Effects of Phenyllactic Acid as Sanitizing Agent for Inactivation of *Listeria monocytogenes* Biofilms

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ABSTRACT

The efficacy of phenyllactic acid (PLA) to inactivate *Listeria monocytogenes* planktonic cells and biofilms was determined and compared with the killing effects of lactic acid (LA), and levulinic acid (LVA) with sodium dodecyl sulfate (SDS). *L. monocytogenes* biofilms of different maturities, i.e., 37 °C for 3 and 7 d and 15 °C for 4 and 7 d, were produced on 24-well flat-bottom polystyrene plates and treated with the sanitizers, including 3% PLA, 1% PLA, 0.5% PLA, 0.25% PLA, 3% LA, 1% LA, and 3% LVA plus 2% SDS for 5, 10, 30, 60 min, respectively. The results of pure culture assays revealed that 1% PLA reduced the population of *L. monocytogenes* by 7 log CFU/ml within 1 min. The biofilms assays revealed that *L. monocytogenes* biofilms can be inactivated to different degrees by the sanitizer treatments. The killing effect of the sanitizers was increased as exposure time and sanitizer concentrations were increased. The sanitizers of 3% PLA, 1% PLA, and 3% LVA plus 2% SDS effectively inactivated the early mature biofilm after a 5-min treatment, whereas 3% PLA was better than all the other sanitizers, including 1% PLA, 3% LA, 3% LVA and 2% SDS for inactivation of the late mature biofilm after a 5-min treatment. Confocal laser scanning microscopy analysis revealed that bacterial cell damages in the biofilm were enhanced as the PLA concentrations and exposure times were increased. These results suggested that PLA was effective in inactivating *L. monocytogenes* and its biofilm, even for the late mature biofilm.

KEYWORDS: *Listeria monocytogenes*, biofilm, phenyllactic acid, lactic acid

1. Introduction

Listeria monocytogenes is an important foodborne pathogen that is able to persist in food processing environments (Farber & Peterkin, 1991; Gandhi & Chikindas, 2007), and can attach to form a biofilm to a variety of surfaces commonly present in a food processing facility (Alonso, Perry, Regeimbal, Regan, & Higgins, 2014; Beresford, Andrew, & Shama, 2001; Gamble & Muriana, 2007; Zhao et al., 2013). Biofilms are defined as matrix-enclosed microorganisms that adhere to a surface and/or to each other, producing a dynamic environment in which the component microbial cells may reach homeostasis, and may optimally organize to make use of all available nutrients (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995). The presence of *Listeria* biofilms in food processing plants is an important hazard because the biofilm can contaminate food products that contact or pass over contaminated surfaces which can lead to an outbreak of listeriosis. Several studies have revealed that *L. monocytogenes* cells in biofilms are difficult to eliminate and can exhibit resistance to chlorine, acid anionic, and quaternary ammonium sanitizers (Frank & Koffi, 1990; Martínez-Suárez, Ortiz, & López-Alonso, 2016). Considering these issues, it is important to develop novel, safe and effective sanitizers to inactivate *L. monocytogenes* biofilms.

3-Phenyllactic acid (2-hydroxy-3-phenylpropanoic acid or β -phenyllactic acid, PLA), an organic acid, has been reported as an antimicrobial compound with broad-spectrum activity against bacteria, including *L. monocytogenes* (Dieuleveux, Van Der Pyl, Chataud, & Gueguen, 1998). Dieuleveux et al. (1998) initially purified and identified the novel anti-*Listeria* compound, PLA, which is produced and excreted by *Geotrichum candidum*. Subsequently, it was determined that PLA is produced by a wide range of lactic acid bacteria species, such as *Lactobacillus*, *Enterococcus*, *Weissella*, and *Leuconostoc*, and has broad antibacterial activity that can inhibit a wide range of Gram-positive bacteria, including *Staphylococcus aureus*, *Enterococcus faecalis*, and *Bacillus cereus*, and Gram-negative bacteria, including *Salmonella*

enterica and *Escherichia coli* (Mu, Yu, Zhu, Zhang, & Jiang, 2012). In addition to its antibacterial activity, PLA can also inhibit yeasts and fungi (Lavermicocca, Valerio, & Visconti, 2003; Lavermicocca, Valerio, Evidente, Lazzaroni, Corsetti, & Gobbetti, 2000; Schwenninger *et al.*, 2008). Hence, PLA may be a natural antibacterial substance that can control pathogens in contaminated food products and processing plants.

