# INACTIVATION OF *LISTERIA MONOCYTOGENES* BIOFILMS BY ELECTROLYZED OXIDIZING WATER

CHYER KIM, YEN-CON HUNG[1], ROBERT E. BRACKETT and JOSEPH F. FRANK

*Department of Food Science and Technology*
*College of Agricultural and Environmental Sciences*
*University of Georgia*
*Griffin, Georgia 30223-1797*

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## ABSTRACT

*This study investigates the resistance of* Listeria monocytogenes *biofilms on stainless steel surfaces to electrolyzed oxidizing (EO) water. A direct agar overlay method was used to estimate the attached bacteria on stainless steel coupons after an EO water treatment. A scraping method was also used to quantify the adherent cell populations after the EO water treatment. The stainless steel surface allowed 10 to 15% of the surface area to be covered by* Listeria *biofilm when the inoculated stainless steel coupon was incubated in 10% tryptic soy broth (TSB) at 23C for 48 h. When the stainless steel coupons containing adherent cells were treated with EO water (56 mg/L of residual chlorine) for 10, 30, 60, 180, and 300 s, adherent cell populations (10.3 $\log_{10}$ CFU/coupon) were reduced with increasing treatment time. Although the direct agar overlay methods do not quantify survival of single bacteria, only one to five cell clumps per coupon survived after 300 s of the EO water treatment. Using the scraping method, the adherent cell population on the stainless steel coupons was reduced by about 9 log cycles after 300 s of EO water treatment.*

## INTRODUCTION

*Listeria monocytogenes* is a psychrotroph and a Gram-positive foodborne pathogen that has been associated with several outbreaks including life-threatening illness in immunocompromised and pregnant people (Ryser and Marth 1991; Fleming *et al.* 1985; Schlech *et al.* 1983). The widespread occurrence and psychrotropic nature of these bacteria could increase the risk that ready-to-eat

[1] Author to whom correspondence should be addressed. TEL: (770)-412-4739; FAX: (770)-229-3216; E-mail: YHUNG@GRIFFIN.PEACHNET.EDU

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foods may serve as vehicles of foodborne illness. The potential for association of *L. monocytogenes* with food, combined with the possibly lethal effects of listeriosis create a potential public health concern when the food is contaminated before consumption (CDC 1985; Ho *et al.* 1986; Schlech *et al.* 1983).

Several scientists (Lee and Frank 1991; Frank and Koffi 1990; Herald and Zottola 1988) observed that *L. monocytogenes* attach to stainless steel, polypropylene and glass, and produce a sanitizer-resistant biofilm on the material. Sattar (1997) indicated that the "dryness" of any surface or the volume of bacterial culture that adheres to a surface is difficult to control. *L. monocytogenes* exposed to unfavorable environments such as starvation or low nutrient conditions can attach to and grow on various surfaces commonly found in a food-processing plant. Several studies have found that *L. monocytogenes* cells in biofilms are resistant to chlorine, acid anionic, and quaternary ammonium sanitizers so that inadequate cleaning and sanitation of food processing surfaces may lead to spread of the pathogen throughout the processing plant (Herald and Zottola 1988; Frank and Koffi 1990; Mafu *et al.* 1990; Lee and Frank 1991; Blackman and Frank 1996).

More recently, electrolyzed oxidizing (EO) water has been reported by scientists (Venkitanarayanan *et al.* 1999; Kim *et al.* 2000b) to have a strong bactericidal effect on many pathogenic bacteria. The generation of EO water involves reactions in a cell containing inert positively charged and negatively charged electrodes separated by a membrane, and through which very diluted salt water passes. By subjecting the electrodes to a DC voltage, two types of water possessing different properties are generated. An electrolyzed basic solution has strong reducing potential, which leads to a reduction of free radicals in biological systems and may be useful in the treatment of organ malfunctions. An electrolyzed acid solution (EO water), which has a high oxidation-reduction potential (ORP) and contains hypochlorous acid (HOCl), has a strong bactericidal effect. The amount of HOCl produced during electrolysis is positively related to the amount of added NaCl (Anon. 1997).

