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Article *in* Journal of food protection · August 2005

DOI: 10.4315/0362-028X-68.7.1375 · Source: PubMed

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Journal of Food Protection, Vol. 68, No. 7, 2005, Pages 1375–1380

Enhancing the Bactericidal Effect of Electrolyzed Water on *Listeria monocytogenes* Biofilms Formed on Stainless Steel

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MS 04-585: Received 29 December 2004/Accepted 2 March 2005

ABSTRACT

Biofilms are potential sources of contamination to food in processing plants, because they frequently survive sanitizer treatments during cleaning. The objective of this research was to investigate the combined use of alkaline and acidic electrolyzed (EO) water in the inactivation of *Listeria monocytogenes* biofilms on stainless steel surfaces. Biofilms were grown on rectangular stainless steel (type 304, no. 4 finish) coupons (2 by 5 cm) in a 1:10 dilution of tryptic soy broth that contained a five-strain mixture of *L. monocytogenes* for 48 h at 25°C. The coupons with biofilms were then treated with acidic EO water or alkaline EO water or with alkaline EO water followed by acidic EO water produced at 14 and 20 A for 30, 60, and 120 s. Alkaline EO water alone did not produce significant reductions in *L. monocytogenes* biofilms when compared with the control. Treatment with acidic EO water only for 30 to 120 s, on the other hand, reduced the viable bacterial populations in the biofilms by 4.3 to 5.2 log CFU per coupon, whereas the combined treatment of alkaline EO water followed by acidic EO water produced an additional 0.3- to 1.2-log CFU per coupon reduction. The population of *L. monocytogenes* reduced by treatments with acidic EO water increased significantly with increasing time of exposure. However, no significant differences occurred between treatments with EO water produced at 14 and 20 A. Results suggest that alkaline and acidic EO water can be used together to achieve a better inactivation of biofilms than when applied individually.

Processing facilities are an important source of pathogens in foods *(2, 25)*. Cross-contamination that involves transfer between surfaces to which pathogens have attached or biofilms have formed are one means by which food becomes contaminated.

Listeria monocytogenes has been implicated in many food-related outbreaks and has caused serious illness in certain high-risk groups, including pregnant women, neonates, immunocompromised patients, and occasionally persons who have no predisposing underlying condition *(36)*. As a result of recurring outbreaks of listeriosis and the associated high mortality rate among those at risk, the U.S. Department of Agriculture's U.S. Food Safety and Inspection Service and the U.S. Food and Drug Administration established zero tolerance policies for the pathogen in ready-to-eat foods *(35)*. *L. monocytogenes* occurs widely in nature *(4)* and can attach to and form biofilms on a wide range of surfaces used in the food industry *(5, 15, 26)*.

Carpentier and Cerf *(7)* defined biofilms as a community of microbes embedded in an organic polymer matrix, adhering to a surface. Several research efforts on the control of biofilms have shown that bacteria in biofilms are protected from the antimicrobial action of sanitizers and are killed only at concentrations orders of magnitude higher than what is required to kill planktonic cells *(13, 27, 33, 37)*. Increased sanitizer resistance of biofilms has been attributed to (i) protection of the underlying organisms by the glycocalyx by limiting the penetration of the sanitizer into the biofilm matrix; (ii) neutralization of the sanitizer inside the matrix; (iii) genetic induction that results in modification to the cell wall; and (iv) slow uptake of antimicrobial agents as a result of the significantly slow growth of biofilm-associated cells *(6, 9, 10, 37)*. The study of the effects of sanitizers on *L. monocytogenes*, planktonic or biofilm form, is of particular interest because of the ubiquitous nature of the microorganism, its isolation from processing plants, and the continuing recalls of processed foods, since food processors work to comply with the federal zero tolerance policy for the pathogen. Although sanitizing chemicals have been developed that are effective against biofilms *(11, 12)*, food processors still have limited sanitation choices for economical biofilm control. Therefore, the evaluation of chemical sanitizers for biofilm control remains an area of active research.

