Efficacy of Chlorine Dioxide against *Listeria monocytogenes* in Brine Chilling Solutions

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ABSTRACT

Chilled brine solutions are used by the food industry to rapidly cool ready-to-eat meat products after cooking and before packaging. Chlorine dioxide (ClO₂) was investigated as an antimicrobial additive to eliminate *Listeria monocytogenes*. Several experiments were performed using brine solutions made of sodium chloride (NaCl) and calcium chloride (CaCl₂) inoculated with *L. monocytogenes* and/or treated with 3 ppm of ClO₂. First, 10 and 20% CaCl₂ and NaCl solutions (pH 7.0) were inoculated with a five-strain cocktail of *L. monocytogenes* to obtain ~7 log CFU/ml and incubated 8 h at 0°C. The results demonstrated that *L. monocytogenes* survived in 10% CaCl₂, 10 and 20% NaCl, and pure water. *L. monocytogenes* levels were reduced ~1.2 log CFU/ml in 20% CaCl₂. Second, inoculated (~7 log CFU/ml) brine solutions (10 and 20% NaCl and 10% CaCl₂) treated with 3 ppm of ClO₂ resulted in a ~4-log reduction of the pathogen within 90 s. The same was not observed in a solution of 20% CaCl₂; further investigation demonstrated that high levels of divalent cations interfere with the disinfectant. Spent brine solutions from hot dog and ham chilling were treated with ClO₂ at concentrations of 3 or 30 ppm. At these concentrations, ClO₂ did not reduce *L. monocytogenes*. Removal of divalent cations and organic material in brine solutions prior to disinfection with ClO₂ should be investigated to improve the efficacy of the compound against *L. monocytogenes*. The information from this study may be useful to processing establishments and researchers who are investigating antimicrobials in chilling brine solutions.

Thermally processed meat and poultry foods are often cooled by means of recirculating brine solutions in order to rapidly chill the product. Since chilled brine solutions are in direct contact with the food product, heat and meat components (protein, fat, and other nutrients), run off into the original solution (11). Thus, these brine solutions have the potential to allow bacteria not only to survive but also grow if held and reused for long periods. The current U.S. Department of Agriculture, Food Safety and Inspection Service regulation (28) allows reuse of chilled brine solutions if they are kept free of fecal coliforms and pathogenic organisms.

Among the pathogens that can survive in chilled brine solutions, *Listeria monocytogenes* is one of the most important associated with thermally processed meat and poultry foods like ready-to-eat (RTE) meats. For this reason, the meat industry is looking for options to control bacterial growth in chilled brine solutions. Ultrafiltration has been reported to reduce microbial counts by more than 5.4 log cycles (22). Citric acid also was reported to be effective against *L. monocytogenes* (20). The use of ultraviolet light against *L. monocytogenes* and lactic acid bacteria species in recycled chill brines resulted in at least a 4.5-log reduction in levels of both the pathogen and spoilage organism (11). The application of electrochemical methods to treat fresh and recycled brine solutions also inhibited *L. monocytogenes* (30). To date, little is known about the performance of chlorine dioxide (ClO₂) in brine solutions contaminated with *L. monocytogenes*.

ClO₂ has been used in the treatment of water supplies in Europe since 1850 (5). In 1967, the U.S. Environmental Protection Agency first registered the liquid form of ClO₂ for use as a disinfectant and sanitizer, and in 1988 the agency registered ClO₂ gas as a sterilant (3). In 1995, ClO₂ was approved as a food additive in poultry chiller water (15). Today, the compound is permitted in a variety of foods for human consumption (2). The disinfection mechanisms, while not fully understood, appear to vary by type of microorganism; ClO₂ is often reported to be a strong oxidizer (1).

Compared with chlorine, ClO₂ has several advantages that have been reported previously. For instance, ClO₂ proved to be seven times more effective than chlorine in controlling aerobic bacteria in poultry chilling water (19). Also, in contrast with chlorine solutions, ClO₂ efficacy is not affected by pH or the presence of ammonia (15). The bactericidal effect of ClO₂ seems to be stable between pH 4 and 8 when tested against *Escherichia coli* (5) and between pH 6 and 10 when tested against simian rotavirus SA11 (6). Furthermore, ClO₂ has also been proven to be safe and effective when used for water disinfection (2, 16, 27).

The meat industry is interested in using ClO₂ as an antimicrobial in brine and chilling water systems used for the cooling of RTE meat and poultry products. According to a recent American Meat Institute petition, “the low levels (3 ppm) proposed for use for brine and chilling water systems used in processing of RTE meat and poultry..."
products are similar to those approved for other food applications" (2).

