glass petri dishes were then placed in desiccators at 20°C and at 100% relative humidity to prevent evaporation of the medium from the chips. After 3 h the chips were removed and washed with 25 ml of sterile potassium phosphate buffer (PPB; 50 mM adjusted to pH 7.0). This wash step removed planktonic cells, leaving behind only attached cells. After washing, 0.1 ml of sterile TSB was added to each chip and the chips were placed back into the desiccators. Every 24 h the wash step was repeated and additional medium was added daily for 4 days (stationary phase).

Ozone treatment of planktonic cells. An infinity corona discharge ozone generator (CD-7, Del Industries, San Luis Obispo, Calif.) and an ozone sensor (1054B, Rosemount Analytical, Ir-

vine, Calif.) were used to generate and detect levels of ozone. The sensor was calibrated in distilled water. When an OD_{600} of 1.0 was obtained, cells were harvested by centrifugation ($16,300 \times g$ for 10 min at 4°C) and suspended into 100 ml of sterile PPB. Ozone was diffused in a 1-liter spinner flask with 900 ml of sterile PPB at the proper ozone concentration. The suspended culture was then added to this flask and exposed to the ozone for 3 min. A sample solution (10 ml) was removed from the flask and placed into 90 ml of 0.1% peptone water and serially diluted and plated on tryptic soy agar plates (TSA; Difco, Becton Dickinson). These plates were incubated at 37°C for 24 h. Results were reported as CFU per milliliter.

Ozone treatment of biofilm cells. Biofilm inoculated chips were submerged in the ozonated PPB for 3 min. After exposure, the chips were immediately swabbed with a sterile cotton-tipped applicator, which was first wetted in sterile 0.1% peptone water (Difco, Becton Dickinson). Removed cells were suspended in 10 ml of 0.1% peptone water and serially diluted and plated on TSA. These plates were incubated at 37°C and were read after 24 h of incubation. Results were reported as CFU per chip.

Chlorine treatment of planktonic and biofilm cells. Levels of chlorine, as calcium hypochlorite (Fisher Scientific), were determined by a colorimetric commercial test kit for both planktonic and biofilm cell experiments (LaMotte Co., Chestertown, Md.). Chlorine solutions (ppm free available chlorine) at the desired concentrations were prepared in distilled water (pH 10.8) or sterile 0.85% saline. The swabbing procedure was used for the removal of cells following chlorine treatment of the chips. The same procedure for cultivating and harvesting cells as described above was used. Exposure to chlorine was carried out in these sets of experiments, but prior to swabbing residual active chlorine was neutralized with 10 ml of a 10% sodium thiosulfate solution. Serial dilutions and plating were performed as described above.

Hydrogen peroxide treatment of planktonic and biofilm cells. Hydrogen peroxide (Fisherbrand, Fisher Scientific) at the desired concentrations was prepared by appropriate dilution of the 30% stock solution in sterile 0.85% saline solution at 4°C . The same procedure for cultivating and harvesting cells as described above was used. Exposure to hydrogen peroxide was carried out in these sets of experiments, but prior to swabbing residual hydrogen peroxide was neutralized by adding the chips to 100 ml of a 1% sodium pyruvate solution. Serial dilutions and plating were performed as described above.

Statistical analyses. Statistical analyses were performed using a StatView 512+ Version 1.2 (Brain Power Inc., Calabasas, Calif.). A one-way analysis of variance was used to determine any significant differences between the tested strains. The means and standard errors of the means of triplicate and quadruplicate experiments are shown in Table 1.

