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

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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/cm² 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*.

Keywords: *Listeria*; *Salmonella*; Biofilm; Cryo-SEM; Lauroyl arginate ethyl

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, 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 hours 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 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/cm²) 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 resource 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 TSB with 0.6% yeast extract

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 μ L 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 μ g/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 μ g/mL that was further diluted in series from 2.5 to 1280 μ g/mL. An aliquot of 100 μ L 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 ($\Delta OD_{595\text{ nm}}$) of < 0.05 .

The MBC was determined by spreading 100 μ L aliquots from negative wells (i.e., $\Delta OD_{595\text{ nm}} < 0.05$) 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 $6000 \times g$ (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 ($1.5\text{ cm} \times 1.5\text{ cm}$; 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. 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 seconds 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 microflora and pathogen levels, if they were present. An inoculated, untreated control was used for comparing untreated vs. treated samples, and an inoculated, water treated control was used to compare water treated vs. LAE treated samples. All the controls, as well as the inoculated and treated rind pieces (2.25 cm²) were mixed with 100 mL of PB in a sterile stomacher bag and homogenized for 4 min using a Stomacher 400 Lab-blender (Seward Limited, London, UK). Serial dilutions were prepared using sterile PB as the diluent. Enumeration of the bacteria present in the supernatant was performed using standard plate count methods. Modified Oxford (MOX) medium was used for *L. monocytogenes*, and Xylose Lysine Deoxycholate (XLD) medium was used for *S. Typhimurium*. Plates were incubated at 37 °C for 24 h, and the colonies were counted with the results expressed as log₁₀ CFU/cm².

2.8. Statistical analysis

For each treatment, six replicates of rind piece were used and the mean values and standard deviations of cell count were determined. The significance levels of differences among the cell counts (in log₁₀ values) of 2, 12, and 24 h incubation samples were assessed using student's t-test (Microsoft Excel Version 15.0).

3. Results and discussion

3.1. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of LAE

LAE is an effective antimicrobial compound against both *L. monocytogenes* and *S. Typhimurium*. MICs and MBCs of LAE against both pathogens were determined to be 20 µg/mL for each. This result was similar to previous studies showing that MICs and MBCs of LAE against *L. monocytogenes*, *E. coli*, and *S. Typhimurium* ranged from 11.8 to 25 µg/mL (Becerril et al., 2013; Brandt et al., 2010; Ma et al., 2013).

3.2. Biofilm formation on cantaloupe rind by *L. monocytogenes* and *S. Typhimurium*

After the initial washing procedure, the bacterial cells on the cantaloupe rind surface were negligible and cannot be detected using cryo-SEM imaging (**Fig. 1**). Due to the lack of visible microflora on the surface of negative control (i.e. washed and uninoculated rind), biofilms observed with inoculated rind samples were believed to originate from the *L. monocytogenes* V7 and *S. Typhimurium* cells applied.

As compared to biofilm observation using conventional scanning electron microscopy (SEM), transmission electron microscopy (TEM), and other methods (Annous et al., 2005; Fett and Cooke, 2003, 2005; Morris et al., 1997), Cryo-SEM minimized the destruction of native biofilm morphology through *in situ* surface sublimation. Without multi-step procedures such as fixation, dehydration, or staining steps, Cryo-SEM observation of biofilms on the cantaloupe surface proved to be a rapid process. The Cryo-SEM results indicated that both *L. monocytogenes* V7 and *S. Typhimurium* ATCC 13311 were able to form biofilm during the 48 h incubation at room temperature following their introduction on the cantaloupe rind (**Fig. 2**

and **Fig. 3**). In addition, there was approximately a $2.00 \log_{10}$ CFU/cm² increase of each pathogen on the surface of the cantaloupe rind according to the microbial enumeration (**Fig. 4** and **Fig. 5**). This increase indicated that the cantaloupe rind was able to support the growth of both microorganisms. Conceivably, the increasing microbial population was associated with the formation of biofilm on cantaloupe rind surface.

As shown in **Fig. 2**, after 2 h of incubation, *S. Typhimurium* was observed to attach on the cantaloupe surface in the planktonic form, and a few cells were arranged and bound together. Some bacterial cells were undergoing binary fission. This was an obvious sign that showed the population increase of *Salmonella* cells during the incubation. After 12 h of incubation, bacterial aggregates were observed within the netting of the cantaloupe rind, which suggested that a biofilm started forming on the surface rapidly. With an extended incubation time of 24 h, large aggregates of *Salmonella* cells held together and formed a sheet-like structure. After 48 h of incubation, filamentous fungi were observed to intersperse with *Salmonella* cells. These results were consistent with the findings of Annous et al. (2005), who demonstrated that two clinical isolates associated with cantaloupe outbreaks, *S. Poona* RM 2350 and *S. Michigan*, were able to form biofilm on whole cantaloupe melons in a short time (Annous et al., 2005).

