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Efficacy of Electrolyzed Water in the Inactivation of Planktonic and Biofilm *Listeria monocytogenes* in the Presence of Organic Matter

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ABSTRACT

The ability of electrolyzed (EO) water to inactivate *Listeria monocytogenes* in suspension and biofilms on stainless steel in the presence of organic matter (sterile filtered chicken serum) was investigated. A five-strain mixture of *L. monocytogenes* was treated with deionized, alkaline EO, and acidic EO water containing chicken serum (0, 5, and 10 ml/liter) for 1 and 5 min. Coupons containing *L. monocytogenes* biofilms were also overlaid with chicken serum (0, 2.5, 5.0, and 7.5 ml/liter) and then treated with deionized water, alkaline EO water, acidic EO water, alkaline EO water followed by acidic EO water, and a sodium hypochlorite solution for 30 and 60 s. Chicken serum decreased the oxidation-reduction potential and chlorine concentration of acidic EO water but did not significantly affect its pH. In the absence of serum, acidic EO water containing chlorine at a concentration of 44 mg/liter produced a >6-log reduction in *L. monocytogenes* in suspension, but its bactericidal activity decreased with increasing serum concentration. Acidic EO water and acidified sodium hypochlorite solution inactivated *L. monocytogenes* biofilms to similar levels, and their bactericidal effect decreased with increasing serum concentration and increased with increasing time of exposure. The sequential 30-s treatment of alkaline EO water followed by acidic EO water produced 4- to 5-log reductions in *L. monocytogenes* biofilms, even in the presence of organic matter.

Cleaning and sanitizing are an important part of the processes that occur in a food processing plant and, if not properly done, may result in the contamination of products leading to foodborne illnesses, recalls, and economic losses. Typical sanitizers that are applied in the food industry include chlorine compounds (hypochlorites and chlorine dioxide), organic acids (peracetic acid), trisodium phosphate, iodophors, and quaternary ammonium compounds. Chlorine compounds are often the most effective and least expensive, although they may be more corrosive and irritating than alternatives, such as iodine and quaternary ammonium compounds *(8, 14).*

Several factors affect the selection of appropriate sanitizers for food processing plants, and these may include the composition and amount of soil present and the types of surfaces to be sanitized, as well as the types of microorganisms that may be found in the plant. The aim of the cleaning and sanitization steps is to remove all food residues present, after processing, as well as to reduce spoilage and pathogenic microorganisms to allowable levels. The cleaning step is important because organic matter reduces the efficiency of some sanitizers, especially those with chlorine as their active component. Typical sanitation programs involve the use of detergents to remove soil and the application of sanitizers to inactivate bacteria and prevent recontamination *(8).* Several reports have been made on the effect of sanitizers on *Listeria monocytogenes* biofilms *(5, 7, 18),* and different success rates have been reported.

Recently, considerable research has been focused on the bactericidal effect of acidic electrolyzed (EO) water on various foodborne pathogens *(10, 11, 17, 21),* with the aim of exploiting its potential for use in the food industry as an alternative chlorine-based sanitizer. Whereas working with other chlorine sanitizers, which involves the handling of high concentrations of the stock chemical, acidic EO water is produced from a dilute solution of sodium chloride (NaCl) and can be generated onsite, making it safe to handle. It is produced by introducing a dilute salt solution (approximately 0.1%) into an EO water generator that contains an electrolytic cell. By subjecting the positively and negatively charged electrodes to a direct current voltage, two types of water are produced: (i) an acidic EO water that contains chlorine in the forms of hypochlorous acid, hypochlorite ion, and chlorine gas and has a strong oxidizing potential (oxidation-reduction potential [ORP] of ~1,100 mV) and a low pH (~2.6) at the anode side of the cell and (ii) an alkaline EO water, which has a strong reducing potential (ORP of about −800 mV) and a high pH (~11) at the cathode side of the cell *(1).* Acidic EO water has been reported to produce significant reductions in *Escherichia coli* O157:H7 and *L. monocytogenes* on kitchen cutting boards *(21)* and *Enterobacter aerogenes* and *Staphylococcus aureus* on glass, stainless steel, glazed ceramic tile, unglazed ceramic tile, and vitreous china *(17).* Kim et al. *(11)* subjected *L. monocytogenes* biofilms on stainless steel cou-

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pons to acidic EO water treatment and found that the bacterial cells were reduced to undetectable levels in 5 min. A recent study showed that treatment of *L. monocytogenes* biofilms with acidic EO water alone for 2 min resulted in a >5-log CFU per coupon reduction, while a sequential treatment that involved treating the biofilms with alkaline EO water followed by acidic EO water produced an additional reduction of 1.2 log CFU per coupon *(2).*

For the control of biofilms, the presence of organic matter presents an additional challenge, as this is an inevitable part of food-processing environments. A recent report by Frank et al. *(6)* showed that when cleaning and sanitizing were employed sequentially with an alkali cleaner and acidified sodium chlorite, *L. monocytogenes* biofilms overlaid with chicken exudates and fat were reduced to nearly undetectable levels, a greater than 7-log reduction. In their research, when only sanitizers were used, the organic load reduced the efficiency of inactivation. Oomori et al. *(16)* determined the effect of nutrient broth, proteose peptone, glycine, glucose, sucrose, and corn oil on the properties of acidic EO water. They reported that when nutrient broth and proteose peptone were added to acidic EO water, the free available chorine in these solutions disappeared quickly. Since there are currently no reports on the effect of EO water on the inactivation of *Listeria* biofilms in the presence of organic matter, the purpose of this study was to determine the effect of organic matter on the properties of EO water and the efficacy of EO water in the inactivation of *L. monocytogenes* (planktonic cells and biofilms) in the presence of organic matter.