While a number of studies have focused on the effectiveness of ClO$_2$ for reducing microbes in meat or poultry (7, 24–26, 29), seafood (15), and water, only one study has investigated survival of *L. monocytogenes* in brine solutions. In that study, researchers demonstrated that *L. monocytogenes* can survive in brine solutions at various pH levels with different concentrations of chlorine (10). These findings demonstrate a need to identify potential interventions to control, inhibit, and prevent the growth of *L. monocytogenes* in brine solutions used for chilling RTE meat products. The objective of this study is to investigate the efficacy of 3 ppm of ClO$_2$ against *L. monocytogenes* in model and spent brine solutions.

**MATERIALS AND METHODS**

**Culture storage, inoculum preparation, enumeration, and enrichment procedures.** Five outbreak-related strains of *L. monocytogenes* (Scott A, J1-116, J1-110, J1-220, and N3-013) were obtained from the Food Microbiology Culture Collection in the Department of Food Science at the Pennsylvania State University (University Park). The isolates were stored at −70°C in tryptic soy broth (Difco Laboratories, Detroit, MI) containing 10% glycerol. Before experiments, *L. monocytogenes* was propagated in tryptic soy broth at 37°C for 24 h. The cultures were maintained on tryptic soy agar (TSA) slants at 4°C and transferred weekly to maintain viability.

One loopful of *L. monocytogenes* was obtained from TSA slants and aerobically incubated in 9 ml of tryptic soy broth at 37°C for 24 h. Following incubation, cells were washed twice by centrifuging at 5,000 × g for 10 min (10), after which the supernatant was decanted, and the cells were resuspended in 1 ml of a sterile saline solution (0.08% NaCl; BDH, West Chester, PA). Cultures prepared in this manner were added to 99 ml of brine solutions to obtain −7 log CFU/ml in all experiments. All samples generated in this study, which were subjected to treatments with ClO$_2$, were enumerated by serially diluting in 0.1% peptone water plus 0.03% sodium thiosulfate (10), plated in duplicate onto TSA plates (experiments 1, 2, 3, 4, and 5), incubated at 37°C for 48 h, and enumerated manually.

**Survival of *L. monocytogenes* in brines (experiment 1).** In order to determine the effect of brine solutions on *L. monocytogenes* in the absence of ClO$_2$, a preliminary experiment was conducted. Calcium chloride (CaCl$_2$) and sodium chloride (NaCl) (VWR, West Chester, PA) solutions (99 ml) at 10 and 20% were made using deionized water, autoclaved to ensure sterility, and chilled to 0°C using a circulating water bath. The pH of the brine was adjusted to 7.0 with HCl (0.1 N) prior to inoculation with the pathogen, when necessary. Overnight cultures of the pathogen were washed as described previously, and 1 ml was added to 99 ml of individual brine solutions to obtain approximately 7 log CFU/ml. Inoculated solutions were stored at 0°C for up to 24 h, and resulting populations were determined every 2 h for 8 h, and again at 12 and 24 h by sampling, serial diluting, and enumerating on TSA plates as described above. The experiment was performed in triplicate, and D-values were calculated.

**Generation of ClO$_2$.** ClO$_2$ was generated in tap water using a ClO$_2$ generator (Halox System, Bridgeport, CT) located in the Department of Food Science (Pennsylvania State University). In order to ensure that the ClO$_2$ concentration remained stable in brine solutions or chill water, ClO$_2$ was generated at approximately 929 ppm, stored in a dark glass container, and chilled immediately to 4°C. Immediately after generation and before experiments, the stock solution was measured for ClO$_2$ concentration with a Chlordioximeter 1000 photometer (Palintest, Gateshead, UK) using the N,N-diethyl-p-phenylenediamine detection method (18).

**Survival of *L. monocytogenes* in model brine solutions treated with 3 ppm of ClO$_2$ (experiment 2).** In order to determine the effect of brine solutions on the pathogen, washed cultures of *L. monocytogenes* were added to 99 ml of sterile, chilled (0°C) water or brine solutions (10 and 20% NaCl and CaCl$_2$ solutions) to achieve a final concentration of 7 log CFU/ml. A volume of the ClO$_2$ stock solution was added to the solutions such that the final concentration was 3 ppm. After treatment with ClO$_2$, brine solutions were held for up to 90 s at 0°C.

A change in color and presence of precipitant was observed during the direct reading of residual ClO$_2$ in brine solutions using the Chlordioximeter photometer. Therefore, determination of ClO$_2$ dosage was made in the brine solutions by adding an equivalent amount of the stock solution of ClO$_2$ to 100 ml of deionized water and taking measurements with the Chlordioximeter photometer.