Acidic electrolyzed (EO) water has been reported *(17, 24, 39)* to exhibit a strong bactericidal effect on various pathogenic bacteria. It has been successfully used as a disinfectant in agriculture, dentistry, and medicine. EO water is produced by subjecting positively and negatively charged electrodes to a DC voltage in the presence of a salt solution. At the anode, an acidic EO water that contains chlorine in the form of hypochlorous acid and has a strong oxidizing potential (oxidation reduction potential [ORP] of approximately 1,100 mV) and a low pH (approximately 2.6) is produced. Alkaline EO water, which has a strong reducing potential (ORP of approximately −800 mV) and a high pH (approximately 11) is produced at the cathode *(1)*. The ef-

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fect of EO water in reducing microflora on fresh vegetables has been investigated. It has been successfully applied to reduce aerobic bacteria, coliforms, *Bacillus cereus, Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7, and *L. monocytogenes* on lettuce *(20, 30, 40); E. coli* O157:H7, *S. enterica* serovar Typhimurium, and *L. monocytogenes* on tomatoes *(3)*; and *Salmonella* spp. on alfalfa seeds and sprouts *(19)*. Fujiwara et al. *(14)* reported that acidic EO water was a more effective disinfectant in cleaning and sanitizing dialysis equipment and pipelines than conventional disinfectants, such as sodium hypochlorite and acetic acid. Acidic EO water also achieved significant reductions in *Campylobacter jejuni* on poultry *(32)*. In studies on the potential of EO water as a sanitizer on various surfaces, acidic EO water produced significant reductions in *E. coli* O157:H7 and *L. monocytogenes* on kitchen cutting boards *(37)* and *Enterobacter aerogenes* and *Staphylococcus aureus* on glass, stainless steel, glazed ceramic tile, unglazed ceramic tile, and vitreous china *(33)*. Kim et al. *(18)* subjected *L. monocytogenes* biofilms on stainless steel coupons to acidic EO water treatment and found that the bacterial cells were reduced to undetectable levels in 5 min. They reported a rapid inactivation of biofilms within 30 s of applying acidic EO water, after which the inactivation rate was significantly reduced. This finding may be due to the inability of acidic EO water to rapidly penetrate to the center of bacterial biofilms after inactivating the bacteria on the surface.

Most of the published research involving the use of EO water in bacterial inactivation has been focused on the acidic fraction of the water. Only limited information exists on the potential application of alkaline EO water in food processing: a report from Japan on its use to make bread with a softer texture than bread made with tap water *(28)* and a report on improving the textural quality of aged rice by cooking with alkaline EO water *(29)*. Preliminary research in our laboratory showed that alkaline EO water could produce a 1-log reduction in a pure suspension of *E. coli* O157:H7 after a 1-min exposure. In a study with shell eggs inoculated with *Salmonella* and *Listeria*, Park et al. *(31)* reported that a 1-min treatment with alkaline EO water followed by a 1-min treatment with acidic EO water that contained 41 mg/liter of chlorine produced a reduction similar to a 1-min treatment with chlorinated water that contained 200 mg/liter of chlorine. Koseki et al. *(21)* also reported that washing lettuce in alkaline EO water for 1 min and then treating with acidic EO water for another minute produced a significant reduction in aerobic bacteria, molds, and yeasts. We hypothesize that alkaline EO water will produce a higher inactivation of adherent bacteria when applied in combination with acidic EO water. The objective of this study was to investigate the efficacy of the combined use of alkaline and acidic EO water in the inactivation of *L. monocytogenes* biofilms on stainless steel.

MATERIALS AND METHODS

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 the study. A loop inoculum of each culture was transferred three times in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) and incubated at 37°C at successive 24-h intervals. A 24-h culture of each bacterial 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 0.1% peptone water (Difco, Becton Dickinson). Each pellet was resuspended in 5 ml of 0.1% peptone water, and the five cultures were combined to form a mixture with a bacteria population of 9 log CFU/ml. Twelve milliliters of the mixture was added to 1.2 liters of sterile 1:10 dilution of TSB (3 g of dry medium per liter of deionized water), and this inoculated low nutrient medium was used for the preparation of biofilms.