MATERIALS AND METHODS

Treatment water. EO water was produced from a ROX-20TA generator (Hoshizaki Electric Inc., Toyoake, Aichi, Japan) at current settings of 14 and 20 A. After a stable amperage reading was achieved, alkaline and acidic EO water were collected from the cathode and anode side, respectively, into separate sterile 1-liter Nalgene beakers, covered to prevent the loss of chlorine, and used within 1 h of production. The ORP and pH of both the alkaline and acidic EO water were measured immediately after preparation with a dual-scale pH meter (Accumet AR50, Fisher Scientific Co., Fair Lawn, N.J.). The residual chlorine content of the acidic EO water was determined by an iodometric method (Hach Co., Ames, Iowa) with a 0.113 N sodium thiosulfate standard solution. A sodium hypochlorite solution (chlorine at a concentration of ~85 mg/liter) was prepared from a 5 to 6% sodium hypochlorite solution (Fisher) to match the chlorine concentration of acidic EO water produced at 20 A. The pH of the solution was then adjusted with 5 N HCl (Labchem Inc., Pittsburgh, Pa.) so that it would be comparable to the pH of the acidic EO water.

Preparation of inocula. Five strains of *L. monocytogenes*—F8027 (celery isolate), F8255 (peach isolate), 101M (beef isolate), H7750 (hot dog isolate), and G3990 (Vacherin Mont d'Or cheese isolate)—were used for this study. A loop inoculum of each culture was transferred three times in tryptic soy broth (TSB; Becton Dickinson, Sparks, Md.) and incubated at 37°C at successive 24-h intervals. A 24-h culture of each bacteria strain was then centrifuged two times for 10 min (3,600 × *g,* 23°C), and the pellet was washed each time with 5 ml of peptone water (1 g of peptone per liter; Becton Dickinson). Each pellet was resuspended in 5 ml of peptone water. The five cultures were combined to form a mixture with a population of approximately 9 log CFU/ml. Ten milliliters of this mixture was added to 1 liter of a sterile 1:10 dilution of TSB (low nutrient medium, with 3 g of dry medium per liter of deionized water). This was used for the preparation of biofilms.

Effect of organic matter on the properties of EO water. Different volumes (0.1 to 1 ml) of sterile filtered chicken serum (Sigma, St. Louis, Mo.) were added to 100 ml of alkaline or acidic EO water in an Erlenmeyer flask. The mixture of EO water and chicken serum was shaken for 5 min at 120 rpm on a platform shaker (model C10, New Brunswick Scientific, Edison, N.J.), and the ORP, pH, and chlorine content of the mixture were determined by the previously described methods.

Treatment of planktonic cells of *L. monocytogenes* with EO water in the presence of organic matter. One milliliter of the previously described five-strain mixture of *L. monocytogenes* (~9 log CFU/ml) was added to 9 ml of deionized, alkaline EO, and acidic EO water containing different concentrations (0, 5, and 10 ml/liter) of sterile filtered chicken serum (Sigma) for 1 and 5 min. Immediately after the exposure time, 1 ml of the bacteria–treatment water mixture was added to 9 ml of a neutralizing buffer solution (neutralizing buffer at 5.2 g/liter; Becton Dickinson). The neutralized mixture was serially diluted, and two 0.1-ml aliquots of the diluents were plated onto tryptic soy agar (TSA; Becton Dickinson). The neutralized mixture was enriched for the presence of surviving *L. monocytogenes* by adding 1 ml to 10 ml of TSB and incubating at 37°C for 24 h. Tubes that exhibited growth were streaked onto modified Oxford agar (Oxoid, Basingstoke, Hampshire, UK) plates containing *Listeria* selective supplement (Oxoid) and incubated at 37°C for 24 to 48 h, and the presence of typical *L. monocytogenes* colonies was noted.

Preparation of stainless steel coupons. New stainless steel sheets (type 304, no. 4 finish, 1-mm thickness; Stewart Stainless Supply Inc., Suwanee, Ga.) were cut into coupons 2 by 5 cm (10 cm^2). They were cleaned in acetone with Kim wipes to remove grease, rinsed in deionized water, and shaken in a 2% solution of Micro-90 soap (International Products Co., Burlington, N.J.) at 120 rpm and 24 ± 2°C for 1 h on a platform shaker (model C10, New Brunswick Scientific). They were then brushed gently with a soft nylon brush, rinsed thoroughly with deionized water, and immersed in 15% phosphoric acid solution for 20 min at room temperature (24 ± 2°C) with shaking at 120 rpm. The coupons were rinsed thoroughly with deionized water, allowed to dry at room temperature, and then autoclaved at 121°C for 15 min.

Preparation of biofilms. The sterile coupons were immersed in the low nutrient medium inoculated with *L. monocytogenes* and incubated at 24 ± 2°C for 4 h to allow bacteria attachment and then rinsed gently in a circular motion for 10 s with peptone water (1 g of peptone per liter) to remove unattached cells. Biofilms were grown by submerging the coupons containing adherent cells in 1 liter of sterile low nutrient medium and incubating for 48 h at 24 ± 2°C to allow further biofilm growth. After the 48-h growth period, the coupons were removed from the spent medium, placed in 1 liter of fresh sterile low nutrient medium, and incubated for another 24-h period to allow further biofilm growth. After incubation, coupons were rinsed with peptone water (1 g of peptone per liter) to remove unattached cells and allowed to dry at room temperature under a biosafety hood for 30 min.