The objective of this study was to determine the effectiveness of EO water for inactivating *L. monocytogenes* biofilms on stainless steel surfaces.

## MATERIALS AND METHODS

### Preparation of Inocula

Five strains of *Listeria monocytogenes* were obtained from the culture collection of the Center for Food Safety and Quality Enhancement, University of Georgia. Strains of *L. monocytogenes* used were Scott A (human isolate from outbreak linked to milk), 108M (beef isolate), LCDC 81-861 (raw cabbage

isolate), V7 (milk isolate), and 101M (beef isolate). Cultures were transferred three times in tryptic soy broth (TSB, pH 7.3, Difco, Detroit, MI) by loop inocula at successive 24-h intervals and incubated at 37C. A 24-h culture of each bacterial strain was centrifuged for 10 min (1,800 × g, 23C) and the pellet was resuspended in 5 mL of phosphate-buffered solution (PBS, 0.3 mM $KH_2PO_4$, J.T. Baker, Phillipsbury, NJ). Two milliliters of each culture were combined to make the five strain mixture ($10^{10}$ CFU/mL). Five milliliters of the mixture were added into 500 mL of diluted (1:10 dilution using deionized water) sterile TSB (low nutrient medium, LNM) and used immediately for preparation of biofilms.

### Preparation of Surfaces for Biofilm Growth

New stainless steel (type 304, No. 4 finish, Washington specialty metal, GA) sheets (ca. 2 mm thickness) were cut into 7.5 by 11 cm pieces (referred to as coupons). Coupons were cleaned in acetone using cheesecloth followed by deionized water rinsing. Thereafter, coupons were placed in a stainless steel pan (53.3 cm × 30.5 cm × 5.1 cm, Delipan, Manning brothers, Athens, GA) with deionized water, and autoclaved at 121C for 15 min.

### EO Water

EO water produced from ROX-20TA (Hoshizaki Electric Inc., Toyoake, Aichi, Japan) at the current setting of 14 A was used for this study. Detailed descriptions of the EO water properties and preparations can be found in Kim *et al.* (2000b). EO water was prepared on as needed basis in a 500-mL sterile Pyrex glass volumetric flask (Fisher Scientific Co., Fair Lawn, NJ). Oxidation-reduction potential (ORP) and pH of EO water were measured with a dual scale pH meter (Accumet® Model 15, Fisher Scientific Co., Fair Lawn, NJ) immediately after preparation. The residual chlorine of EO water was determined by an iodometric method (Hach Co., Ames, IA) with 0.113 N of sodium thiosulfate standard solution.

### Preparation of Biofilms

Sterile stainless steel coupons were immersed for 4 h at 23C in 500 mL of LNM. Coupons were then dipped in 1 L of sterile PBS to remove unattached cells. Biofilms were prepared by submerging the coupons containing the adherent cells in 500 mL of sterile TSB and incubating for 48 h at 23C. On termination of biofilm formation, coupons were rinsed by dipping in 1 L of sterile PBS and used for EO water treatment.

## Bacterial Analysis

The number of *L. monocytogenes* attached on coupons before the EO water treatment was estimated using both the direct agar overlay method and the scraping method (Frank and Koffi 1990). For the direct agar overlay method, coupons were placed in 150 × 15-mm petri dishes. *Listeria* selective modified Oxford agar (MOX, Oxoid, Basingstroke, UK) was poured directly onto the coupon. The selective agar was contained to a 49.5 $cm^2$ (5.5 × 9 cm) area on the coupon by a sterile Teflon template (Frank and Chmielewski 1997). For the scraping method, a Teflon scraper (Policeman, 15 cm, Fisher Scientific Co., Fair Lawn, NJ) was used to remove attached cells from stainless steel coupons and collecting them in 100 mL of PBS as described by Frank and Koffi (1990). Appropriate dilutions of the cell suspensions were surface plated on MOX agar and then incubated at 37C for 24 h before colonies were counted.