Preparation of stainless steel coupons. New stainless steel (type 304, no. 4 finish) sheets (1 mm thickness) (Stewart Stainless Supply Inc., Suwanee, Ga.) were cut into rectangular coupons (2 by 5 cm). Coupons were cleaned in acetone using Kim wipes to remove grease, rinsed in deionized water, and shaken in a 2% solution of Micro-90 soap (International Products Co., Burlington, NJ) at 120 rpm at 24 ± 2°C for 1 h on a platform shaker (model C10, New Brunswick Scientific, Edison, NJ). 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 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 in a stainless steel pan (53.3 by 30.5 by 5.1 cm; Delipan, Manning Brothers, Athens, Ga.).

Preparation of biofilms. The sterile coupons were immersed in the low nutrient medium inoculated with *L. monocytogenes* and incubated at 25°C for 4 h to allow bacterial attachment and then rinsed gently in a circular motion for 10 s with 0.1% peptone water to remove unattached cells. Biofilms were grown by submerging the coupons that contained adherent cells in 1.2 liters of sterile low nutrient medium and incubating for 48 h at 25°C to allow further biofilm growth. After incubation, coupons were rinsed gently in a circular motion for 10 s with 0.1% peptone water to remove unattached cells and subjected to EO water treatment.

EO water. EO water produced from a ROX-20TA generator (Hoshizaki Electric Inc., Toyoake, Aichi, Japan) at current settings of 14 and 20 A was used for this study. After a stable amperage reading was achieved, alkaline and acidic EO water were collected from the cathode and anode sides, 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 the 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.

Treatment of biofilms with EO water. Coupons that contain biofilms were immersed in 150 ml of alkaline EO water, acidic EO water, or alkaline EO water followed by acidic EO water for 30, 60, and 120 s at room temperature (24 ± 2°C). To ensure that each treatment went through the same number of rinses, the coupons were treated with sterile 0.1% peptone water when one treatment had to be omitted. After treatment, the coupons were immediately immersed in a neutralizing buffer solution (5.2 g/liter of neutralizing buffer; Difco, Becton Dickinson) for 10 s and then subjected to microbiological analysis.

TABLE 1. *Properties of EO water used for treatment*

Water	Amperage (A)	pH	ORP (mV)[a]	Chlorine (mg/liter)
Acidic EO	14	2.40 ± 0.08	1,163 ± 7	47.12 ± 2.38
Alkaline EO		11.15 ± 0.10	−868 ± 5	0.00
Acidic EO	20	2.38 ± 0.07	1,169 ± 1	84.68 ± 9.41
Alkaline EO		11.26 ± 0.04	−874 ± 7	0.00

[a] ORP, oxidation reduction potential.

Microbiological analysis. To enumerate *L. monocytogenes*, the coupons were placed in sterile Nalgene bottles (8 oz) that contained 20 ml of sterile 0.1% peptone water and 3 g of acid-washed glass beads (425 to 600 μm; Sigma-Aldrich Co., St Louis, Mo.). 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. For the untreated coupons and those treated with alkaline EO water only, the surviving bacteria were enumerated by spread plating 0.1 ml of the diluent on tryptic soy agar (TSA; Difco, Becton Dickinson) plates. Bacteria in biofilms from treatments that involved acidic EO water were enumerated by pour plating 1 ml of the diluent with TSA at 45°C or spread plating 1 ml on four TSA plates (0.25 ml per plate). Plates were incubated at 37°C for 48 h, and colonies were counted and recorded as log CFU per coupon. Treated samples that did not show any growth on TSA plates 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 incubated at 37°C for 24 h. Tubes that exhibited growth were streaked onto modified Oxford agar (Oxoid, Basingstoke, Hampshire, England) plates that contained *Listeria* selective supplement (Oxoid) and incubated at 37°C for 24 to 48 h to confirm the presence of *Listeria.*

Data analysis. Experiments were replicated five times with duplicate treatments in each replication. Data were analyzed using the general linear model procedures of the Statistical Analysis System (SAS Institute Inc., Cary, N.C.). Comparisons of means were performed using Duncan's multiple range tests.