Soiling of coupons with organic matter. Chicken serum was chosen to represent protein soil that may be found in food processing plants. Various amounts (0, 0.125, 0.25, and 0.375 ml)

of the sterile chicken serum were added to the top surface of coupons prepared as previously described; these amounts were selected such that when the coupons with serum were subsequently subjected to treatment with 50 ml of treatment solution, the resulting concentration of serum in the water would be 0, 2.5, 5.0, and 7.5 ml/liter, respectively. Only one side of the coupons was overlaid with chicken serum to allow precise control of the amount of organic load. All the coupons, including those with no serum, were incubated at 45°C for 30 min, to fix the protein to the coupons, and then dried for 1 h under a biosafety hood before being subjected to EO water treatment. Before treatment, two coupons were selected from each serum concentration (i.e., 0, 2.5, 5, and 7.5 ml/liter), and the populations of *L. monocytogenes* recovered from these were used as controls for the respective serum levels.

Treatment of biofilms with EO water. Coupons containing biofilms were immersed in 50 ml of deionized water, alkaline EO water, acidic EO water, alkaline EO water followed by acidic EO water (sequential treatment), and acidified sodium hypochlorite solution for 30 and 60 s at room temperature (24 ± 2°C). For the sequential treatment, the coupons were immersed in alkaline EO water for the specific treatment time, removed, and then rinsed for 10 s in 0.1% peptone water to remove any excess alkaline EO water before being immersed in the acidic EO water for the selected treatment time, i.e., 30 or 60 s. After treatment, the coupons were immediately immersed in 50 ml of neutralizing buffer solution for 40 s and then subjected to microbiological analysis. For treatments with acidic EO water and sodium hypochlorite solution, at the end of the treatment time, 5 ml of the treatment water was added to 5 ml of double-strength neutralizing buffer solution (neutralizing buffer at 10.4 g/liter; Becton Dickinson).

Microbiological analysis. To enumerate *L. monocytogenes,* the coupons (soiled and unsoiled) were placed in sterile Nalgene bottles (8 oz [ca. 237 ml]) containing 20 ml of sterile peptone water (1 g of peptone per liter) and 3 g of acid-washed glass beads (425 to 600 μm; Sigma-Aldrich Co., St. Louis, Mo.) as described by Hassan et al. *(9)* with some modifications. The bottles were then shaken for 10 min on an orbital incubator shaker (model C24, New Brunswick Scientific) at 400 rpm to remove the bacteria from the coupons. Serial dilutions of the peptone water were made after shaking. The surviving bacteria from the control and treated coupons were enumerated by spread plating 0.1 ml of the diluents on TSA. The plates were incubated at 37°C for 48 h, and colonies were counted and recorded as log CFU per coupon. For microbiological analysis of the treatment water, serial dilutions of deionized and alkaline EO water were done without neutralization, enumerated on TSA plates, and then incubated at 37°C for 48 h. For acidic EO water and sodium hypochlorite solution, 1 ml of the double-strength neutralized solution was enumerated by plating 0.25 ml of this water on four TSA plates and incubating at 37°C for 48 h. Treated coupons were subjected to enrichment by adding 10 ml of the peptone water used for removing bacteria from the coupons to 10 ml of TSB and incubating at 37°C for 24 to 48 h. Enrichment of the treatment water was also done by adding 1 ml of the neutralized treatment solution to 10 ml of TSB and incubating at 37°C for 24 h. The presence of *L. monocytogenes* was confirmed as previously described.

Data analysis. Experiments were replicated three times with duplicate treatments in each replication. Data were analyzed by the general linear models procedure of the Statistical Analysis System (SAS Institute, Cary, N.C.). Comparisons of means were calculated with Tukey-Kramer multiple range tests.

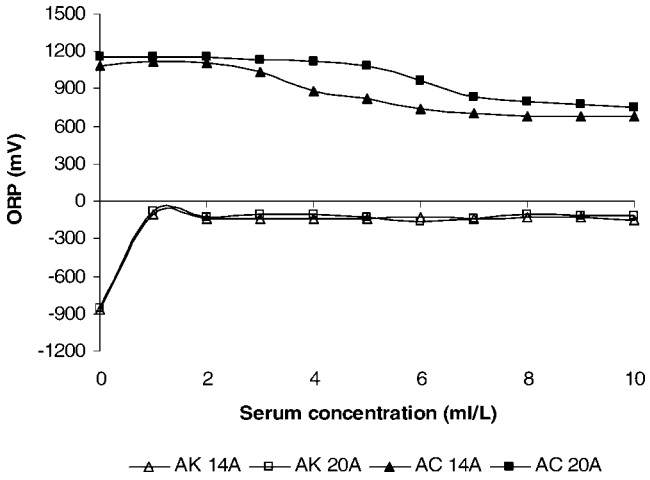

FIGURE 1. *Oxidation-reduction potential of electrolyzed water after treating with different concentrations of chicken serum for 5 min. AK, alkaline EO water; AC, acidic EO water.*

RESULTS AND DISCUSSION

Effect of organic matter on the properties of EO water. The addition of chicken serum of up to 10 ml/liter in treatment water did not significantly affect the pH of alkaline and acidic EO water produced at either 14 or 20 A. In other words, the pH, which was originally 11.03 and 11.23 for alkaline EO water and 2.50 and 2.37 for acidic EO water produced at 14 and 20 A, respectively, remained essentially the same at all levels (1 to 10 ml/liter) of serum concentration (data not shown).

