**Control of pathogens in biofilms on the surface of stainless steel by levulinic acid plus
sodium dodecyl sulfate**

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ABSTRACT

The efficacy of levulinic acid (LVA) plus sodium dodecyl sulfate (SDS) to remove or inactivate *Listeria monocytogenes*, *Salmonella* Typhimurium, and Shiga toxin-producing *Escherichia coli* (STEC) in biofilms on the surface of stainless steel coupons was evaluated. Five- or six-strain mixtures (ca. 9.0 log CFU/ml) of the three pathogens were separately inoculated on stainless steel coupons. After incubation at 21°C for 72 h, the coupons were treated for 10 min by different concentrations of LVA plus SDS (0.5% LVA + 0.05% SDS, 1% LVA + 0.1% SDS, and 3% LVA + 2% SDS) and other commonly used sanitizers, including a commercial quaternary ammonium-based sanitizer (150 ppm), lactic acid (3%), sodium hypochlorite (100 ppm), and hydrogen peroxide (2%). The pathogens grew in the biofilms to ca. 8.6 to 9.3 log CFU/coupon after 72 h of incubation. The combined activity of LVA with SDS was bactericidal in biofilms for cells of the three pathogens evaluated, with the highest concentrations (3% LVA + 2% SDS) providing the greatest log reduction. Microscopic images indicated that cells were detached from the biofilm matrix and the integrity of cell envelopes were decreased after the treatment of LVA plus SDS. This study is conducive to better understanding the antimicrobial behavior of LVA plus SDS to the foodborne pathogens within biofilms.

Key words: biofilm, levulinic acid, sodium dodecyl sulfate, *Listeria*, *Salmonella*, *Escherichia coli*

1. Introduction

Foodborne pathogens such as *Listeria monocytogenes*, *Salmonella*, and Shiga toxin-producing *Escherichia coli* (STEC) are major food safety concerns. *L. monocytogenes* causes listeriosis, a disease that mainly affects immunocompromised individuals, the elderly and pregnant women (Kathariou, 2002). The symptoms of listeriosis include encephalitis, meningitis, and abortion (Schlech, 2000). *Salmonella* and STEC collectively cause in the United States an estimated 1.6 million foodborne illnesses annually (Scallan et al., 2011). *Salmonella* causes fever, diarrhea and abdominal cramps 8 to 72 h after infection (Li et al., 2013), whereas STEC has been implicated in numerous outbreaks, with symptoms including bloody diarrhea and hemolytic uremic syndrome (HUS) (Durso et al., 2005).

In food processing facilities, some surfaces such as dead-end microscopic cracks in gaskets, drip pan within refrigerators, and damp walls and ceilings due to condensation are favorable sites for bacteria to grow in static biofilms (Chmielewski and Frank, 2004). Biofilms are single or multi layers of microorganisms embedded in their own extracellular polymeric substances (EPS) which associate with a solid surface (Donlan and Costerton, 2002). It has been suggested that biofilms are the predominant matrix resulting from bacterial growth, and approximately 80% of all bacterial infections are biofilm-associated (de la Fuente-Nunez et al., 2012; Janssens et al., 2008). Biofilms formed by foodborne pathogens can pose a substantial hygienic risk for the food industry because biofilms with pathogens can serve as a contamination source and have an enhanced resistance to mechanical actions and commonly used sanitizers (Carpentier and Cerf, 1993). Corcoran et al. (2014) reported that commonly used disinfectants, including sodium hypochlorite (500 ppm), sodium hydroxide (1 M), and benzalkonium chloride (0.02%), failed to eradicate *Salmonella* biofilms on food contact surfaces. The sanitizer applied