Biofilms on untreated coupons were also viewed using an epifluorescent microscope (Nikon LABOPHOT, Southern Microinstruments, Atlanta, GA). Cells attached to coupons were stained by submersion in Bacto acridine orange (0.1 g/L, Difco, Detroit, MI) for 2 min, followed by rinsing in water (Difco manual 1984). After drying for 10 min, the coupons were observed under the microscope with immersion oil using a 405 nm excitation filter and a 515 nm emission filter.

## Treatment of Biofilm Formed Coupon with EO Water

Biofilm containing coupons were submerged in 500 mL of EO water and continuously stirred during the treatment using an Orbit shaker (Model 3520, Lab-line Instruments, Inc., Melrose Park, IL) at 100 rpm. Bacterial populations after 60 s and 300 s treatment were determined by direct agar overlay method as previously described. The extent of biofilm formation on these coupons was determined by epifluorescence photographs using BIO-RAD gel Doc® 2000 (BIO-RAD Laboratories, Segrate, Milan, Italy). Microbial data were averaged and presented as CFU/coupon (82.5 $cm^2$).

Due to the difficulty of estimating actual bacterial numbers using the direct agar overlay method, the scraping method was also used to enumerate the number of surviving bacteria after coupons were treated in 500 mL EO water for 10, 30, 60, 180, and 300 s at 23C. On termination of the EO water treatment, coupons were immediately placed into sterile neutralizing buffer solution (5.2 g/L, neutralizing buffer, Difco, Detroit, MI) containing a mixture of 0.0043% monopotassium phosphate, 0.016% sodium thiosulfate and 0.5% aryl sulfonate complex for 10 s to neutralize residual chlorine in EO water on the coupons. Control coupons were placed directly into neutralizing buffer solution without exposure to EO water. The coupons rinsed with neutralizing buffer solution were subjected to a bacterial assay using the scraping method as

previously described. Populations were converted to $\log_{10}$ CFU/coupon and plotted versus treatment time. Five independent replicate trials were conducted for the treatment.

### Data Analysis

Data were analyzed using the general linear model (GLM) procedures (SAS 1985). Comparisons of means were performed using Duncan's multiple range test.

## RESULTS AND DISCUSSION

ROX EO water, at the most effective concentration from a previous study on *Bacillus cereus* spores (Kim *et al.* 2000a), was used for this study. EO water used for this study had a pH of 2.6, ORP of 1,160 mV and residual chlorine of 56 mg/L. Plate counts of cells scraped from biofilms after 48 h of incubation in a TSB culture indicated that these biofilms produced on the surface of stainless steel coupons contained $1.9 \times 10^{10}$ CFU/82.5 $cm^2$ (Table 1). Stainless steel allowed an accumulation of *Listeria* biofilm at 10 to 15% of the total area after 48 h of incubation. The epifluorescent photomicrograph of the 48 h biofilms provides clear evidence of microcolony formation, indicating that significant growth occurred on the surface (Fig. 1). These results indicate that stainless steel coupons supported a significant accumulation of the bacteria during 48 h of incubation in TSB at 23C. Blackman and Frank (1996) reported that cell coverage on stainless steel was estimated at 25% using epifluorescence measurement, whereas by using scanning electron microscope (SEM) measurements it was estimated at 20%.

Although the direct agar overlay method does not quantify individual bacteria surviving after the treatment time, it gives a sensitive indication of the effectiveness of the treatment. The direct agar overlay method showed that *Listeria* biofilms were inactivated to a level of 11 CFU/coupon after 60 s of EO water treatment (Table 1). Only one to five clumps (CFU) of bacteria per coupon were still observed after 300 s of the EO water treatment.

The scraping method was applied to quantify the populations of individual bacteria that survived the EO water treatment. The *L. monocytogenes* biofilms were inactivated by EO water from 10.4 $\log_{10}$ CFU/coupon to 4.6 $\log_{10}$ CFU/coupon within 10 s treatment (Fig. 2). In the pure culture study, however, strains of *L. monocytogenes* were completely inactivated within 10 s after application of the EO water (Kim *et al.* 2000b). A fast inactivation rate of biofilms was observed within 30 s after application of the EO water. The inactivation rate of biofilms was reduced significantly after the first 30 s

treatment. This is probably due to a longer time required for EO water to penetrate to the inside of the bacteria clumps whereas microorganisms outside of the bacteria clumps will react with the EO water immediately. Extending the EO water treatment time to 300 s resulted in inactivation to below detectable levels (5 CFU/coupon).