Immediately after inoculation, samples were taken for plating and enumeration purposes. Following the addition of ClO$_2$, samples were obtained every 30 s by aseptically removing 1 ml of inoculated treated brine solutions and serial diluting them in 0.1% peptone water containing 0.03% sodium thiosulfate for neutralization of the ClO$_2$, followed by direct plating on TSA in duplicate as described previously. The experiment was performed in triplicate, and D-values also were calculated.

**Assessment of stress response on *L. monocytogenes* (experiment 3).** Cells were pretreated in chilled brine solution or water as negative control, transferred to chilled sterile distilled water, and then treated with ClO$_2$ as follows. An overnight culture of *L. monocytogenes* was centrifuged at 5,000 × g for 10 min, supernatant decanted, and cells resuspended in 1 ml of chilled water and brine solutions (10 and 20% NaCl and CaCl$_2$ solutions) to achieve a final concentration of 7 log CFU/ml. After centrifugation, the supernatant was removed and cells were resuspended in 1 ml of sterile deionized water and added to 99 ml of chilled distilled water to obtain approximately 7 log CFU/ml. The stock solution of ClO$_2$ was added to the inoculated water solutions to obtain ~3 ppm. All samples were exposed for 60 s, and resulting bacterial populations were serially diluted and plated in duplicate on TSA as described above. The experiment was performed in triplicate.

**Assessment of ClO$_2$ depletion (experiment 4).** To address the hypothesis that divalent cations (i.e., calcium) maybe responsible for the depletion of ClO$_2$, a modification of experiment 2 was performed as follows. Washed cultures of *L. monocytogenes* were added to 99 ml of sterile, chilled (0°C) water and brine solutions (20% NaCl, MgCl$_2$, and CaCl$_2$ solutions) to achieve a final concentration of 7 log CFU/ml. Immediately after inoculation with the pathogen into the brine solutions, a sample was taken for initial enumeration purposes. Then, a stock solution of ClO$_2$ was added to the test solutions such that the final concentration in the inoculated brine solutions was equivalent to 3 ppm in water. After treatment with ClO$_2$, brine solutions were held for up to 30 s, and resulting bacterial populations were serially diluted and plated in duplicate on TSA as described above. The mean reduction was
Inoculation of spent brine solutions with *L. monocytogenes* (experiment 5). Using a format similar to that used in experiment 2, spent brine solutions from hot dogs and hams were obtained directly from a meat processor and stored at 4°C for approximately 2 months until experiments were conducted. During that time, compositional analyses were performed on the solutions. Spent solutions from hams contained 3% organic material with a final concentration of ~6% NaCl. Spent brine solutions from hot dogs contained 1% organic material with a final concentration of ~10% NaCl. The organic material was estimated as the difference between the weight of a dry sample and the weight of the ash obtained after heating for 12 h at 600°C (13).

Before experiments were performed, spent brine solutions were transferred to sterile test tubes and chilled to 0°C in a circulating water bath. *L. monocytogenes* was inoculated into the spent brine solutions to achieve 7 log CFU/ml. After inoculation with the pathogen, the stock solution of ClO₂ was added to the test solutions such that the final concentration was approximately 3 or 30 ppm and held at 0°C. Samples were taken every 30 s for up to 180 s and enumerated as described above. Equivalent ClO₂ levels in water were determined using the approach mentioned previously, and results were recorded. Each trial was repeated in triplicate.

**Calculation of D-values.** The inactivation rate was measured by determining the decimal reduction time (D-value) calculated from the average of the inverse slope of the three survival curves of every treatment, including the control. The curves were obtained by plotting log values versus the corresponding time intervals and using linear regression to determine the slope.

**Determination of water activity.** Water activity of brine solutions was measured with a calibrated Decagon CX-1 (Decagon Devices, Pullman, WA) water activity meter following the manufacturer’s instructions. The measurement temperatures were between 17.1 and 18.1°C. The experiment was performed in triplicate.

**Statistical analysis.** Microbial populations obtained from duplicate TSA plates were averaged and converted to log CFU per milliliter. Each repetition provided a curve, and the average of the points from these three curves was determined. The slope of each curve was obtained using linear regression, and the D-value was obtained using the inverse slope of the equation. The D-values were analyzed using Statistical Software, release 15.00 (Minitab Inc., State College, PA). Comparisons of D-values were done using one-way analysis of variance followed by Tukey’s multiple comparison test. Statistical significance was set at *P* < 0.05.