Although it has been reported that PLA can inhibit *L. monocytogenes* growth in culture medium and milk (Dieuleveux & Gueguen, 1998), there are few reports that address its activity against *L. monocytogenes* in biofilms. The *L. monocytogenes* strains isolated from food and patients were previously typed to four major serotypes (1/2a, 1/2b, 1/2c, and 4b) (Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004). Although *L. monocytogenes* serotype 4b is considered the most virulent, serotype 1/2c is the strongest biofilm former among the most frequently occurring serotypes in food and food processing environments (Norwood & Gilmour, 1999; Lundén, Miettinen, Autio, & Korkeala, 2000, Harvey, Keenan, & Gilmour, 2007). Hence, *Listeria monocytogenes* ATCC 5779 (serotype 1/2c, cheese isolate) was used in this study. The objective of this study was to evaluate the effectiveness of PLA to inactivate *L. monocytogenes* in biofilms of different maturities.

2. Materials and methods

2.1. Bacterial strain and preparation of a single-species culture

Listeria monocytogenes ATCC 5779 (serotype 1/2c, cheese isolate) was stored at -30 °C. Before each experiment, the culture was thawed and first grown at 37 °C in Brain Heart Infusion broth (BHI, Becton Dickinson, Sparks, MD) for 24 h, and then streaked on BHI agar and grown at 37 °C for 24 h. A single bacterial colony was inoculated into a test tube containing 10 ml of BHI medium, and transferred at least three times at 24-h intervals before use. The final bacteria were grown to the exponential phase to obtain a bacterial count of about 10⁹ CFU/ml, with the number of listeria confirmed by plating serial dilutions (1:10) on BHI agar.

2.2 Preparation of sanitizers

The sanitizers, including PLA, LA, and LVA and SDS (Sigma, St. Louis, MO) were diluted in deionized water to the concentrations (w/v) to be evaluated in each experiment. The pH of the sanitizer solutions is shown in Table 1.

2.3 Sanitizer sensitivity assay

The protocol of the Association of Official Analytical Chemists was used to determine the sensitivity of *L. monocytogenes* ATCC 5779 to PLA and other sanitizers as described in a previous report (Zhao, Doyle, Zhao, Blake, & Wu, 2001). *L. monocytogenes* ATCC 5779 suspension (1 ml) was added to 199 mL of sanitizer solution (1% PLA, 0.5% PLA, 0.25% PLA, 3% LA, 1% LA, 1% LA+ 0.5% PLA, 1% LA+ 0.25% PLA, 0.5% LA+ 0.25% PLA, 0.5% LA+ 0.5% PLA, 3% LVA+ 2% SDS) being stirred with a magnetic stir bar at 100 rpm in a 500-ml Erlenmeyer flask. At predetermined sampling times, 1.0 ml of the treated bacterial suspension was removed and mixed with 9.0 ml of 0.1 M phosphate buffer (PBS) to neutralize the pH. Then, the solution was serially (1:10) diluted in 0.1% peptone water, and 0.1 ml of each dilution was surface-plated on BHI agar plates in duplicate. The plates were held at 37 °C for 48 h and *L. monocytogenes* colonies were enumerated. If no *Listeria* colonies were present by the direct plating method, a selective enrichment was applied. A 1.0-ml portion of bacterial suspension from each tube was added to a test tube containing 9 ml of BHI broth and the tubes were incubated at 37 °C for 24 h. A loopful of broth was surface streaked on each of duplicate MOX agar plates that were then held at 37 °C for 24 h, and up to 5 colonies typical of *L. monocytogenes* (black) were confirmed by biochemical and immunological methods as we described previously (Zhao, Doyle, & Zhao, 2004). All experiments were duplicated, and averages of results are reported