RESULTS AND DISCUSSION

Ozone inactivation of planktonic and biofilm cells. As shown in Table 1, both strains of *L. monocytogenes* were sensitive to ozone in both the planktonic and biofilm states. In the unattached state, strain Scott A was completely destroyed by exposure to 0.25 ppm O_3 (8.29-log reduction, CFU per milliliter). Ozone's destruction of Scott A biofilms increased, when the concentration was increased, with complete elimination at 4.00 ppm O_3 (8.07-log reduction, CFU per chip). A 16-fold increase in sanitizer concentration was required to destroy attached cells of *L. monocytogenes* ver-

TABLE 1. Log reduction of *Listeria monocytogenes* cells after 3 min of exposure to varying levels of ozone in PPB at 24°C

Ozone concentration (ppm)	Mean log reduction (CFU/chip or CFU/ml) \pm SEM			
	Scott A		10403S	
	Biofilm	Unattached	Biofilm	Unattached
0.25	1.48 \pm 0.09	8.29 \pm 0.05 ^a	2.65 \pm 0.11	1.93 \pm 0.21
0.50	4.03 \pm 0.24	ND ^{a,b}	2.75 \pm 0.57	3.47 \pm 0.06
1.00	4.34 \pm 0.08	ND ^a	4.02 \pm 0.54	8.16 \pm 0.13 ^a
2.00	4.51 \pm 0.03	ND ^a	3.97 \pm 0.21	ND ^a
4.00	8.07 \pm 0.04 ^a	ND ^a	7.47 \pm 0.07 ^a	ND ^a

^a Complete elimination was observed.

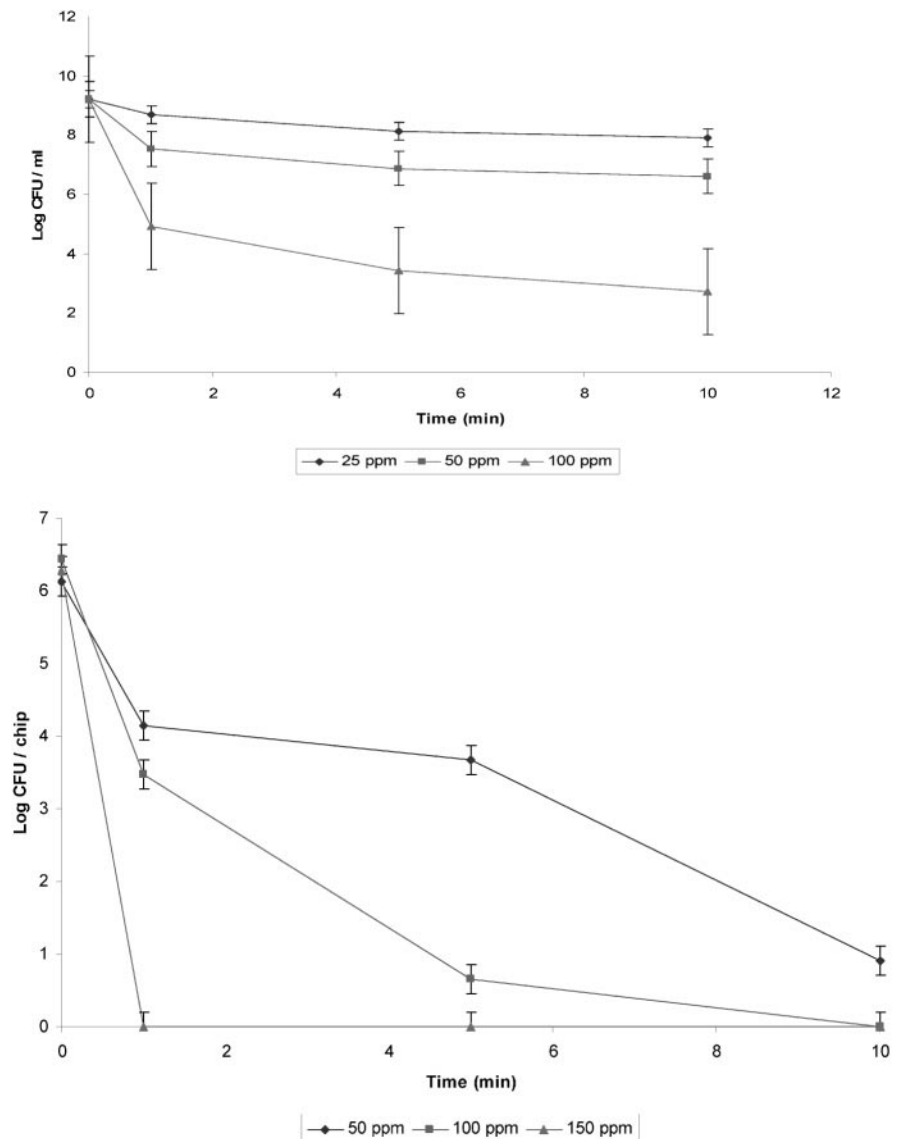
^b ND, not detected.