RESULTS AND DISCUSSION

Properties of EO water. The properties of acidic and alkaline EO water used in the study are presented in Table 1. At 14 and 20 A, the acidic EO water generated had chlorine concentrations of approximately 47 and 85 mg/liter, respectively. Alkaline EO water did not contain any chlorine. Changing the amperage of the EO water generator did not significantly alter the pH and ORP of the water produced at the electrodes. At both amperages, acidic EO water had an average pH and ORP of approximately 2.4 and 1,160 mV, respectively, and the alkaline EO water had a pH and ORP of approximately 11 and −870 mV, respectively (Table 1).

Method of enumeration. In spread plating normally 0.1 ml of diluent is plated, and in cases where the chemical treatment has been very effective, the probability of recovering injured survivors by plating 0.1 ml is low. On the other hand, in pour plating 1 ml of the diluent, the probability of recovering bacteria that survived the acidic EO water treatment is increased. However, a concern exists that already injured cells from the acidic EO water treatment may die as a result of the temperature (45°C) of the molten agar used in pour plating. For the current study, similar counts were obtained using these two methods ($P > 0.05$) (Table 2). The bacteria population that survived after treatment with acidic EO water alone or alkaline EO water followed by acidic EO water decreased as exposure time increased. For any given treatment time, the population of *L. monocytogenes* that survived after the combined treatment was lower than what survived after treatment with acidic EO water alone. Since there were no significant differences between pour plating and spread plating in this study (Table 2), pour plating was chosen as the method of enumeration for subsequent experiments.

Treatment of biofilms with EO water. Recovery of cells from the biofilms, by shaking with glass beads, yielded reproducible results throughout the study. Control coupons, which were treated with deionized water, had an average population of 8 log CFU per coupon (Table 3) regardless of treatment time. Alkaline EO water produced at

TABLE 2. *Population of* Listeria monocytogenes *recovered from coupons using pour and spread plating*

Treatment[a]	Time (s)	Mean population (log CFU/coupon)[b]	
		Pour plating	Spread plating
Acidic EO water	30	3.44 ± 0.54 A	3.56 ± 0.65 A
	60	3.20 ± 0.29 AB	3.35 ± 0.33 AB
	120	2.52 ± 0.40 B	2.67 ± 0.30 B
Combined[c]	30	3.12 ± 0.32 A	3.14 ± 0.34 A
	60	2.48 ± 0.64 B	2.62 ± 0.63 A
	120	1.60 ± 0.30 C	1.70 ± 0.37 B

[a] EO water used for treatment was produced at 20 A.
[b] Means followed by the same letters in the same column within each treatment are not significantly ($P > 0.05$) different.
[c] Alkaline EO water followed by acidic EO water.

TABLE 3. *Survival of* Listeria monocytogenes *biofilms following exposure to EO water*