2.4 Biofilm formation and sanitizer treatment

Approximately 10^7 CFU (1 ml) of *L. monocytogenes* were added into each well of 24-well flat-bottom polystyrene plates (Costar, Corning, NY). The plates were incubated

statically at 37 °C for 3 d and at 15 °C for 4 d or at 37 °C and at 15 °C for 7 d. To maintain bacterial viability, BHI broth was changed every 24 h by aspirating old medium along the walls of each well, the well was rinsed three times with 0.1% peptone to remove the unbounded cells, and fresh medium was then added into each well. After incubation, the supernatant fluid was removed, and the wells were rinsed three times with 2 ml of 0.01 M phosphate buffer saline (PBS, Sigma, St. Louis, MO). The plates were then air dried for 5 min at 21 °C. Four concentrations of PLA (3% PLA, 1% PLA, 0.5% PLA, 0.25% PLA) and two concentrations of LA (3% LA, 1% LA) were used in this study as sanitizer treatments. Sterile water was the negative control and the sanitizer of 3% LVA and 2% SDS was used as positive control. The water or chemicals were removed by aspiration after the 5, 10, 30, or 60-min treatment. After sanitizer treatment, the wells were filled with 2 ml of N/E neutralizing broth (Becton Dickinson) for 10 min before aspiration, and then, 1 ml of PBS was added into each well. The content of each well was harvested by scraping the surface thoroughly with a sterile polyester-tipped swab (15.2 cm, Fisher Scientific, Pittsburgh, PA). The PBS containing released biofilm cells and the swab were added to 9 ml of 0.01 M PBS, pH 7.25, and vigorously agitated by a Vortex (G-560, Scientific Industries, Bohemia, NY) at 250 rpm for 1 min. Cell suspension was serially (1:10) diluted and a volume of 0.1 ml from each diluted tube were spread-plated in duplicate onto BHI plates. The plates were incubated at 37°C for 48 h for bacterial enumeration. The level of inactivation was expressed as the $\log_{10}$ reduction in the cell survival ratio for the chemical treatments ($\log N/N_0$). N refers to the bacterial counts after chemical treatments, whereas N_0 refers to the bacterial counts following water incubation (negative control biofilms).

2.5 Confocal laser scanning microscopy (CLSM)

For CLSM, a 1% diluted exponential phase culture of *L. monocytogenes* ATCC 5779 was added to 8-well chamber slides (Nunc™ Lab-Tek™, Fisher Scientific) at 400 µl per well. The plates were incubated at 37°C for 3 d before exposure to sanitizer treatments. The medium

was changed every 24 h to maintain bacterial viability. Prior to the treatment step, the wells were washed with 800 µl of 0.01 M PBS and air-dried for 10 min, and after treatments they were filled with N/E neutralizing broth to neutralize the residual sanitizer solutions. To visualize cells, fluorescent dyes were used by applying the LIVE/DEAD BacLight™ viability kit (Molecular Probes, Life Technologies, Eugene, OR). The staining mixture was prepared by adding 3 µl of component A and 3 µl of component B per 1 mL of sterile saline. Freshly prepared staining mixture (400 µl) was added to each well and incubated at 21 °C for 15 min in the dark. After incubation, the wells were washed with PBS as described above. The wells were further filled with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (Sigma) and incubated for 30 min at room temperature to fix the specimen. The slides were removed from the chambers and then covered with coverslips with the use of BacLight™ Mounting Oil (Molecular Probes, Life Technologies, Eugene, OR). The samples were analyzed with a Zeiss LSM700 CLSM (Carl Zeiss Microscopy, Thornwood, NY) using Plan-Apochromat 63 oil-immersion, numerical aperture 1.4.

2.6 Statistical analysis

The sensitivity of *L. monocytogenes* ATCC 5779 biofilms to PLA and the other sanitizers was determined by three independent trials with duplicate plating (n = 6). Data were analyzed for analysis of variance (ANOVA) by SPSS software (version 19.0, IBM-SPSS Inc., Armonk, NY) to determine least significant differences ($p < 0.05$) among the treatments. Principal component analysis (PCA) was conducted using the Unscrambler 9.7 software (Camo, USA) to discriminate the influence of different factors: culture temperature, growth time, exposure time and sanitizers on biofilm inactivation.

3. Results

3.1 Bacterial inactivation assay

The results of the inactivation of *L. monocytogenes* ATCC 5779 planktonic bacterial by PLA, LA and the other sanitizers are shown in Table 1. The 3% LVA plus 2% SDS and 1%

PLA treatments inactivated *L. monocytogenes* ATCC 5779 to undetectable levels within 1 min, whereas 3% LA required at least 20 min to inactivate the same cell populations of listeria. PLA of 0.5% and 0.25% inactivated approximately 10^7 CFU/ml of *L. monocytogenes* in 30 min and 60 min, respectively, whereas viable strains were still detected by enrichment culture after treatment with 1% LA for 180 min. Interestingly, both the combined treatment of 1% LA + 0.5% PLA, and 0.5% LA + 0.5% PLA inactivated about 10^7 CFU/ml of *L. monocytogenes* in 20 min, which is more effective than the treatment of 3% LA only. The combined treatment of 1% LA + 0.25% PLA, or 0.5% LA + 0.25% PLA, inactivated approximately 10^7 CFU/ml of *L. monocytogenes* in 30 min, and 60 min, respectively.