Similar to *S. Typhimurium*, the ability of *L. monocytogenes* to form biofilm on the surface of cantaloupe rind was also demonstrated (**Fig. 3**). Noticeably, *Listeria* cells seemingly attached and formed aggregates on the rind surface earlier than that of *Salmonella* cells. After 2 h of incubation at room temperature, the bacterial cells tended to crowd together to form biofilm. After 12 h of incubation, a large number of *Listeria* cells were reproduced and covered

the cantaloupe surface in the form of aggregates. According to other studies, these aggregates were likely to be biofilms with the bacteria bound to each other and to the cantaloupe surface by microbial exopolymeric materials (Fett, 2000; Fett and Cooke, 2003). When incubated for 24 h and 48 h, a more regular biofilm structure was seen on the surface. Additionally, some native cocci-shaped bacteria were also detected with 24 h and 48 h samples.

Our study showed that both *S. Typhimurium* and *L. monocytogenes* were capable of forming biofilms on the cantaloupe rind surface. Moreover, the biofilm formation occurred rapidly after the inoculation of foodborne pathogens. For *S. Typhimurium*, it took 12 h after inoculation to start forming aggregates and biofilm. In contrast, *L. monocytogenes* only needs 2 h of incubation to form aggregates on the surface. Within 24 h incubation, both pathogens formed noticeable biofilm structure on the rind of the cantaloupe. Despite a lack of considerable thickness, aggregates and biofilms formed by both pathogens on rind surface could protect them from adverse environmental conditions.

3.3. Efficacies of LAE treatments against S. Typhimurium and L. monocytogenes on cantaloupe rind surface

In order to evaluate the impact of biofilm formation on the efficacy of aqueous sanitizers, LAE as the model antimicrobial material was applied on rind samples at various incubation times after inoculation. The antimicrobial activity of LAE is not affected by the inoculum dose (Becerril, et al., 2013). In liquid medium, the MIC and MBC values of LAE were both 20 µg/mL against *L. monocytogenes* and *S. Typhimurium*, thus providing a suitable reference to evaluate the resistance of biofilms.

Fig. 4 shows the impact of LAE treatment on the cell count reduction of the *S.*

Typhimurium strain used. The positive control, for which no washing step was applied, showed that the count of *S. Typhimurium* on rind surface increased to over 10^8 CFU/cm² during the 24 h incubation. Such an increase in bacterial count contributed to cell aggregation and biofilm formation on the rind surface. In contrast, water washing showed minor impacts on bacterial reduction. For rind samples with 2, 12, or 24 h incubation, the count of viable *Salmonella* cells on the water-washed rind was slightly lower than that of the positive control (< 1.00 log reduction), showing an evident whereas limited effect of washing with water.

The use of LAE solutions resulted in much greater cell count reduction than water washing. After 2 h incubation, LAE concentrations of 200, 400, or 800 µg/mL were all able to achieve a log reduction close to or over 2.00 compared with the water-washed samples. When the LAE concentration was 1600 or 2000 µg/mL, *S. Typhimurium* was undetectable after treatment.

With 12 and 24 h incubation samples, however, *S. Typhimurium* showed a strong resistance to LAE treatment. For example, with the 2 h incubation sample, 1600 µg/mL LAE was able to achieve a complete removal of bacterial cells. For the 12 h incubation samples, in contrast, a 2.42 log reduction was realized, leaving a cell count of over 10^4 CFU/cm² on the rind surface after 1600 µg/mL LAE treatment.

Compared with *S. Typhimurium*, *L. monocytogenes* was much more resistant to LAE treatments of cantaloupe rind. As shown in **Fig. 5**, the positive control indicated a cell count reaching $10^{7.85}$ CFU/cm² after 24 h incubation. When treated with water or LAE, however, the *Listeria* cells showed much higher retention than *Salmonella* cells. For any of 2, 12, or 24 h incubation rind samples, none of LAE solutions applied (200 – 2000 µg/mL) was able to

reduce the cell count by over 1.00 log compared with the water-washed samples.

