Food Microbiology 64 (2017) 139–144

Contents lists available at ScienceDirect

Food Microbiology

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

Pathogen biofilm formation on cantaloupe surface and its impact on the antibacterial effect of lauroyl arginate ethyl

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ARTICLE INFO

Article history:
Received 24 January 2016
Received in revised form
27 December 2016
Accepted 27 December 2016
Available online 29 December 2016

Keywords:
Listeria
Salmonella
Biofilm
Cryo-SEM
Lauroyl arginate ethyl

ABSTRACT

Pathogen biofilm at fruit surface may pose a particular risk to food safety. In this study, the biofilms of *Listeria monocytogenes* V7 and *Salmonella enterica* serovar Typhimurium ATCC 13311 on cantaloupe fruit surface were visualized, and the resistance of biofilms against lauroyl arginate ethyl (LAE, an antibacterial compound) was evaluated. Each bacterium was inoculated on isolated cantaloupe rind surfaces at 10^5 -10^6 CFU/cm^2 and after incubation for 2, 12, 24, and 48 h, the surfaces were imaged using cryo-scanning electron microscopy (Cryo-SEM). The images showed that both pathogens formed biofilms on rind surfaces, with *S.* Typhimurium forming biofilm in 12 h and *L. monocytogenes* cells starting to aggregate in 2 h. For the inoculated rind surfaces treated with LAE, the cell counts were affected by both the incubation time and LAE concentration. For rind surface with 2 h incubation of *S.* Typhimurium, 400 and 800 μg/mL LAE was able to achieve >2.00 log reduction; however, 12 h incubation required 1600 and 2000 μg/mL LAE for >2.00 log reduction. In contrast, even the highest LAE concentration (2000 μg/mL) was unable to cause 1.00 log reduction for *L. monocytogenes* regardless the incubation time applied. The results showed that the biofilms of both bacteria substantially reduced LAE efficacy, and that the biofilm of *L. monocytogenes* was more resistant than that of *S.* Typhimurium.

1. Introduction

In the past decade, cantaloupes have been implicated as a source of foodborne outbreaks from *Salmonella enterica* serovar Poona, *S.* Chester, *S.* Oranienburg, *S.* Saphra, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Campylobacter* and Norovirus (Walsh et al., 2014). Cantaloupes are susceptible to contamination at any point during growth, harvesting, transportation, and distribution (Upadhyay et al., 2014). In 2011, a multistate outbreak of listeriosis linked to whole cantaloupes resulted in 147 illnesses (143 hospitalizations, 33 deaths) in 28 states across the U.S. (CDC, 2012a). In 2012, another multistate outbreak of *S.* Typhimurium and *S.* Newport infections linked to whole cantaloupes led to 261 illnesses (94 hospitalizations, 3 deaths) in 24 states (CDC, 2012b). These outbreaks highlight the susceptibility of cantaloupes to microbial contaminations, and the mesh-like external structure of the rind provides a coarse surface that helps the bacterial attachment (Ukuku, 2006). In addition, the potential of bacterial biofilm formation on cantaloupe surface may also reduce the efficacy of antimicrobial solutions by providing protection to the cells (Annous et al., 2005). Together, there is a large food safety concern with whole cantaloupe.

A biofilm is defined as an aggregate of microorganisms in which cells are embedded within a matrix of extracellular polymeric substance (EPS) and adhere to each other and/or to a surface (Vert et al., 2012). It is a self-protection growth pattern of bacteria, which is different from their planktonic counterparts. Due to the presence of EPS, cells in biofilms are capable of withstanding harsh environments and more resistant to disinfectants (Flemming and Wingender, 2010; Hall-Stoodley et al., 2004; Upadhyay et al., 2013). For example, it was shown that aqueous sanitizers were not sufficient to reduce bacteria on plant tissues (Annous et al., 2006; Yaron and Römling, 2014). For cantaloupe, Annous et al. (2005) reported that biofilm formation on rind by *S.* Poona and *S.* Michigan occurred in 24 h following the inoculation. The biofilm formed on cantaloupe surface may make it more difficult to remove bacteria cells using conventional aqueous sanitizers (Annous et al., 2004; Ukuku and Sapers, 2001).