on biofilms should not only possess antimicrobial activity, but also should be able to penetrate the EPS barrier such that with sufficient concentration and exposure time it will contact all of the cells in the biofilm. The efficacy of many sanitizers used in food processing facilities is reduced when organic matter is present, whereby their usefulness as an antimicrobial is mitigated (Simpson Beauchamp et al., 2012). Effective sanitizers that are practical, efficacious, and safe to use are needed to control biofilms in food processing. Levulinic acid (LVA) with sodium dodecyl sulfate (SDS) has been reported previously to be an effective sanitizer for inactivating foodborne pathogens in the presence of organic matter (Magnone et al., 2013; Zhao et al., 2011; Zhao et al., 2009), as this treatment can reduce cell populations in biofilms by > 6 log within 1 min (Wang et al., 2012; Zhao et al., 2011). To our knowledge, no studies have evaluated the antimicrobial efficacy of a LVA with SDS combination on inactivating and removing the foodborne pathogens *L. monocytogenes* and STEC growing as biofilms on stainless steel. Hence, the goal of this study was to determine the effectiveness of LVA plus SDS for inactivating *L. monocytogenes*, *Salmonella*, and STEC cells in biofilms formed on stainless steel coupons.

2. Materials and Methods

2.1. Bacterial strains

Five strains of *L. monocytogenes*, including LM101 (serotype 4b, salami isolate), LM112 (serotype 4b, salami isolate), LM113 (serotype 4b, pepperoni isolate), H9666 (serotype 1/2c, human isolate), and ATCC 5779 (serotype 1/2c, cheese isolate); five isolates of *S. Typhimurium* DT104, including H2662 (cattle isolate), 11942A (cattle isolate), 13068A (cattle isolate), 152N17-1 (dairy isolate), and H3279 (human isolate); and six strains of STEC, including O26:H11 (DEC10B, cattle isolate), O45:H2 (human isolate), O103:H2 (human isolate),

O111:NM (0944-95, cattle isolate), O121-Hunt (human isolate), and O157:H7 (932, human isolate), were used. The cultures were collected and incubated as described previously (Chen et al., 2014). Briefly, each strain of the three pathogens was cultured in 10 ml of BHI (*L. monocytogenes*) or TSB (*Salmonella* and STEC) individually, and incubated at 37°C for 20 h. The cultures were then washed three times with 0.1 M phosphate buffered saline (PBS, pH 7.2, Sigma, St. Louis, MO) by centrifugation at $3,000 \times g$ for 10 min at 4°C and re-suspended in BHI or TSB medium. For each pathogen, the optical density (OD) of each strain was adjusted in a spectrophotometer (model 4001/4, Spectronic Instruments, Rochester, NY) with BHI or TSB to an OD reading of 0.9 (ca. $9.0 \log \text{CFU/ml}$) at 630 nm. Approximately the same cell number of each strain of the pathogen was combined to obtain three bacterial mixtures. Cell numbers in the mixtures were determined by spread plating serial dilutions (1:10 in 0.1 M PBS) onto TSA plates. The plates were incubated at 37°C for 24 (*Salmonella* and STEC) to 48 h (*L. monocytogenes*), and typical colonies were counted.

2.2. Preparation of stainless steel coupons

Stainless steel (type 304; Tull Metals Company, Atlanta, GA) coupons (4 cm $\times$ 2.5 cm) were prepared according to the protocol described by Zhao et al. (2004), with minor modifications. Prior to use, the coupons were washed by a 12-h immersion in 1,000 ml of an aqueous 2% RBS 35 detergent concentrate solution (20 ml of RBS 35 concentrate per liter of sterile distilled water at 21°C; Pierce, Rockford, IL), and rinsed three times by a 10-min immersion in 1,000 ml of sterile distilled water at 21°C. The washed stainless steel coupons were air dried, and an area 1.27 cm in diameter was encircled by a permanent marker. The coupons were then wrapped individually with aluminum foil and autoclaved at 121°C for 15 min.