### RESULTS AND DISCUSSION

In this study the regulatory limit of ≤3 ppm of residual ClO₂ in water as specified by the U.S. Environmental Protection Agency, 21 CFR 173.300 (b) (1), was used. The 3-ppm level represents the concentration of ClO₂ remaining in the water used in poultry processing after contact with the food, rather than an upper limit of ClO₂ introduced into the system.

**Experiment 1.** This experiment was performed to determine the survivability of *L. monocytogenes* in the saline solutions without the ClO₂ treatment. Saline solutions of 10 and 20% NaCl and 10% CaCl₂ did not have a significant effect (*P* > 0.05) on the population of *L. monocytogenes* over 8 h of evaluation. However, a significant reduction (*P* < 0.05) in the population (~1.5 log CFU/ml) of *L. monocytogenes* was observed when inoculated in the 20% CaCl₂ solution after 8 h (Fig. 1). The same was observed 24 h (at 0°C) after reaching a reduction of ~3 log CFU/ml (data not shown). These findings support other studies where *L. monocytogenes* was found to be halotolerant, including one study that reported the survival of *L. monocytogenes* for up to 30 days at −12°C in 20% NaCl (23). Another study has also demonstrated the survival of *L. monocytogenes* in commercial cheese brines after 259 days (17). Interestingly, these findings are apparently the first report addressing the survival of *L. monocytogenes* in chilled brine solutions.

The D-values obtained for brine solutions inoculated with *L. monocytogenes* after 8 h were 597, 466, 294, 78, and 11 h for water, 10% NaCl, 20% NaCl, 10% CaCl₂, and 20% CaCl₂, respectively. The D-value obtained for the 20% CaCl₂ solution was significantly different (*P* < 0.05) than those D-values observed for water and NaCl solutions. No significant difference (*P* > 0.05) was observed between the D-values from 10% and 20% CaCl₂ brine solutions. Even though there were no significant differences (*P* > 0.05) among the D-values from water or 10 and 20% NaCl, it was possible to observe an inverse, dose-dependent response described by the inverse relationship between salt concentration and the time needed for *L. monocytogenes* inactivation. The same is also true if the D-values obtained from water and 10 and 20% CaCl₂ solutions were compared. In this case, a significant difference (*P* < 0.05) was observed.

**Experiment 2.** In these experiments, chilled brine solutions inoculated with *L. monocytogenes* were treated with ~3 ppm of ClO₂ (as determined by adding an equivalent amount to water). The addition of ClO₂ had a significant (*P* < 0.05) and immediate effect on the pathogen population, reducing the population by more than 3.5 log

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**FIGURE 1. Survival of *L. monocytogenes* in brine solutions. Experiment was performed in triplicate; the lines represent simple linear regression.
CFU/ml, when sampled for up to 90 s in 10% NaCl, 20% NaCl, and 10% CaCl₂ brine solutions (Fig. 2). However, the addition of ClO₂ to the 20% CaCl₂ solution inoculated with the pathogen only reduced the population of L. monocytogenes by less than 2 log CFU/ml, suggesting that this brine solution provided a protective effect. The average D-values for brine treatments inoculated with L. monocytogenes and treated with 3 ppm of ClO₂ were 50.9, 26.2, 23.5, 23, and 21.9 s for water, 10% NaCl, 20% NaCl, 10% CaCl₂, and 20% CaCl₂, respectively. The D-value from 20% CaCl₂ was significantly different (P < 0.05) from the other treatments and control.

Interestingly, a slight change in color was observed and a precipitate was formed when 3 ppm of ClO₂ was added to the 20% CaCl₂ brine solutions. Given these results, it was hypothesized that the precipitate and/or the brine solution interfered with the antimicrobial activity of the ClO₂ by either affording some protection to the pathogen or by binding the available ClO₂ so that its concentration could not inhibit the pathogen.

**Experiment 3.** From experiment 2, an increased survivability of L. monocytogenes was observed when suspended in 20% CaCl₂ brine treated with 3 ppm of ClO₂. However, it was not clear if these observations were due to an unknown stress-response effect, or if there was a depletion of ClO₂ by the CaCl₂ brine solution. In the water treatment industry, ClO₂ is used to precipitate soluble forms of iron and manganese followed by sedimentation and filtration (1, 14). However, there is no research that addresses the role of inorganic compounds and their potential interference in chilled brines and, ultimately, the performance of ClO₂ treatments against L. monocytogenes in brine chilling solutions.