versus unattached cells of strain Scott A. Strain 10403S required an ozone concentration of 1.00 ppm to eliminate planktonic cells (8.16-log reduction, CFU per milliliter). Attached cells of the same strain were eliminated at a concentration of 4.00 ppm O₃ (7.47-log reduction, CFU per chip). A fourfold increase in sanitizer concentration was

required to destroy biofilm cells of *L. monocytogenes* versus unattached cells of strain 10403S.

Chlorine inactivation of planktonic and biofilm cells. The results for the chlorine inactivation of planktonic and biofilm cells of *L. monocytogenes* 10403S are shown

FIGURE 1. (Top) Effects of three concentrations of calcium hypochlorite on planktonic cells of *Listeria monocytogenes* 10403S treated at 20°C. (Bottom) Effects of three concentrations of calcium hypochlorite on biofilm cells of *Listeria monocytogenes* 10403S treated at 20°C.



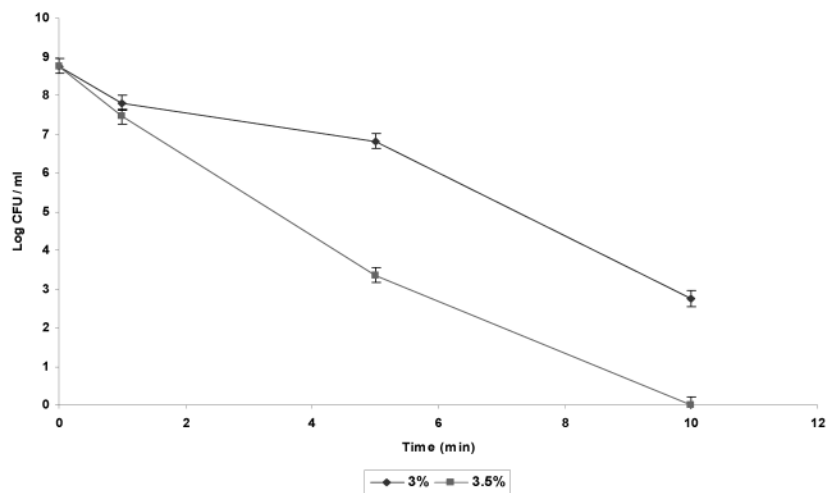
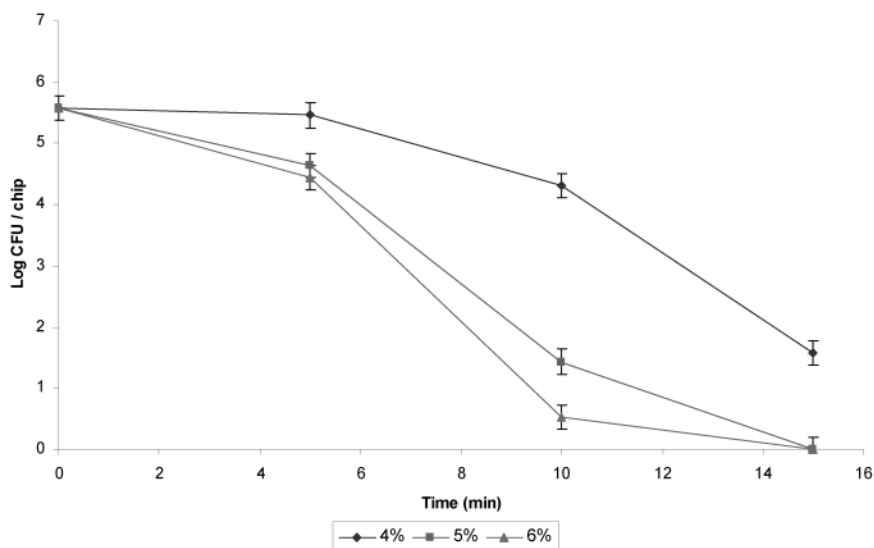