The efficacy of various antimicrobial agents such as hydrogen peroxide, chlorine, nisin, potassium sorbate, sodium lactate, and chitosan, has been investigated in controlling foodborne pathogens on cantaloupes (Chen et al., 2012; Ukuku and Sapers, 2001; Ukuku, 2004). As a convenient and cost-effective sanitizer, chlorine is a

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http://dx.doi.org/10.1016/j.fm.2016.12.020
0740-0020/

most widely used sanitizer in the food industry (Parish et al., 2003). It is also allowed for washing raw fruits and vegetables according to the FDA's regulation (FDA, 2003). In practice, chlorine at 100–150 μg/mL is used for washing cantaloupes (Suslow, 1997). However, studies have shown that such chlorine concentrations may result in low reductions (1–1.5 log CFU/cm^2) in pathogen counts (Upadhyay et al., 2016). The limited efficacy is more obvious when the interval between the inoculation and the treatment exceeds 24 h (Ukuku et al., 2001). Another concern with using chlorine as a washing treatment is its potential to generate harmful chemical byproducts such as chloramines and trihalomethanes when interacting with organic materials (Richardson et al., 1998). Thus, there is an increasing interest in finding safe and effective antimicrobials for controlling pathogens on cantaloupes.

The objective of this study was to monitor the biofilm formation on the rind of cantaloupes by selected foodborne pathogens (i.e. *L. monocytogenes* and *S.* Typhimurium). Due to the limitations of traditional methods for observing biofilms, Cryo-SEM was used in this study to visualize biofilms. Furthermore, lauroyl arginate ethyl (LAE), a relatively new antimicrobial compound registered with the FDA as GRAS (generally recognized as safe; FDA, 2005), was tested as a model for its efficacy against bacteria on the rind surface. It should be noted, however, that in the US the use of sanitizers is regulated by the US Environmental Protection Agency (EPA), not FDA.

2. Materials and methods

2.1. Cantaloupes

Cantaloupe fruits (*Cucumis melo* L. var. *reticulatus*; Martori Farms, Scottsdale, AZ, USA) were purchased at a local grocery store the day before experiment and stored at 4 °C until use. Cantaloupes were washed under running distilled water and scrubbed with a clean brush for 2 min, then air-dried at 22 °C for 30 min in a class II biosafety cabinet (Labconco Corporation, Kansas City, MO, USA) to remove excessive moisture.

2.2. Bacteria and growth conditions

L. monocytogenes V7 (originally isolated from raw milk; Stelma et al., 1987) and *S.* Typhimurium ATCC 13311 (originally isolated from mutton; ATCC® 13311™) were used to represent Gram-positive and Gram-negative foodborne pathogens, respectively. Since manure-related contamination of irrigation water is a major source of pathogen contamination of cantaloupe fruits, both strains are associated with the food safety of cantaloupe. The cultures were maintained on tryptic soy agar (TSA) with 0.6% yeast extract (TSAYE; Becton Dickinson, Sparks, MD, USA) at 4 °C. Prior to use, the culture was subjected to two successive transfers by loop inocula in 5 mL tryptic soy broth (TSB) with 0.6% yeast extract (TSBYE). A third transfer of 200 μL was made into 20 mL TSBYE and incubated at 37 °C for 18–24 h with continuous agitation (100 rpm) on a MaxQ 2000 platform shaker (Barnstead Lab-line, Melrose, IL, USA). The initial concentration of each bacterial culture was approximately 10^8 CFU/mL.