3.2 Inactivation of *L. monocytogenes* in Biofilms

The effectiveness of PLA, lactic acid (LA) and the other sanitizers including levulinic acid (LVA) and sodium dodecyl sulfate (SDS) on the inactivation of *L. monocytogenes* cells in biofilms grown to different maturities is revealed in Fig. 1 and Fig. 2, respectively. The results revealed that the listeria in the biofilms could not be completely inactivated by all of the sanitizers even when treated for 60 min.

The effectiveness of the sanitizers on the inactivation of listeria in biofilms grown at 37 °C for 3 d is revealed in Fig. 1A. Significant ($P<0.05$) reductions of 4.06, 3.94 and 3.32 log CFU/ml were observed for 3% PLA, 1% PLA and 3% LVA + 2% SDS treatments, respectively, compared with the other sanitizers, including 3% LA after a 5-min treatment (Fig. 1A). As the treatment time was increased, the effectiveness of 0.5% PLA, 0.25% PLA, 3% LA and 1% LA significantly increased ($P<0.05$) (Fig. 1A). Treatment with 0.5% PLA for 30 and 60 min, reduced listeria populations by 3.99 and 5.10 log, respectively, for which there was no significant difference ($P>0.05$) for treatments of 3% PLA, 1% PLA, 3% LA, and 3% LVA and 2% SDS.

The effectiveness of the sanitizers on the inactivation of listeria cells in biofilms grown at 37°C for 7 d is revealed in Fig. 1B. The initial *Listeria* counts in these biofilms were

approximately 10 log CFU/ml, indicating that these biofilms were more densely populated with listeria than those treated in the studies described above. The trends of the inactivation of listeria populations in these late mature biofilms were similar. 3% PLA reduced 3.40 log CFU ($P<0.05$)/ml in the biofilm after a 5-min treatment, which was a significantly greater reduction of listeria than treatment with 1% PLA, 0.5% PLA, 0.25% PLA, 3% LA, 1% LA, and 3% LVA + 2% SDS which reduced 0.92, 0.33, 0.20, 0.88, 0.28, and 2.04 log CFU/ml bacterial cells, respectively (Fig. 1B). Increasing the treatment time, significantly increased ($P<0.05$) the effectiveness of 1% PLA, or 3% LVA and 2% SDS (Fig. 1B). A 30- or 60-min treatment with 3% PLA, 1% PLA or 3% LVA plus 2% SDS were not significantly different in inactivating listeria in the late mature biofilms, and these treatments were significantly more effective than the other sanitizers, including 3% LA and 0.5% PLA (Fig. 1B).

Inactivation trends of listeria in biofilms grown at 15°C for 4 d and 7d are revealed in Fig. 2 and are similar to the results shown in Fig. 1. 3% PLA, 1% PLA, and 3% LVA + 2% SDS were significantly ($P<0.05$) more effective with reductions of 4.51, 4.07 and 4.19 log CFU/ml, respectively, after 5 min treatment (Fig. 2A), compared to 0.5% PLA, 0.25% PLA, 3% LA and 1% LA which reduced the listeria populations by 1.39, 0.24, 2.07 and 0.36 log CFU/ml, respectively (Fig. 2A). The effectiveness of PLA, LA and the other sanitizer combinations on the inactivation of listeria in biofilms grown at 15°C for 7 d is revealed in Fig. 2B. 3% PLA reduced 3.51 log CFU of listeria/ml after a 5-min treatment, which was significantly ($P<0.05$) more effective than the other sanitizers of 1% PLA, 0.5% PLA, 0.25% PLA, 3% LA, 1% LA, and 3% LVA plus 2% SDS which reduced 1.09, 0.48, 0.35, 0.94, 0.36, and 2.48 log CFU of listeria/ml, respectively (Fig. 2B).