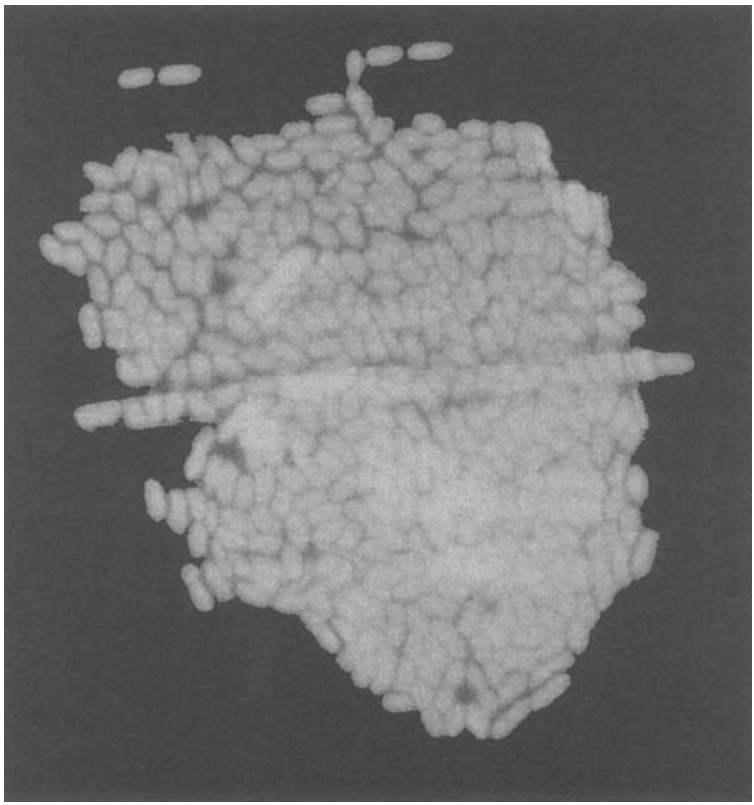

FIG. 1. EPIFLUORESCENT PHOTOMICROGRAPH OF *LISTERIA MONOCYTOGENES* BIOFILMS ON STAINLESS STEEL COUPON AFTER 48 H INCUBATION IN TRYPTIC SOY BROTH
Magnification × 1,000.

TABLE 1.
VIABILITY OF *LISTERIA MONOCYTOGENES* BIOFILMS AFTER TREATMENT WITH EO WATER AS DETERMINED BY AGAR OVERLAY METHOD

| Treatment | Exposure Time (s) | Population Survived[1] (CFU/coupon)* |
|---|---|---|
| Control | | $1.9 \times 10^{10}$ |
| EO water | 60 | $<1.1 \times 10^{1}$ |
| EO water | 300 | <1 - 5 |

[1]Initial population of inoculum was 10.1 $\log_{10}$ CFU/mL.
*Results are expresses as CFU counts per total area of coupon (82.5 $cm^2$).