Amperage (A)	Treatment	Mean population and reduction (log CFU/coupon) for time of exposure[a]					
		30 s		60 s		120 s	
		Population	Reduction	Population	Reduction	Population	Reduction
14	DW	A 8.02 (0.06) A		A 8.06 (0.01) A		A 7.90 (0.05) A	
	Alkaline EO water	A 7.72 (0.07) B	0.30	A 8.02 (0.03) A	0.04	A 7.68 (0.17) B	0.22
	Acidic EO water	B 3.69 (0.02) A	4.33	B 3.41 (0.22) A	4.65	B 2.69 (0.07) B	5.21
	Combined	C 3.17 (0.39) A	4.85	C 2.32 (0.01) B	5.74	C 1.49 (0.29) C	6.41
20	DW	A 8.06 (0.03) A		A 7.98 (0.06) A		A 7.90 (0.01) A	
	Alkaline EO water	A 7.90 (0.00) A	0.16	A 7.78 (0.11) A	0.20	A 7.75 (0.02) A	0.15
	Acidic EO water	B 3.50 (0.15) A	4.56	B 3.17 (0.04) AB	4.81	B 2.77 (0.03) B	5.13
	Combined	C 3.19 (0.10) A	4.87	C 2.57 (0.18) B	5.41	C 1.80 (0.21) C	6.10

[a] Means preceded by the same letters in the same column within each amperage are not significantly ($P > 0.05$) different. Means followed by the same letters in the same row are not significantly ($P > 0.05$) different. Initial biofilm population = 8.90 log CFU per coupon. Numbers shown in parentheses are standard deviation values. DW, deionized water (control); combined, alkaline EO water treatment followed by acidic EO water treatment.

14 and 20 A reduced viable populations of *Listeria* biofilms by 0.04 to 0.30 log CFU per coupon, and its effect was independent of treatment time. Overall, the population of *L. monocytogenes* recovered from coupons treated for 30 to 120 s with alkaline EO water produced at 14 or 20 A was not significantly different ($P > 0.05$) from the control (Table 3).

Treatment of *L. monocytogenes* biofilms with acidic EO water produced at 14 A for 30 s reduced the bacteria population from 8.0 to 3.7 log CFU per coupon. Extending the treatment times resulted in 4.7- and 5.2-log CFU per coupon reductions after 60 and 120 s, respectively (Table 3).

Although the surviving population after 30-s exposure to acidic EO water was slightly higher than those exposed for 60 s, the difference between these was not statistically significant ($P > 0.05$). Treatment for 120 s with acidic EO water reduced cell populations to significantly ($P \leq 0.05$) lower levels than the surviving population after 30- and 60-s treatment. This indicates that the amount of time allowed for the acidic EO water to penetrate the biofilm is important in determining its efficiency in inactivating adherent bacteria. A similar trend was obtained using EO water produced at 20 A, where treatment of adherent *L. monocytogenes* cells for 30, 60, and 120 s achieved 4.6-, 4.8-, and 5.1-log CFU per coupon reductions, respectively (Table 3).

At both 14 and 20 A, the surviving population after exposure of biofilms to alkaline EO water followed by acidic EO water (combined treatment) was significantly lower ($P \leq 0.05$) than the population that survived after treatment with acidic EO water alone (Table 3). The survival of *L. monocytogenes* after exposure to alkaline EO water followed by acidic EO water was also time dependent, with treatments for 120 s resulting in the highest inactivation. The combined treatment using EO water produced at 14 A for 30, 60, and 120 s achieved 4.9-, 5.7-, and 6.4-log CFU per coupon reductions, respectively. When EO water was produced at 20 A, the combined treatment achieved 4.9-, 5.4-, and 6.1-log CFU per coupon reductions in bacteria population after biofilms were exposed for 30, 60, and 120 s, respectively. The combined treatment produced 0.3- to 1.2-log reductions more than the corresponding treatment with acidic EO water alone. For the combined treatment, the surviving populations after exposure of biofilms to EO water produced at 14 A for 60 and 120 s were lower than the surviving population after treatment with EO water produced at 20 A. However, these differences between EO water produced at 14 and 20 A were not statistically significant ($P > 0.05$). For any particular set of treatments (i.e., alkaline EO water alone or acidic EO water alone or the combined treatments), no significant differences with respect to amperage were observed, even though acidic EO water produced at 20 A contained approximately twice as much chlorine as that produced at 14 A.