The effectiveness of the combined treatment of 1% LA + 0.5% PLA, and 1% LA + 0.25% PLA on the inactivation of listeria biofilms was also studied. 1% LA + 0.5% PLA had the same effectiveness on inactivating listeria in biofilms as that of 3% LA for all the treatment exposure times (Fig. 1 and Fig. 2). The effectiveness of the sanitizers of 1% LA + 0.5% PLA,

and 1% LA + 0.25% PLA significantly increased with increased treatment times (Fig. 1 and Fig. 2).

3.3 Biofilm formation characteristics as determined by CLSM analysis

CLSM images revealed that *L. monocytogenes* was able to form three-dimensional structures during the incubation time that was used. The *L. monocytogenes* biofilm formed on the chamber was uneven. The biofilm located near the center of the chamber was thinner, with an average height of ca. 2 μm after 3 d at 37°C than that located along the walls of the chamber that had an average height of ca. 6 μm . The biofilm formed at the corners of the chamber was the thickest, at up to 8~10 μm .

Disintegration of the bacterial cell membrane of the listeria in the biofilm of ca. 6 μm following treatment with the sanitizers for 10 min and 60 min was revealed in Fig. 3. The red brightness of the CLSM images was enhanced when the treatment concentrations of PLA and treatment time were increased, revealing that more bacterial cells in the biofilm were damaged, which was in concordance with the bacterial counts of viable cells in the biofilms after being treated with PLA. Although the layers of the biofilm treated with 1% PLA, 3% PLA and 3% LVA and 2% SDS for 60 min appeared almost entirely red, several locations within the biofilm were green indicating that some bacterial cells were still intact and viable.

Discussion

Many studies revealed that there are different stages in biofilm development (de la Fuente-Núñez, Reffuveille, Fernández, & Hancock, 2013; Monds & O'Toole, 2009; Thuptimdang P, Limpiyakorn, McEvoy, Prüß, & Khan, 2015). There are at least four stages in biofilm formation: planktonic, attachment, maturation, and dispersion. According to the features of biofilm development, mature biofilms are significantly more resistant to sanitizers and they can disperse planktonic bacterial cells to the environment. Hence, four different *L. monocytogenes* biofilms of different maturities that were grown at different temperatures and times were used to evaluate the effectiveness of the sanitizers. A principal component analysis

(PCA) was to determine if the maturity of the biofilm and sanitizer exposure time influenced biofilm inactivation of listeria (Fig. 4A). The PCA diagram of three clusters revealed that culture time and sanitizer exposure time can affect biofilm inactivation (Fig. 4A). Samples for Cluster 3 were all treated with the sanitizer for up to 30 and 60 min, regardless of the maturity of the biofilm. These results revealed that given enough exposure time the chemicals could penetrate the exopolysaccharide (EPS) barrier to inactivate the bacterial cells in the biofilm. Samples for Cluster 1 were of biofilms grown at 37°C for 3 d and 15°C for 4 d that were treated with sanitizers for 5 min, and samples for Cluster 2 were of biofilms grown at 37°C and 15°C for 7 d that were treated with sanitizers for 5 min and 10 min, respectively. These results revealed that the bacterial cells in these biofilms were more difficult to inactivate as biofilm maturity increased. The increased resistance of biofilms with increased maturity may be due to the thickness of the matrix of glycoproteins, EPS and other compounds (glycocalyx) surrounding biofilm cells which can act to protect cells physically from surface-active agents (Uhlich, Cooke, & Solomon, 2006). Similar conclusions were reported in previous studies. Lee & Frank (1991) reported that listeria cells in biofilms grown on stainless steel surfaces for 8 days were twice as resistant to hypochlorite than the cells grown for 4 days and 100 times more resistant as cells that were grown for only 4 h. Yang, Kendall, Medeiros, & Sofos (2009) determined that the nine sanitizers they evaluated were more effective for reducing bacterial cells in 7-day-old biofilms grown on smooth surface high-density polyethylene coupons than the cells in 21-day-old biofilms. Beauchamp et al. (2012) determined that *E. coli* O157:H7 cells in biofilms became less sensitive to most sanitizer treatments as biofilm age increased.