2.3. Biofilm formation

Each sterile stainless steel coupon was individually transferred into a tissue culture dish base (60 mm × 15 mm, Falcon, Franklin Lakes, NJ) which was then placed in an extra-deep Petri dish (100 mm × 25 mm, Thermo Scientific, Rochester, NY) containing 10 ml of sterile water. An inoculum of 0.1 ml of the mixtures (ca. 9.0 log CFU/ml) of *L. monocytogenes*, *S. Typhimurium*, or STEC was deposited within the marked area of the stainless steel coupon and incubated at 21°C. Every 24 h, the marked area was aspirated to remove spent media, washed five times with 0.1 ml of 0.1 M PBS to remove unattached cells, and replaced with fresh BHI (*L. monocytogenes*) or TSB (*Salmonella* and STEC) medium. All the biofilms in this study were grown for 72 h before sampling.

2.4. Efficacy of sanitizer treatments

Before the coupons were treated with sanitizers, the marked area on coupons was aspirated and washed five times with 0.1 M PBS as described above, to remove unattached cells. The sanitizers evaluated included a commercial quaternary ammonium-based sanitizer (QAC, 150 ppm) containing a mixture of dimethylammonium chlorides with various even-numbered alkyl chain lengths as active ingredients, lactic acid (LA, 3%; Sigma), sodium hypochlorite (SHC, 100 ppm; Becton Dickinson, Sparks, MD), hydrogen peroxide (HP, 2%; Becton Dickinson), levulinic acid (LVA, 3%; Sigma), sodium dodecyl sulfate (SDS, 2%; Sigma), and three different concentrations of LVA plus SDS (0.5% LVA + 0.05% SDS, 1% LVA + 0.1% SDS, and 3% LVA + 2% SDS). Sterile distilled water was used as the control. All of the sanitizers were prepared according to the manufacturers' instructions, immediately before use. After rinsing with 0.1 M PBS as described previously and air dried for 5 min, 0.1 ml of sanitizer was placed on the

marked area on each coupon. After exposure to the sanitizer for 10 min, the marked area was aspirated to remove the sanitizers and unattached cells. The residual sanitizers were neutralized with 0.1 ml of neutralizing buffer (Becton Dickinson) for 10 min. After aspiration, the coupons were subject to bacterial enumeration or added with 0.1 ml of BHI or TSB medium in the encircled area as a 24-h enrichment culture.

2.5. Bacterial enumeration

Each coupon bearing pathogenic bacteria in biofilms was washed with 0.1 ml of 0.1 M PBS as described previously and then individually placed in a 50-ml centrifuge tube containing 9.9 ml of 0.1 M PBS and 30 glass beads (5-mm diameter; Fisher Scientific, Norcross, GA). The tubes were agitated by a Vortex mixer (Fisher Scientific) for 2 min to detach the cells from the stainless steel surface. One milliliter of the suspension and 0.1 ml of serial dilutions (1:10 in PBS) were plated in duplicate on TSA plates. The plates were incubated at 37°C for 24 (*Salmonella* and STEC) to 48 h (*L. monocytogenes*) before bacterial counts.

2.6. Scanning electron microscopy (SEM)

Biofilm formation by the three pathogens on the surface of stainless steel coupons after treatment with LVA plus SDS was visualized using scanning electron microscopy (SEM). Biofilms were grown at 21°C for 72 h and then treated with sterile distilled water (control) and different concentrations of LVA plus SDS for 10 min as described previously. After adding with neutralizing buffer, the coupons were fixed with 2% glutaraldehyde (Sigma) for 1 h at room temperature, rinsed three times for 15 min each with PBS, air dried for 30 min, and sputter coated with gold (model 11428-AB, Spi Supplies, West Chester, PA). The samples were

subsequently examined with a Zeiss 1450EP scanning electron microscope (Zeiss, Scotts Valley, CA).