Data on the effect of the serum concentration on the ORP of EO water are presented in Figure 1. When chicken serum was added to achieve a concentration of 1 ml/liter, the ORP of alkaline EO water increased sharply from −861 to −102 mV and then remained about the same with the addition of serum up to 10 ml/liter (Fig. 1). Alkaline EO water produced at 20 A followed the same trend as that for 14 A, and there were no significant differences between the two. The ORP of acidic EO water produced at 14 A was 1,084 mV, and with the addition of chicken serum to achieve a concentration of 1 to 3 ml/liter, the ORP remained the same and then decreased gradually between 4 and 7 ml/liter to about 700 mV. Beyond this point, any further addition of chicken serum to achieve a higher concentration of up to 10 ml/liter did not significantly change the ORP. A similar trend was observed for acidic EO water produced at 20 A (Fig. 1). The changes observed in the ORP of acidic and alkaline EO water show that the organic matter had reduced its oxidizing and reducing properties, respectively.

The addition of chicken serum to acidic EO water caused a decrease in the chlorine content (Fig. 2). As the serum concentration increased, the chlorine concentration decreased steadily from 43 to 3 mg/liter and from 82 to 11 mg/liter for acidic EO water produced at 14 and 20 A, respectively (Fig. 2). White *(22)* reported that proteins react with chlorine to form organochloramines, which could modify the properties of EO water. The reduction in the oxidizing property of acidic EO water could be due to a reduction in hypochlorous acid. The neutralization of chlo-

TABLE 1. *Properties of electrolyzed water used for the treatment of planktonic cells and biofilms of* Listeria monocytogenes

Amperage (A)	Nature of organism	Treatment water	pH	ORP (mV)	Total chlorine (mg/liter)
14	Planktonic cells	Acidic	2.33	1,166	44
		Alkaline	10.94	−864	0
20		Acidic	2.40	1,169	94
		Alkaline	11.23	−882	0
20	Biofilms	Acidic	2.29	1,163	85
		Alkaline	11.20	−885	0
		NaOCl	2.54	1,138	86

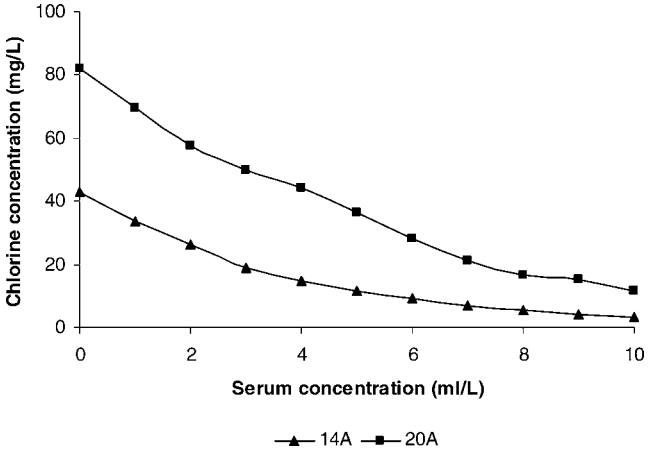

FIGURE 2. *Available chlorine concentration of acidic electrolyzed water after treating with different concentrations of chicken serum for 5 min.*

rine in acidic EO water and other sanitizers by organic matter has also been demonstrated by other studies. As was observed in this study, El-Kest and Marth *(4)* reported that the higher the amount of organic matter present, the greater the decrease in the concentration of available chlorine.

Treatment of planktonic cells of *L. monocytogenes* with EO water in the presence of organic matter. The properties of the EO water used for this study are presented in Table 1. Even though the pH of the alkaline EO water produced at 14 and 20 A was high (10.94 and 11.23, respectively), *L. monocytogenes* cells in suspension survived this treatment, and the populations recovered were not significantly ($P > 0.05$) different from the control, irrespective of the serum concentration and time of exposure (Table 2). Taormina and Beuchat *(19)* also observed that *L. monocytogenes* survived a 30-min exposure to alkaline cleaners with a pH range of 10.4 to 11.6.

In the absence of serum, acidic EO water generated at 14 and 20 A produced a >6-log CFU/ml reduction in *L. monocytogenes* after a 1-min treatment, and no cells were recovered upon enrichment (Table 2). Our earlier study indicated that 1 min of EO water treatment reduced the initial population (8.90 log CFU per coupon) of *Listeria* biofilms by approximately 5 log CFU per coupon *(2).* With serum at a concentration of 5 ml/liter, however, a 1-min treatment with acidic EO water produced at 14 A reduced the population of *L. monocytogenes* by only 0.33 log. As the serum concentration increased, the survival of *L. monocytogenes* after treatment with acidic EO water generally increased (Table 2).