Several factors may contribute to the unusually high resistance of bacterial cells to LAE treatment. One factor could be related to the bacterial load. Indeed, the 12 and 24 h incubation rind samples hosted much higher cell counts than the 2 h incubation samples, and the high bacterial load could cause a higher burden for LAE treatment. However, we consider that the bacterial load was a minor factor, since Becerril et al. (2013) showed that the efficacy of LAE was not substantially affected by the bacterial load. In addition, the *Listeria*-inoculated, 2 h incubation samples also showed high resistance to LAE, even though their cell count was almost as low as that of the *Salmonella*-inoculated, 2 h incubation samples.

This study showed that the formation of biofilm was a major factor for the resistance of *S. Typhimurium* and *L. monocytogenes* to LAE. For *S. Typhimurium*, 12 and 24 h incubation rind samples displayed much higher resistance than the 2 h incubation samples (**Fig. 4**), which was related to the formation of biofilms (**Fig. 2**). For *L. monocytogenes*, the 2 h incubation rind samples showed resistance comparable with that of 12 and 24 h, and in general the resistance was higher than that for *S. Typhimurium*. In liquid medium, *L. monocytogenes* and *S. Typhimurium* were equally susceptible to LAE (with the same MBC values), suggesting that the difference of their resistance to LAE on rind surface could be related to specific cell-rind interactions and biofilm EPS structures.

Conclusions

In this study, Cryo-SEM was used to observe biofilms at cantaloupe surface, and the images showed that both *L. monocytogenes* V7 and *S. Typhimurium* ATCC 13311 were able to

form biofilms on the cantaloupe rind surface during the 48 h incubation. Previous studies suggested that hydrophobic interactions were the major factor for bacterial cell attachment with plant tissues and such interactions could be disrupted by surfactants (Ölmez and Temur, 2010). However, as a positively charged surfactant, LAE even at high concentration failed to eradicate *Listeria* and *Salmonella* cells from cantaloupe rind surface, highlighting the resistance of both bacteria due to the presence of biofilm protection. To effectively reduce the bacterial load on cantaloupe surface, sufficient disruption of biofilm would be necessary to allow the antimicrobial compounds to access individual cells.

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Figure captions

Figure 1. The cantaloupe rind (left image) shows a mesh-like network, which provides a rough surface that helps in bacterial attachment. Cryo-SEM micrograph of a water-washed,

uninoculated cantaloupe rind surface shows extensive, deeply fissured region of the surface (netting). Blue arrow indicates the enlargement of the cracks (right-top image), which forms the characteristic netting on cantaloupes. Red arrow shows the cantaloupe surface between the netting (right-bottom image). The arrows are conceptual indications that do not reflect the actual image magnifications of particular locations.

Figure 2. Cryo-SEM images of 2, 12, 24, and 48 h incubation rind samples inoculated with *Salmonella enterica* serovar Typhimurium ATCC 13311. Blue arrows show single bacterial cells. Note that some cells were undergoing binary fission. Red arrow in the 12 h incubation image shows that cells were arranged to form aggregates and film-like structures. Red arrows in 48 h incubation image show the filamentous fungi interspersed with *Salmonella* cells.

Figure 3. Cryo-SEM images of 2, 12, 24, and 48 h incubation rind samples inoculated with *Listeria monocytogenes* V7. Blue arrows in the 2 h image indicate single bacterial cells, and red arrows show arranged bacterial cells that started to form aggregates. Compared to the 2 h incubation, the 12 h incubation led to substantial cell multiplication and aggregation. In the 24 and 48 h images, red arrows show biofilm formed, and blue arrows indicate native cocci.

Figure 4. The viable cell counts ($\log_{10}$ CFU/cm²) of *Salmonella enterica* serovar Typhimurium ATCC 13311 on cantaloupe rind surface treated with 200, 400, 800, 1600, and 2000 μ g/mL of lauroyl arginate ethyl for 5 min after incubation for 2, 12, and 24 h at 22 °C. Each data point represents the mean value of six replicates with standard deviation as error bar. The asterisk

indicates cells undetectable. For each treatment, the significant differences ($P<0.05$) among the cell counts of 2, 12, and 24 h incubation samples are indicated using different letters.

Figure 5. The viable cell counts ($\log_{10}$ CFU/cm²) of *L. monocytogenes* V7 on cantaloupe rind surface treated with 200, 400, 800, 1600, and 2000 μ g/mL of lauroyl arginate ethyl for 5 min after incubation for 2, 12, and 24 h at 22 °C. Each data point represents the mean value of six replicates with standard deviation as error bar. For each treatment, the significant differences ($P<0.05$) among the cell counts of 2, 12, and 24 h incubation samples are indicated using different letters.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5