Cross-protection is a phenomenon observed when cells survive an otherwise lethal form of stress after adapting to a different sublethal condition (12). Cross-protection has been reported for L. monocytogenes. In one report, adapting L. monocytogenes cells to mildly acidic conditions rendered cultures more resistant to relatively high (25 to 30%) carbon dioxide atmospheres (9). In another study, the prior adaptation to sublethal levels of bile acids or heterologous stresses, such as acid, heat, salt, or sodium dodecyl sulfate, significantly enhanced bile resistance (4). And finally, exposure to diverse stresses including starvation and treatment with hydrogen peroxide, ethanol, and low pH treatments increased the thermotolerance of L. monocytogenes (21). Low NaCl concentration (2 to 3.5%) also has been shown to have a protective effect for L. monocytogenes against the food preservative nisin at low temperatures (8).

Following this line of reasoning, the possibility that cross-protection might be occurring in our experiments was examined.

In this experiment, L. monocytogenes was exposed to the various brine solutions at 0°C, transferred to water at the same temperature, and subsequently treated with 3 ppm of ClO₂ for 30 s. It was observed that, regardless of exposure, populations of the pathogen were reduced significantly (P < 0.05) in ~3 log CFU/ml (Fig. 3). These results suggest that prior exposure to brine solutions does not offer any protection to the pathogen when it is exposed ultimately to ClO₂.

**Experiment 4.** Results from experiment 3 demonstrated that the increased survivability of L. monocytogenes when suspended in 20% CaCl₂ and treated with 3 ppm of ClO₂ was not due to a stress-response effect. However, it was not clear if there was depletion of the compound in the solution. It is hypothesized that divalent cations [Ca²⁺] may be responsible for the depletion of the compound. To address this hypothesis, a modification of experiment 2 was performed. The results demonstrated that neither of the brine solutions with divalent cations (MgCl₂ and CaCl₂) treated with ClO₂ decreased the populations of the pathogen significantly (P > 0.05) as compared with treatments with the monovalent cation (NaCl; P < 0.05; data not shown). These findings suggest that high levels of divalent cations in brine solutions have the potential to interfere with ClO₂...
when used against *L. monocytogenes*. Interestingly, brine solutions made with magnesium chloride and treated with the antimicrobial did not result in visible precipitation as was seen with CaCl₂ experiments.

**Experiment 5.** This experiment was performed in order to evaluate the efficacy of ClO₂ in reducing pathogen populations in spent brine solutions from processed meat chillers. Levels of *L. monocytogenes* inoculated into spent brine solutions from hot dog chillers and ham chillers were not reduced after treatment with 3 and 30 ppm of ClO₂. This finding suggests that the spent brine solutions, with as little as 1% organic material, appear to have a high ClO₂ demand, due in part to the organic material suspended in the brine. The ClO₂ appears to be depleted by the organic material and may not be available to affect reductions against the pathogen. Since no significant reductions were observed, D-values for this experiment could not be calculated.

Based on these preliminary findings, the following recommendations should be considered. Before ClO₂ is selected for use as a primary disinfectant in spent brine systems, an oxidant demand study should be completed. If the oxidant demand is significant, the removal of organic material should be employed first to improve the efficacy of ClO₂ against pathogens in brine solutions.

This study has demonstrated that *L. monocytogenes* is able to survive in brine solutions composed of 10 and 20% NaCl and 10% CaCl₂ after 8 h at 0°C. These findings are in agreement with other published studies. However, *L. monocytogenes* was reduced ~1.2 log CFU/ml when suspended in 20% CaCl₂ brine solutions 8 h at 0°C. A ~3 log CFU/ml reduction was observed 24 h following exposure to the compound. To our knowledge, this information has not been reported previously. The efficacy of ClO₂ treatment in reducing *L. monocytogenes* populations was higher when used in both NaCl and 10% CaCl₂ solutions, reaching reductions of up to 4 log CFU/ml in 90 s. The same results were not possible to achieve when ClO₂ was used with 20% CaCl₂.

Subsequent experiments demonstrated that previous exposure of *L. monocytogenes* to brine solutions does not impart protection or enhanced resistance against ClO₂. It appears that the brine composed of 20% CaCl₂ interferes with the activity of ClO₂, possibly by binding the compound, interfering with activity, or diminishing the oxidizing capacity. As such, the use of CaCl₂ brine solutions at a level of 20% should be carefully evaluated for use in combination with 3 ppm of ClO₂. The presence of high levels of divalent cations in brine solutions also has the potential to interfere with ClO₂ action when used against *L. monocytogenes*. Finally, the addition of 3 and 30 ppm of ClO₂, as described in this article, is not effective for reducing *L. monocytogenes* in spent brine solutions from hot dog and ham chillers, due to the presence of organic and, possibly, inorganic material. Interventions before disinfection with ClO₂ should be investigated in order to improve the efficacy of this compound against *L. monocytogenes*.

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