Contrary to what was observed with acidic EO water produced at 14 A, in the presence of serum at a concentration of 5 ml/liter, acidic EO water produced at 20 A achieved a >6-log CFU/ml reduction in *L. monocytogenes* after 1- and 5-min exposures (Table 2). The higher reduction observed for acidic EO water produced at 20 A may

TABLE 2. *Survival of* Listeria monocytogenes *following exposure to electrolyzed water in the presence of organic matter*

Amperage (A)	Time (min)	Treatment[a]	Population (log CFU/ml)[b] 0 ml/liter serum concn in treatment water	En (no. positive/ no. analyzed)[c]	5 ml/liter serum concn in treatment water	En (no. positive/ no. analyzed)[c]	10 ml/liter serum concn in treatment water
14	1	DI water	A 7.69 ± 0.18 A		A 7.73 ± 0.22 A		A 7.67 ± 0.14 A
		Alkaline EO	A 7.71 ± 0.15 A		A 7.77 ± 0.15 A		A 7.73 ± 0.23 A
		Acidic EO	B <1.00 B	0/6	A 7.40 ± 0.34 A		A 7.65 ± 0.25 A
	5	DI water	A 7.75 ± 0.15 A		A 7.72 ± 0.15 A		A 7.71 ± 0.25 A
		Alkaline EO	A 7.69 ± 0.17 A		A 7.73 ± 0.17 A		A 7.69 ± 0.14 A
		Acidic EO	B <1.00 C	0/6	B 7.26 ± 0.18 B		A 7.60 ± 0.17 A
20	1	DI water	A 7.76 ± 0.06 A		A 7.70 ± 0.07 A		A 7.78 ± 0.11 A
		Alkaline EO	A 7.76 ± 0.11 A		A 7.76 ± 0.10 A		A 7.79 ± 0.11 A
		Acidic EO	B <1.00 B	0/6	B <1.00 B	3/6	A 7.56 ± 0.26 A
	5	DI water	A 7.84 ± 0.15 A		A 7.71 ± 0.13 A		A 7.69 ± 0.07 A
		Alkaline EO	A 7.76 ± 0.12 A		A 7.77 ± 0.06 A		A 7.78 ± 0.11 A
		Acidic EO	B <1.00 B	0/6	B <1.00 B	2/6	B 6.55 ± 0.90 A

[a] DI water, deionized water; alkaline EO, alkaline electrolyzed water; acidic EO, acidic electrolyzed water.

[b] Means preceded by the same letters in the same column within each amperage, time of exposure, and serum concentration are not significantly different ($P > 0.05$). Means followed by the same letters in the same row within each treatment, time of exposure, and amperage are not significantly different ($P > 0.05$).

[c] Treatments that were positive for *L. monocytogenes,* of the number of treatments analyzed by enrichment. Initial bacteria population used for treatments was approximately 8.90 log CFU/ml.

be attributed to the higher concentration of chlorine at that serum level. In the presence of organic matter, the chlorine concentration measured by the iodometric titration method represents the total available chlorine present, and this is made up of both free available and combined forms of chlorine. The reduced ability of acidic EO water produced at 14 A with serum (5 ml/liter) in inactivating *L. monocytogenes* may be because most of the chlorine present is in the combined unavailable chlorine form. Oomori et al. *(16)* reported a decrease in the available chlorine concentration of acidic EO water after the addition of nutrient broth, proteose peptone, glycine, corn oil, cow's milk, and minced meat. In another study, Park et al. *(17)* reported that free chlorine at a concentration of 1 mg/liter in acidic EO water was sufficient to completely inactivate the initial population (8 log CFU/ml) of *L. monocytogenes* within 30 s of treatment. To predict the concentration of available chlorine in the presence of serum, a regression model was developed to fit the experimental data to the general equation (equation 1) by a stepwise regression procedure of the Statistical Analysis System (release 8.02, SAS Institute).

$$Y = -9.33 + 1.02 \cdot X_1 - 0.085 \cdot X_1 \cdot X_2 \ (R^2 = 0.95) \quad (1)$$

where Y is the available chlorine concentration (in milligrams per liter) after being subjected to organic matter treatment (serum), X_1 is the initial chlorine concentration (in milligrams per liter), and X_2 is the concentration of organic matter (in milligrams per liter).

The population of *L. monocytogenes* recovered after a 1-min exposure to acidic EO water produced at 20 A, containing serum at 10 ml/liter, was about the same as that from the deionized water treatment (Table 2). When the exposure time was extended to 5 min, a significantly lower population (6.55 log CFU/ml) was recovered. Therefore, longer exposure times may compensate for the presence of limited amounts of chlorine to achieve a bactericidal effect on *Listeria.* It is interesting to note that acidic EO water produced at 14 A containing serum at a concentration of 5 ml/liter also had a chlorine concentration of 11 mg/liter (Fig. 2) but did not produce the ~1-log CFU/ml reduction in *Listeria* observed with acidic EO water produced at 20 A containing serum at a concentration of 10 ml/liter when the time of exposure was increased from 1 to 5 min (Table 2). These differences in the ability to inactivate *L. monocytogenes* could be due to differences in the proportions of free and combined chlorine in the chlorine concentrations (11 mg/liter) measured, i.e., for acidic EO water produced at 14 A containing serum at 5 ml/liter and for acidic EO water produced at 20 A containing serum at 10 ml/liter; however, this is not clear.

The survival of *L. monocytogenes* after treatments with acidic EO water containing chicken serum is a result of the lower bactericidal activity of combined chlorine as opposed to free available chlorine, since in the absence of serum, acidic EO water completely inactivated 8.9 log CFU of *L. monocytogenes* per ml (Table 2). In their study on disinfection by acidic EO water in the presence of organic materials, Oomori et al. *(16)* showed that the bactericidal activity of acidic EO water against *E. coli* K-12 was reduced when peptone, glycine, and nutrient broth were added to the treatment water. Van de Weyer et al. *(20)* also tested the efficacy of several disinfectants on *Listeria* in the presence of organic matter and reported that the bactericidal activity of the chlorine containing disinfectant was diminished in the presence of proteins.