Compared with the increasing interest in the use of acidic EO water as an antimicrobial solution, potential uses of alkaline EO water are limited. It has previously been used in combination with acidic EO water or other sanitizers to increase the antimicrobial effect of the treatment *(21, 31, 38)*. This study confirms that alkaline EO water by itself has no significant antimicrobial activity (Table 3). Several researchers have demonstrated that *L. monocytogenes* in biofilms on stainless steels exist in the form of clumps, clusters with channels within the biofilm, or multilayered microcolonies that may be protected from immediate inactivation by sanitizers *(8, 18, 23)*. Longer times of exposure to sanitizer are therefore required to achieve inactivation. Kim et al. *(16, 18)* reported that although 10-s exposure of planktonic cells of *L. monocytogenes* to acidic EO water resulted in complete inactivation, 10-s exposure of *L. monocytogenes* biofilms to acidic EO water only reduced bacteria by 5.8 log CFU per coupon. Although they reported a higher reduction for their 10-s acidic EO water treatment than that obtained for the 30-s treatment in the current study, a larger surface area was used for biofilm formation in their case (82.5 versus 21.4 cm^2) and hence the higher initial bacteria population and log reduction.

Increasing the amperage at which the EO water was generated from 14 to 20 A increased the chlorine concentration of acidic EO water from 47 to 85 mg/liter. However,

no significant differences occurred in the population of bacteria inactivated by these two EO waters, regardless of exposure time (Table 3). Lee and Frank *(23)* observed only an approximately 0.25-log CFU/cm^2 difference for the inactivation of surface-adherent *L. monocytogenes* when treated with sodium hypochlorite with chlorine concentrations of 100 and 150 mg/liter. Similar results were also reported by Rossoni and Gaylarde *(34)*, who found no significant differences between counts after treatment of *S. aureus* and *Pseudomonas fluorescens* biofilms with 100 and 200 mg/liter of sodium hypochlorite. From the results of this study and others, there seems to be a threshold chlorine concentration beyond which a further increase does not result in greater efficacy when applied to biofilms. However, increasing time of exposure at this threshold concentration may achieve additional biofilm inactivation. Research by Kim et al. *(18)* on the inactivation of *L. monocytogenes* biofilms for up to 5 min also supports this conclusion.

Treatments of biofilms with alkaline EO water followed by acidic EO water achieved a statistically significantly ($P \leq 0.05$) higher inactivation of *L. monocytogenes* than when acidic EO water was used alone (Table 3). Alkaline EO water by itself is not an effective bactericide; however, it may condition the biofilm to facilitate the antibacterial action of the acidic EO water. Being primarily made of sodium hydroxide, which is a saponifier that can react with fats and proteins, alkaline EO water may destabilize or dissolve the extracellular polymeric substances that surround the attached cells, thereby facilitating the penetration of the active components of acidic EO water. Frank et al. *(12)* reported that alkali cleaners can remove *L. monocytogenes* biofilms, although the concentration of alkali used in this study was much greater than the alkaline EO water. Koseki et al. *(21)* also reported a higher efficiency in bacteria inactivation when alkaline and acidic EO water were used in combination. They found that treatment of lettuce with alkaline EO water for 1 min followed by treatment with acidic EO water for 1 min resulted in a 2-log CFU/g reduction in aerobic counts, which was the same reduction obtained when lettuce was treated with acidic EO water alone for 10 min. Recent work that used both fractions of EO water also showed that pretreatment of lettuce inoculated with *E. coli* O157:H7 and *Salmonella* spp. with alkaline EO water and subsequent treatment with acidic EO water resulted in a greater microbial reduction than what was obtained using other pretreatment solutions *(22)*. Since both the acidic and alkaline portions of EO water are always produced together during electrolysis, the additional log reduction achieved when alkaline EO water is applied in combination with acidic EO water provides for a more efficient use of the EO water equipment, with no additional cost.

ACKNOWLEDGMENTS

We thank Dr. Revis Chmielewski, Mrs. Julie Ehlers, and Dr. Chyer Kim for training and technical support for this work.

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