International Journal of Food Microbiology 207 (2015) 1–7

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

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

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ARTICLE INFO

Article history:
Received 26 November 2014
Received in revised form 8 April 2015
Accepted 18 April 2015
Available online 24 April 2015

Keywords:
Biofilm
Levulinic acid
Sodium dodecyl sulfate
Listeria
Salmonella
Escherichia coli

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 the 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.

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 (EPSs) 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., 2009, 2011), 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

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.04.026
0168-1605/

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), 152 N17-1 (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 3000 ×*g* for 10 min at 4 °C and resuspended 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 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 × 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 1000 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 1000 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% OsO_4 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