Treatment of *L. monocytogenes* biofilms with EO water in the presence of organic matter. Higher numbers of *L. monocytogenes* were recovered from control coupons and water-rinsed coupons with added serum than from those without serum (Table 3). This may be because of *Listeria* growth after the addition of the serum. A lower initial population (5.98 log CFU per coupon) of *Listeria* in the biofilms was also recovered from control coupons than in our earlier study (8.90 log CFU per coupon *(2)*) (Table 3). This difference can be attributed to the death of cells as a result of desiccation of the biofilm during the 30-min incubation at 45°C as previously described.

The recovery of *L. monocytogenes* from biofilms treated with alkaline EO water was similar to the recovery when treatment was with deionized water (Table 3). In both cases, a higher population was recovered as the concentration of serum increased. No significant ($P > 0.05$) differences in survival were detected with respect to time of exposure to alkaline EO water.

Fewer survivors were recovered after treatment of biofilms with acidic EO water than with alkaline EO water and deionized water. The survival of *L. monocytogenes* biofilms overlaid with serum, however, depended on the amount of serum applied. The population of *L. monocytogenes* recovered from coupons overlaid with serum at 7.5 and 5.0 ml/liter and exposed to acidic EO water for 30 s was significantly higher ($P < 0.05$) than those with serum at 2.5 and 0 ml/liter in that order (Table 3). For coupons with serum applied at 0 and 2.5 ml/liter, a >3-log reduction in *L. monocytogenes* biofilms was achieved in 30 s of exposure to acidic EO water; however, higher serum concentrations (5.0 and 7.5 ml/liter) resulted in a <3-log reduction (Table 3). As the time of exposure was increased from 30 to 60 s for coupons overlaid with chicken serum at 5.0 and 7.5 ml/liter, a significantly ($P < 0.05$) lower number of survivors was also recovered. In the presence of organic matter, a longer time of exposure (>30 s) may therefore be required for acidic EO water to achieve significant inactivation of pathogenic bacteria. A sanitizer must reduce a microbial population in suspension by 5 log cycles after a 30-s exposure and an attached or biofilm population by 3 log units or more to be considered effective *(3, 13, 15, 18, 20).* Acidic EO water reduced *L. monocytogenes* in suspension by >6 log and biofilms by <4 log in the absence of serum and can therefore be considered an effective sanitizer. As demonstrated by other studies *(6, 13, 15),* our results showed that biofilms are more resistant to sanitizers than planktonic cells.

Irrespective of organic loads, the sequential treatment of biofilms with alkaline EO water followed by acidic EO water was effective, producing a greater than 5-log reduction in *Listeria* biofilm populations (Table 3). Statistical

TABLE 3. *Survival of* Listeria monocytogenes *in biofilms after treatment with electrolyzed water in the presence of organic matter*

Treatment water[a]	Serum concn in treatment water (ml/liter)	Populations recovered from coupons (log CFU/coupon) and time of exposure[b]			
		30 s	En (no. positive/no. analyzed)[c]	60 s	En (no. positive/no. analyzed)[c]
Control	0	5.98 ± 0.56		5.92 ± 0.69	
	2.5	6.47 ± 1.16		6.66 ± 0.34	
	5.0	6.77 ± 0.76		7.07 ± 0.49	
	7.5	7.17 ± 0.60		7.30 ± 0.50	
DI water	0	b 5.19 ± 0.63 a A		b 5.24 ± 0.59 a A	
	2.5	ab 5.61 ± 0.72 a A		ab 5.98 ± 0.38 a A	
	5.0	ab 5.73 ± 0.83 a A		ab 5.66 ± 0.44 a A	
	7.5	a 6.41 ± 0.52 a A		a 6.13 ± 0.51 a A	
Alkaline EO	0	b 4.91 ± 0.57 a A		a 4.73 ± 0.62 a A	
	2.5	ab 5.27 ± 0.66 a A		a 4.88 ± 0.32 a B	
	5.0	ab 5.36 ± 0.78 a AB		a 5.43 ± 0.25 a A	
	7.5	a 6.10 ± 0.49 a AB		a 5.23 ± 0.53 b A	
Acidic EO	0	b 1.45 ± 0.29 a B	4/6	a 1.55 ± 0.53 a B	4/6
	2.5	b 2.71 ± 0.91 a B		a 2.31 ± 1.31 a C	4/6
	5.0	a 4.05 ± 0.97 a BC		a 1.85 ± 0.64 b B	
	7.5	a 5.13 ± 0.85 a B		a 2.97 ± 1.37 b B	
Sequential	0	a 1.51 ± 0.43 a B	3/6	a <1.30 a B	4/6
	2.5	a 1.51 ± 0.50 a C	2/6	a <1.30 a C	2/6
	5.0	a 1.55 ± 0.43 a D	4/6	a 1.35 ± 0.12 a B	1/6
	7.5	a 2.00 ± 1.17 a C	5/6	a 1.36 ± 0.14 a C	2/6
NaOCl	0	c 1.55 ± 0.36 a B	4/6	a 1.51 ± 0.50 a B	4/6
	2.5	bc 2.58 ± 0.45 a BC		a 1.62 ± 0.35 b C	5/6
	5.0	b 3.42 ± 1.14 a C		a 2.31 ± 1.08 a B	
	7.5	a 5.40 ± 0.49 a AB		a 2.70 ± 0.87 b BC	5/6

[a] DI water, deionized water; alkaline EO, alkaline electrolyzed water; acidic EO, acidic electrolyzed water; sequential, alkaline electrolyzed water followed by acidic electrolyzed water.