2.7. Transmission electron microscopy (TEM)

The effect of LVA plus SDS on the structures of the pathogens in biofilms was investigated to determine this treatment's influence on cell viability and cellular injury. After treatment with LVA plus SDS, the coupons bearing pathogenic bacteria in biofilms were aspirated to remove chemicals and neutralizing buffer was added on the marked area as described previously. Each coupon was swabbed with a sterile 6-inch (15.2 cm) polyester-tipped swab (Fisher Scientific), and the swab was then dipped in 900 µl of 0.1 M PBS, pH 7.2. After agitation by a Vortex mixer for 2 min, the suspensions were fixed with 2% glutaraldehyde for 1 h at room temperature, rinsed three times with PBS for 15 min each time, then secondarily fixed with 1% OsO₄ for 1 h at room temperature, and dehydrated in an ethanol series of 25%, 50%, 75%, 100%, 100% and 100% for 15 min each. The samples were then soaked in 50% (PO:ethanol) and 100% propylene oxide (PO) for 5 min each, and infiltrated with EmBed 812 resin (EMS, Hatfield, PA) in a series of 25%, 50% and 75% (resin:PO) for 1 h each. The samples were then allowed to polymerize in 100% resin overnight at 60°C. Hardened blocks were trimmed and sectioned on an ultramicrotome (RMC/Boekeler, Tuscon, AZ) to a thickness of approximately 50 nm collected on a copper grid. Sections were post-stained with 2% uranyl acetate for 30 min and lead citrate for 5 min. The samples were washed with distilled water and air dried, then examined using a Technai 20 transmission electron microscope (FEI, Eindhoven, Netherlands).

2.8. Statistical analysis

Each experiment was repeated three times, with duplicate samples each time. The mean population of pathogens per ml or coupon was converted to log CFU/ml or log CFU/coupon. Data were analyzed for one-way analysis of variance (ANOVA) by SAS software (SAS 9.3, SAS Institute, Cary, NC) to determine least significant differences at $P < 0.05$ among the treatments.

3. Results and discussion

After static incubation at 21°C for 72 h, *L. monocytogenes*, *S. Typhimurium*, and STEC grew to ca. 8.6, 9.0, and 9.3 log CFU/coupon, respectively, in biofilms on the surface of the stainless steel coupons (Table 1). For all of the sanitizers tested, complete elimination/inactivation of the pathogens in biofilms did not occur, except for the combination of the highest concentrations of LVA plus SDS (3% LVA + 2% SDS) on STEC in biofilms. There is a synergistic antimicrobial effect between LVA and SDS since the application of LVA or SDS individually at equivalent concentrations is considerably less effective, with only a 0.2 to 1.2-log CFU/coupon reduction. Dramatically enhanced antimicrobial efficacy when combining LVA with SDS was also observed previously (Zhao et al., 2014; Zhao et al., 2009, 2010). Surfactants, like SDS, can act as antiadhesive agents and LVA may assist in removal of the attachment polymer by chelating divalent cations required to link the polymer at the surface (Frank, 2001). Hence, the combination of an organic acid and surfactant may promote each other to detach bacterial cells from a biofilm matrix. Once the cells are directly exposed to the chemicals without the protection of EPS, SDS can chelate divalent cations, such as Ca^{2+} and Mg^{2+} , leading to instability of outer membrane of Gram-negative bacteria (Hancock and Rozek, 2002). LVA, in addition to its antimicrobial activity due to lowering pH, may also damage cell membranes by releasing lipopolysaccharide from the outer membrane of Gram-negative bacteria (Alakomi et al., 2000; Ricke, 2003). LVA and SDS are both permeabilizers that complement each other enabling

penetration of cells, leading to increased susceptibility (Alakomi et al., 2000; Helander et al., 1997).