2.3. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of LAE in vitro

The MIC was determined by the microbroth dilution method (Branen and Davidson, 2004; Ma et al., 2013). The bacterial culture was diluted to 10^7 CFU/mL in TSBYE, and 100 μL of the diluted culture was added into wells of a 96-well microtiter plate (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA). Antimicrobial stock solutions (2560 μg/mL) were prepared by dissolving 0.256 g of 10% lauroyl arginate ethyl (LAE; A & B ingredients Inc., Fairfield, NJ, USA) in 10.0 mL sterile distilled water. The working antimicrobial solution was prepared by diluting the stock solution in TSBYE to 1280 μg/mL that was further diluted in series from 2.5 to 1280 μg/mL. An aliquot of 100 μL of the antimicrobial solution was mixed with the bacterial culture in each well, and the plates were incubated at 37 °C for 24 h. The MIC was defined as the lowest antimicrobial concentration corresponding to an optical density change at 595 nm ($\Delta OD_{595\ nm}$) of <0.05.

The MBC was determined by spreading 100 μL aliquots from negative wells (i.e., $\Delta OD_{595\ nm}$ <0.05) on TSAYE, followed by incubation at 37 °C for 24 h. MBC was defined as the antimicrobial concentration corresponding to at least a 3.00 log reduction of viable cells (Branen and Davidson, 2004).

2.4. Inoculation of cantaloupe rind

A 10 mL of each bacterial culture was centrifuged at 6000 ×*g* (Rotor JA-14, Beckman, Palo Alto, CA, USA) for 10 min. Afterwards, the cell pellets were washed twice in phosphate buffer (PB; 0.1 M, pH 7.0). The supernatant was discarded and the cell pellets were washed and resuspended in 10 mL sterile PB (about 10^8 CFU/mL) and further diluted by 10 times as the inoculation fluid for isolated rind pieces.

To prepare rind pieces, cantaloupe rind was first cut into small square pieces (1.5 cm × 1.5 cm; 2.25 cm^2) using a sterile knife. For each piece, the flesh portion was removed and the rind (about 5 mm in thickness) was placed in a sterile 12-well microtiter plate (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA). On each rind piece, 100 μL inoculation fluid was spotted to yield a cell count of about 10^5–10^6 CFU/cm^2, and the inoculated rind pieces were air-dried at 22 °C for 1 h in a biosafety cabinet and then placed for up to 48 h for Cryo-SEM imaging and LAE treatment.

2.5. Cryo-SEM imaging of biofilm formation

In order to determine the ability of both pathogens to form biofilm on cantaloupe surface, the cantaloupe rind pieces inoculated as described above were sampled at 2, 12, 24, and 48 h following inoculation. After sampling, the cantaloupe rind pieces were immediately immersed in 5% glutaraldehyde (PB as solvent) for 10 min to inactivate pathogens. In a Cryo-SEM (FEI Company, Hillsboro, OR, USA) facility, the specimens (1 cm^2 for each) were cut from the inoculated rind pieces, and each was attached to a sample holder using carbon tape and plunged into liquid nitrogen slush. A vacuum was pulled and the samples were transferred to a GAtan Alto 2500 pre-chamber cooled to −180 °C where they were sublimated at −90 °C for 5 min followed by sputter coating for 90 s with platinum. The samples were imaged using the Everhart-Thornley detector operating at 5 kV accelerating voltage, 4.0–5.0 working distance, and 30-μm aperture. The collected images were cropped and reduced in resolution using Adobe Photoshop CS3.

2.6. Treatment of inoculated cantaloupe rind with LAE

The inoculated rind pieces were exposed to different concentrations of LAE solutions (200, 400, 800, 1600, 2000 μg/mL, 10 mL) each in a stomacher bag for 5 min at 22 °C, and then each transferred to another stomacher bag that contained 100 mL of PB.

2.7. Microbial enumeration

Three controls were used in this study. An uninoculated, untreated control was used to determine inherent background