[b] Means preceded by the same lowercase letters in the same column within each treatment and time of exposure are not significantly different ($P > 0.05$); means followed by the same lowercase letters in the same row within each treatment are not significantly different ($P > 0.05$); means followed by the same capital letters in the same column within each serum concentration and time of exposure are not significantly different ($P > 0.05$); detection limit = 1.3 log CFU per coupon.

[c] Number of treated coupons positive for *L. monocytogenes,* as detected by enrichment, of the number of coupons analyzed by enrichment.

analysis of the data indicated that at each level of added serum, significantly ($P < 0.05$) lower numbers of survivors were recovered from the sequential treatment than from the treatment with acidic EO water alone (Table 3). In their research on the removal of *L. monocytogenes* biofilms with chemical cleaning and sanitizing agents, Frank et al. *(6)* observed that the presence of an organic load reduced microbial inactivation when sanitizers were used without previous cleaning. For practical purposes and effective sanitization, gross amounts of organic soil in food-processing facilities should be removed before application of alkaline EO and acidic EO water. In the application of EO water as a sanitizer for the food industry, the sequential treatment should be used if organic matter may be present, as the alkaline EO water may remove food residues and possibly modify the biofilm structure, making the adherent bacteria more susceptible to the acidic EO water *(2).*

Taormina and Beuchat *(19)* observed that *L. monocytogenes* exposed to alkaline cleaners for 30 min became sensitive to subsequent chlorine exposure. Frank et al. *(6)* reported that when cleaning and sanitizing were employed sequentially with an alkali cleaner (10-min exposure) and acidified sodium chlorite (30 min), adherent *L. monocytogenes* populations were reduced to nearly undetectable levels with a >7-log reduction. Somers and Wong *(18)* also reported that treatment of *L. monocytogenes* biofilms on various surface materials with a solvated alkaline product (10-min exposure) followed by a hypochlorite sanitizer (1 min) achieved their target 3-log reduction. In the presence of meat and fat residue, however, the target 3-log reduction was achieved only 77% of the time. Recent studies utilizing both fractions of EO water have also shown that pretreatment of lettuce inoculated with *E. coli* O157:H7 and *Salmonella* with alkaline EO water followed by acidic EO water resulted in a greater microbial reduction than what was obtained with other pretreatment solutions *(12).*

The results from the treatment of *L. monocytogenes* biofilms with acidified sodium hypochlorite followed a trend similar to that for treatment with acidic EO water (Table 3). In the absence of serum and at a serum concen-

TABLE 4. *Populations of* Listeria monocytogenes *recovered from treatment water after exposure of biofilms contained on stainless steel coupons to electrolyzed water in the presence of organic matter*

Treatment water[a]	Serum concn in treatment water (ml/liter)	Populations recovered from treatment water (log CFU/ml) and time of exposure[b]			
		30 s	En (no. positive/ no. analyzed)[c]	60 s	En (no. positive/ no. analyzed)[c]
Dl water	0	B 3.18 ± 0.90		B 3.38 ± 0.57	
	2.5	AB 4.08 ± 1.02		A 4.71 ± 0.24	
	5.0	A 5.17 ± 0.65		A 4.86 ± 0.62	
	7.5	A 5.23 ± 0.69		A 4.87 ± 0.74	
Alkaline EO	0	C 2.99 ± 0.72		B 3.80 ± 0.54	
	2.5	BC 3.71 ± 0.74		AB 4.01 ± 0.28	
	5.0	AB 4.72 ± 0.61		A 4.77 ± 0.45	
	7.5	A 5.04 ± 0.76		A 4.72 ± 0.71	
Acidic EO	0	ND	0/6	ND	0/6
	2.5	ND	0/6	ND	0/6
	5.0	ND	0/6	ND	0/6
	7.5	ND	0/6	ND	0/6
Sequential	0	ND	0/6	ND	0/6
	2.5	ND	0/6	ND	0/6
	5.0	ND	0/6	ND	0/6
	7.5	ND	0/6	ND	0/6
NaOCl	0	ND	0/6	ND	0/6
	2.5	ND	0/6	ND	0/6
	5.0	ND	0/6	ND	0/6
	7.5	ND	0/6	ND	0/6

[a] DI water, deionized water; alkaline EO, alkaline electrolyzed water; acidic EO, acidic electrolyzed water; sequential, alkaline electrolyzed water followed by acidic electrolyzed water.

[b] Means preceded by the same letters in the same column within each treatment and time of exposure are not significantly different ($P > 0.05$); ND, not detectable on direct plate count and negative on enrichment.

[c] Number of treated coupons positive for *L. monocytogenes,* as detected by enrichment, of the number of coupons analyzed by enrichment.

tration of less than 5 ml/liter, acidified sodium hypochlorite produced a greater than 3-log reduction in 30 s. However, at a serum concentration of 7.5 ml/liter, a less than 2-log reduction was achieved (Table 3). Best et al. *(3)* also reported that in the presence of human serum, sodium hypochlorite at 60 μg/ml achieved a <1-log reduction of *L. monocytogenes* on stainless steel disks after a 1-min exposure.