FIGURE 2. (Top) Effects of two concentrations of hydrogen peroxide on planktonic cells of *Listeria monocytogenes* 10403S treated at 20°C. (Bottom) Effects of three concentrations of hydrogen peroxide on biofilm cells of *Listeria monocytogenes* 10403S treated at 20°C.



in Figure 1. At 100 ppm chlorine at 20°C, the number of planktonic cells was reduced by 5.77 log CFU/ml after 5 min of exposure and by 6.49 log CFU/ml after 10 min of exposure. Biofilm cells of *L. monocytogenes* 10403S were reduced by 5.79 log CFU per chip following exposure to 100 ppm chlorine at 20°C for 5 min, with complete elimination (6.27 log CFU per chip) after exposure to 150 ppm at 20°C for 1 min. Norwood and Gilmour (16) found that 10 ppm chlorine was sufficient to inactivate 10⁷ CFU/ml *L. monocytogenes* Scott A planktonic cells, but that 500 ppm chlorine was required to cause a statistically significant fall in number of biofilm-associated cells. The discrepancy in the chlorine concentration necessary to inactivate planktonic cells in this study may be explained by the pH of the chlorine solution. In the present study, the pH of the chlorine solution in distilled water was 10.8, while in the Norwood and Gilmour (16) study, the planktonic inactivation was performed in phosphate-buffered saline, at an undefined pH. The discrepancy in the chlorine concentration necessary to inactivate biofilm cells may be explained by use of a different strain (Scott A versus 10403S) and the

use of a multispecies biofilm. The results of this study concur with studies by Mustapha and Liewen (15) and Stopforth et al. (19), who found 100 and 200 ppm chlorine (as sodium hypochlorite) to be effective in the inactivation of *L. monocytogenes* biofilms attached to stainless steel.

Hydrogen peroxide inactivation of planktonic and biofilm cells. The results for the hydrogen peroxide inactivation of planktonic and biofilm cells of *L. monocytogenes* Scott A are shown in Figure 2. A 3% H₂O₂ solution reduced the initial concentration by 6.0 log CFU/ml after 10 min of exposure at 20°C, and a 3.5% H₂O₂ solution reduced the planktonic population by 5.4 and 8.7 log CFU/ml (complete elimination) after 5 and 10 min of exposure at 20°C, respectively. Exposure of *L. monocytogenes* Scott A cells grown as biofilms on stainless steel chips to 5% H₂O₂ resulted in a 4.14-log CFU per chip reduction after 10 min of exposure at 20°C and in a 5.58-log CFU per chip reduction (complete elimination) after 15 min of exposure. Six percent H₂O₂ caused 5.06 and 5.58 log CFU per chip (complete elimination) reductions following exposure for

10 and 15 min, respectively, at 20°C. Unlike chlorine, the efficacy of hydrogen peroxide treatment is relatively unaffected by high organic loads. Lin et al. (12) showed that treatment of iceberg lettuce with 2% H₂O₂ at 50°C resulted in a 3-log reduction of *L. monocytogenes*.

Results from this study show that ozone, chlorine, and hydrogen peroxide were able to kill both planktonic and biofilm cells of *L. monocytogenes*. In general, biofilm cells were more resistant to the three disinfectants than were planktonic cells.

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