The other evaluated chemical sanitizers, including 150 ppm QAC, 2% lactic acid, 100 ppm sodium hypochlorite, and 2% hydrogen peroxide, were not effective against all the three pathogens in the biofilms on stainless steel. QAC predominantly target cell's membrane, and Gram-positive bacteria are more sensitive to QACs at low concentrations than Gram-negative (McDonnell and Russell, 1999). The undissociated form of lactic acid can freely diffuse across the bacterial cell membrane and lower the cytoplasmic pH upon dissociation (Virto et al., 2005). Lactic acid is generally effective to inactivating enteric bacterial pathogens (Zhao et al., 2014). In hypochlorite solution, hypochlorous acid readily reacts with organic matters in cells and is more bactericidal to Gram-negative (Corcoran et al., 2014; Virto et al., 2005). Hydrogen peroxide acts as an oxidant by producing hydroxyl free radicals attacking essential cell components (McDonnell and Russell, 1999). All three of the pathogens used in this study can produce catalase, which provides protection to the embedded cells by preventing full penetration of hydrogen peroxide into the biofilm (Stewart et al., 2000). These commonly used sanitizers failed to fully inactivate bacterial cells within biofilms, perhaps because the cells in the biofilm matrix do not contact these sanitizers due to insufficient penetration and neutralization with constituents of the biofilms (Corcoran et al., 2014; Stewart et al., 2001; Stewart et al., 2000).

The SEM images (Figures 1 - 3) revealed that most of the cells in the biofilms were detached from the biofilm matrix after a 10-min treatment with LVA plus SDS. Once the bacterial cells detach from the biofilm matrix, they are more vulnerable to antimicrobial agents (Kumar and Anand, 1998). The SEM images revealed the occurrence of abundant EPS in the biofilms formed by the three pathogens, and that a greater number of the pathogens in biofilms

were inactivated when the treatment concentrations of LVA and SDS were increased. The TEM analysis revealed structural changes to bacterial cells treated with LVA plus SDS (Figures 4 - 6). The untreated cells of *L. monocytogenes* (Figure 4A), *S. Typhimurium* (Figure 5A), and STEC (Figure 6A) had smooth and well-defined cell walls or outer membranes. Treatment of *L. monocytogenes* with 0.5% LVA + 0.05% SDS produced a loss of definition in the cell wall and aggregation of cytoplasmic cellular component. We did not observe any cells in the *L. monocytogenes* samples ($n = 12$) that were treated with higher concentrations of LVA plus SDS, likely because of cellular lysis and loss of cells during the sample preparation for TEM. For *S. Typhimurium* and STEC, aggregation of the cytoplasmic components of cells followed by less defined cell morphology was observed as the treatment concentrations of LVA plus SDS were increased. Cellular leakage of STEC was observed (Figure 6B), indicating the cell membrane was damaged in the presence of low concentrations of LVA plus SDS. Bacterial cell counts by the plating method performed in this study revealed that *L. monocytogenes* was more susceptible to LVA plus SDS compared to the other two Gram-negative pathogens. This finding is consistent with our previous study (Chen et al., 2014). Unlike Gram-negative bacteria, Gram-positives such as *L. monocytogenes* do not possess an outer membrane. Hence, they are more susceptible to the action of chemicals interfering with the transport of ions across the cell membrane (Feliciano et al., 2012; Skrivanova et al., 2006).

In conclusion, the combination of LVA and SDS was most effective against the three pathogens in biofilms compared to the other evaluated sanitizers, with the highest concentrations (3% LVA + 2% SDS) providing the greatest log reduction. SEM images confirmed that the combination of LVA and SDS was effective in inactivating bacterial cells in biofilms, and the TEM images revealed that LVA with SDS cause changes to the permeability of the cell

membrane and aggregation of the cytoplasmic components. Results of this study may be useful to mitigate the presence of biofilms in food processing facilities.

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TABLE 1 Inactivation of 72-h biofilms of *L. monocytogenes*, *S. Typhimurium*, and STEC formed on stainless steel after exposure to a sanitizer for 10 min.