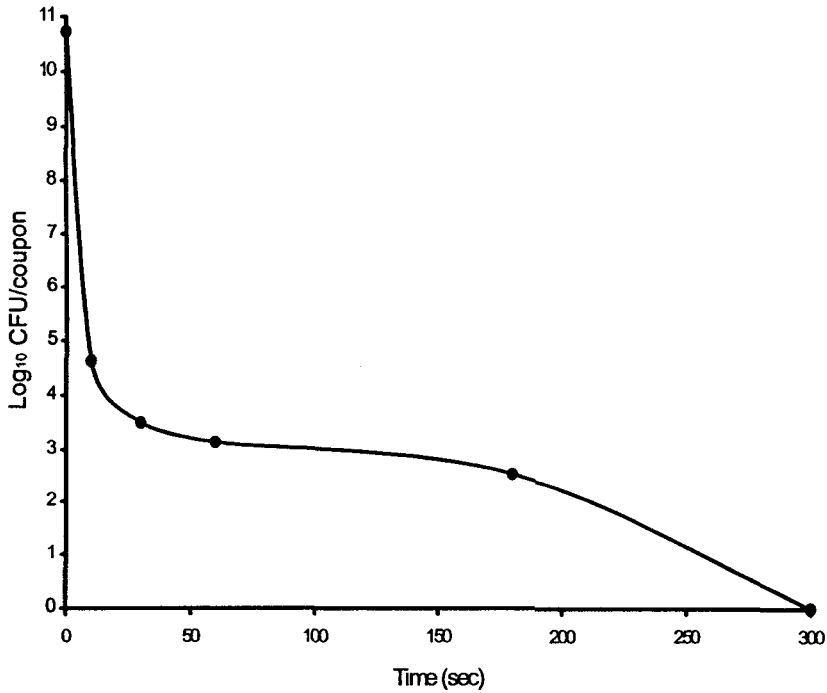


FIG. 2. SURVIVING *LISTERIA MONOCYTOGENES* ON COUPONS EXPOSED TO EO WATER AS DETERMINED BY SCRAPING METHOD
*Initial population of inoculum was 10.4 $\log_{10}$ CFU/mL.

Exposure of adherent *Listeria* to EO water for 60 s decreased the microorganism population to 11 CFU/coupon as demonstrated by the direct agar overlay method (Table 1), whereas the scraping method recovered approximately 3.1 $\log_{10}$ CFU/coupon of bacteria (Fig. 2). In other words, a comparison of the direct agar overlay method with results from the scraping method indicated that the surviving adherent microcolonies (clumps) consist of about 100 cells each. Lee and Frank (1991) observed that up to 300 s of exposure to 200 ppm chlorine solution was required to achieve *Listeria* inactivation on contaminated stainless steel surfaces. For the current study, 300 s of exposure to EO water containing 56 ppm (56 mg/L) residual chlorine reduced the *Listeria* biofilms on stainless steel surface from $1.9 \times 10^{10}$ CFU/82.5 $cm^2$ to below detection levels. Frank and Koffi (1990) also reported that the use of 100 to 800 n-alkyl (50% $Cl_4$, 40% $Cl_2$, 10% $Cl_6$) dimethyl dichlorobenzyl ammonium chloride (Benzalkonium chloride, BAC) reduced biofilm populations of *Listeria* by 100- to 1000-fold in 30 s, whereas planktonic cells were completely inactivated (over a 6-log cycle decrease) during the exposure to 400 ppm BAC for 300 s. Several other studies (Albrich and Hurst 1982; Clapp *et al.* 1994; McKenna and Davies 1988; Weiss and Regiani 1984) also indicated that the bactericidal action of strong oxidizing agents results from free radical production inside the cell. Results of this study demonstrated the ability of *L. monocytogenes* to accumulate as a biofilm on a hydrophilic stainless steel surface commonly found in food-processing plants. Several scientists (Herald and Zottola 1988; Mostellar and Bishop 1993; Helke *et al.* 1993; Mafu *et al.* 1990) previously reported that *Listeria* has the ability to adhere to various surfaces; including stainless steel, Teflon® and polypropylene. Blackman and Frank (1996) also reported that development of a biofilm is a result of both adherence and growth following adherence. They observed bacterial colonies on many surfaces (stainless steel, Teflon® and polypropylene) when incubated with TSB, indicating that surface growth as well as attachment had occurred. In our study, microcolonies were also observed on a stainless steel surface incubated with TSB and bacteria growth was followed.

In summary, results obtained from the scraping method indicated that each single CFU observed from the direct agar overlay method was likely to represent numerous viable cells. Hence, direct agar overlay method may be underestimating actual cell numbers on the coupons. Results obtained from this study demonstrated that 300 s EO water treatment on stainless steel surfaces significantly reduced the number of biofilm-forming bacteria from $1.9 \times 10^{10}$ CFU/82.5 $cm^2$ to below detection levels. EO water treatment may be an effective means of inactivating biofilm forming bacteria on equipment surface during cleaning.

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