Survival of *L. monocytogenes* in treatment water. After exposure of the biofilm-containing coupons to the various treatments, the water used for the treatments was tested for the presence of *L. monocytogenes* to determine the cross-contamination potential of the treatment solution. The populations of *L. monocytogenes* recovered from the deionized water and alkaline EO water after treatment of coupons without serum were significantly lower than those recovered after treatment of coupons that had been overlaid with a serum concentration of more than 5.0 ml/liter (Table 4). The recovery of *L. monocytogenes* from the deionized and alkaline EO water used for treatment confirms their lack of bactericidal activity and their potential for recontamination of equipment (Table 4). No *L. monocytogenes* populations were recovered from the acidic EO water and acidified sodium hypochlorite posttreatment waters, and all enrichments were negative for *L. monocytogenes* (Table 4). Therefore, rinsing equipment with these solutions is unlikely to result in recontamination.

In conclusion, acidic EO water is an effective sanitizer that may have application in food-processing facilities. The sequential use of alkaline EO water followed by acidic EO water achieved a >4-log reduction on *L. monocytogenes* biofilms, even with the presence of organic matter of up to 7.5 ml/liter.

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REFERENCES

1. Anonymous. 1997. Electrolyzed water. Central laboratory report. Hoshizaki Electric Co., Toyoake, Aichi, Japan.
2. Ayebah, B., Y.-C. Hung, and J. F. Frank. 2005. Enhancing the bactericidal effect of electrolyzed water on *Listeria monocytogenes* biofilms formed on stainless steel. *J. Food Prot.* 68:1375–1380.
3. Best, M., M. E. Kennedy, and F. Coates. 1990. Efficacy of a variety of disinfectants against *Listeria* spp. *Appl. Environ. Microbiol.* 56: 377–380.

4. El-Kest, S. E., and E. H. Marth. 1988. Inactivation of *Listeria monocytogenes* by chlorine. *J. Food Prot.* 51:520–524.
5. Fatemi, P., and J. F. Frank. 1999. Inactivation of *Listeria monocytogenes*/*Pseudomonas* biofilms by peracid sanitizers. *J. Food Prot.* 62:761–765.
6. Frank, J. F., J. Ehlers, and L. Wicker. 2003. Removal of *Listeria monocytogenes* and poultry soil–containing biofilms using chemical cleaning and sanitizing agents under static conditions. *Food Prot. Trends* 23:654–663.
7. Frank, J. F., and R. A. Koffi. 1990. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *J. Food Prot.* 53:550–554.
8. Giese, J. H. 1991. Sanitation: the key to food safety and public health. *Food Technol.* 45:74–80.
9. Hassan, A. N., D. M. Birt, and J. F. Frank. Behaviour of *Listeria monocytogenes* in a *Pseudomonas putida* biofilm on a condensate-forming surface. *J. Food Prot.* 67:322–327.
10. Kim, C., Y.-C. Hung, and R. E. Brackett. 2000. Efficacy of electrolyzed oxidizing (EO) and chemically modified water on different types of food borne pathogens. *Int. J. Food Microbiol.* 61:199–207.
11. Kim, C., Y.-C. Hung, R. E. Brackett, and J. F. Frank. 2001. Inactivation of *Listeria monocytogenes* biofilms by electrolyzed oxidizing water. *J. Food Proc. Preserv.* 25:91–100.
12. Koseki, S., K. Yoshida, Y. Kamitani, S. Isobe, and K. Itoh. 2004. Effect of mild heat pre-treatment with alkaline electrolyzed water on the efficacy of acidic electrolyzed water against *Escherichia coli* O157:H7 and *Salmonella* on lettuce. *Food Microbiol.* 21:559–566.
13. Lindsay, D., and A. von Holy. 1999. Different responses of planktonic and attached *Bacillus subtilis* and *Pseudomonas fluorescens* to sanitizer treatment. *J. Food Prot.* 62:368–379.
14. Marriot, N. G. 1999. Sanitizers, p. 139–157. *In* Principles of food sanitation, 4th ed. Spiral Biotech, Gaithersburg, Md.
15. Mosteller, T. M., and J. R. Bishop. 1993. Sanitizer efficacy against attached bacteria in a milk biofilm. *J. Food Prot.* 56:34–41.
16. Oomori, T., T. Oka, T. Inuta, and Y. Arata. 2000. The efficiency of disinfection of acidic electrolyzed water in the presence of organic materials. *Anal. Sci.* 16:365–369.
17. Park, H., Y.-C. Hung, and C. Kim. 2002. Effectiveness of electrolyzed water as a sanitizer for treating different surfaces. *J. Food Prot.* 65:1276–1280.
18. Somers, E. B., and A. C. Wong. 2004. Efficacy of two cleaning and sanitizing combinations on *Listeria monocytogenes* biofilms formed at low temperature on a variety of materials in the presence of ready-to-eat meat residue. *J. Food Prot.* 67:2218–2229.
19. Taormina, P. J., and L. R. Beuchat. 2002. Survival of *Listeria monocytogenes* in commercial food-processing equipment cleaning solutions and subsequent sensitivity to sanitizers and heat. *J. Appl. Microbiol.* 92:71–80.
20. Van de Weyer, A., M.-J. Devleeschouwer, and J. Dony. 1993. Bactericidal activity of disinfectants on *Listeria. J. Appl. Bacteriol.* 74: 480–483.
21. Venkitanarayanan, K. S., G. O. I. Ezeike, Y.-C. Hung, and M. P. Doyle. 1999. Efficacy of electrolyzed oxidizing water for inactivation of *Escherichia coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes. Appl Environ. Microbiol.* 65:4276–4279.
22. White, G. C. 1999. Chemistry of chlorination, p. 212–287. *In* Handbook of chlorination and alternative disinfectants, 4th ed. John Wiley and Sons, New York.