Treatment ^a	Counts (log CFU/coupon) ^b		
	<i>L. monocytogenes</i>	<i>S. Typhimurium</i>	STEC
DW (control)	8.6 ± 0.2a ^c	9.0 ± 0.0a	9.3 ± 0.1a
QAC (150 ppm)	4.2 ± 0.9d	8.2 ± 0.3a	7.6 ± 0.5c
LA (3%)	4.4 ± 0.2d	3.1 ± 0.0de	5.7 ± 0.1e
SHC (100 ppm)	7.9 ± 0.3bc	4.4 ± 0.9c	7.8 ± 0.0c
HP (2%)	8.3 ± 0.0ab	8.3 ± 0.2a	7.8 ± 0.6c
LVA (3%)	8.3 ± 0.2ab	8.6 ± 0.2a	8.8 ± 0.1b
SDS (2%)	7.4 ± 0.5c	8.8 ± 0.0a	9.0 ± 0.0ab
LVA (0.5%) + SDS (0.05%)	1.9 ± 0.3e	5.8 ± 0.5b	7.0 ± 0.3d
LVA (1%) + SDS (0.1%)	+ e	3.1 ± 0.8d	6.1 ± 0.1e
LVA (3%) + SDS (2%)	+ e	2.1 ± 0.5e	- f

^a DW, distilled water; QAC, quaternary ammonium compound; LA, lactic acid; SHC, sodium

hypochlorite; HP, hydrogen peroxide; LVA, levulinic acid; SDS, sodium dodecyl sulfate.

^b Values are means ± standard deviations. The minimum detection limit by the direct plating method was 1.7 log CFU/coupon. “+” indicates that pathogen was not detected by the direct plating method but was enrichment culture-positive. “-” indicates that both the direct plating method and enrichment cultures were negative.

^c Values in the same column that are not followed by the same lower case letters are significantly different ($P < 0.05$).

FIGURE LEGENDS

FIGURE 1. Representative photomicrographs by SEM of biofilms formed by *Listeria monocytogenes* after a 10-min treatment with water (control, A), 0.5% levulinic acid + 0.05% SDS (B), 1% levulinic acid + 0.1% SDS (C), or 3% levulinic acid + 2% SDS (D). Scale bar = 2 μm .

FIGURE 2. Representative photomicrographs by SEM of biofilms formed by *Salmonella* Typhimurium after a 10-min treatment with water (control, A), 0.5% levulinic acid + 0.05% SDS (B), 1% levulinic acid + 0.1% SDS (C), or 3% levulinic acid + 2% SDS (D). Scale bar = 2 μm .

FIGURE 3. Representative photomicrographs by SEM of biofilms formed by STEC after a 10-min treatment with water (control, A), 0.5% levulinic acid + 0.05% SDS (B), 1% levulinic acid + 0.1% SDS (C), or 3% levulinic acid + 2% SDS (D). Scale bar = 2 μm .

FIGURE 4. Representative photomicrographs by TEM of biofilms formed by *Listeria monocytogenes* after a 10-min treatment with water (control, A), or 0.5% levulinic acid + 0.05% SDS (B). The black arrow highlights the morphological differences after the 10-min treatment of levulinic acid plus SDS. Scale bar = 100 nm.

FIGURE 5. Representative photomicrographs by TEM of biofilms formed by *Salmonella* Typhimurium after a 10-min treatment with water (control, A), 0.5% levulinic acid + 0.05% SDS (B), 1% levulinic acid + 0.1% SDS (C), or 3% levulinic acid + 2% SDS (D). Scale bar = 100 nm.

FIGURE 6. Representative photomicrographs by TEM of biofilms formed by STEC after a 10-min treatment with water (control, A), 0.5% levulinic acid + 0.05% SDS (B), 1% levulinic acid + 0.1% SDS (C), or 3% levulinic acid + 2% SDS (D). The black arrow highlights the

385 morphological differences after the 10-min treatment of levulinic acid plus SDS. Scale bar = 100
386 nm.

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FIGURES

Fig. 1

Fig. 2

Fig. 3

454 **Fig. 4**

Fig. 5

481 **Fig. 6**

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