Levulinic acid (LVA) with sodium dodecyl sulfate (SDS) has been reported previously to be an effective sanitizer for inactivating foodborne pathogens (Magnone, Marek, Sulakvelidze, & Senecal, 2013; Zhao, Zhao, & Cannon, Doyle, 2011; Zhao, Zhao, & Doyle, 2009). Chen, Zhao, & Doyle (2015) reported that different concentrations of LVA plus SDS (0.5% LVA + 0.05% SDS, 1% LVA + 0.1% SDS, and 3% LVA + 2% SDS) were all more effective against

the three pathogens in biofilms grown on stainless steel compared to the other commonly used sanitizers, including a commercial quaternary ammonium-based sanitizer (150 ppm), lactic acid (3%), sodium hypochlorite (100 ppm), and hydrogen peroxide (2%). Hence, 3 % LVA and 2% SDS was used in this study as the positive control to compare against the effectiveness of PLA. The effectiveness of the sanitizers to biofilms of different maturities was comprehensively analyzed for different treatment times by PCA methods (Fig. 5B, 5C, 5D). Fig. 4B classified the sanitizers into three different clusters based on the bacterial reduction to the samples of early mature biofilm (3-d old biofilm at 37 °C and 4-d old biofilm at 15 °C) treated for 5 min and 10 min. The PCA diagram was segregated into three clusters. Cluster 3 included the sanitizers, i.e., 3% PLA, 1% PLA, and 3% LVA and 2% SDS, indicating that these three sanitizers were similar in their effectiveness to inactivate the biofilms of early maturity with a short sanitizer exposure time. Cluster 2 included the sanitizers 3% LA, 0.5% PLA, 1% LA and 0.5% PLA, and 1% LA, and 0.25% PLA. Based on listeria reduction in late mature biofilms (7-d old biofilm at 37°C and 15°C) treated for 5 min and 10 min, the sanitizers were distributed into two different clusters and two different points (Fig. 4C). Results revealed that the effectiveness of the sanitizers to eliminate listeria in late mature biofilms occurred in the following order: 3% PLA, 3% LVA and 2% SDS, and then the sanitizers discussed in Clusters 1 and 2. The sanitizers were distributed in Fig. 4D into two different clusters based on the listeria reduction in all the biofilm samples treated for 30 min and 60 min. Cluster 1 included the sanitizers of 3% PLA, 3% LVA+2% SDS, 1% PLA, 3% LA, 0.5% PLA, 1% LA+0.5% PLA, and 1% LA+0.25% PLA, indicating that all of the above sanitizers had similar efficacy in reducing listeria with long exposure times. The sanitizers in low concentrations can gradually inactivate listeria as the exposure time was increased. de Oliveiraa, Brugneraa, Cardosob, Alvesc, & Piccolia (2010) reported that dead cells inactivated by the sanitizers can protect other *Listeria* cells in the biofilm, and this may help explain why the effectiveness of high concentrations of sanitizers did not increase with

increased exposure times. Based on the above results, we can conclude that PLA was most effective in reducing listeria on biofilms compared with the other sanitizers evaluated, and may be a useful sanitizer in food processing facilities. A combination of 1% LA plus 0.5% PLA provides the same degree of effectiveness of 3% LA, which may enable the use of lower amounts of PLA in sanitizer preparations.

Biofilm development of listeria is influenced by the material characteristics of the matrix to which the listeria attached as well as surface roughness of this material. (Teughels, Van Assche, Sliepen, & Quirynen, 2006). Chen, Zhao, & Doyle (2015) reported that exposure to 3% LVA plus 2% SDS for 10 min can reduce listeria populations by ca. 8 log CFU/ml. In this study, 3% LVA + 2% SDS for 10 min only reduced listeria populations by ca. 4 log reduction. The difference for this log reduction may be attributed to the different materials on which the biofilms were grown. Since stainless steel that was used only had a flat surface structure, the biofilms that were formed along the walls of the 24-well flat-bottom polystyrene plates were thicker than those grown on the stainless steel, hence it was more difficult for the chemicals to penetrate the EPS barrier to inactivate the listeria cells in the biofilm. Gomes, Moreira, Simões, Melo, & Mergulhão (2014) reported that biofilms were mainly formed on the walls and not on the bottom of the wells in 96-well microtiter plates. In our study, it was also determined by CLSM analysis that the biofilms grown along the corners of each well was much thicker than those grown on the bottom. This may help explain why the biofilms existing in the crevices of equipment in food processing facilities are more difficult to eliminate than those grown on equipment surfaces. These results suggest that equipment design could be improved by eliminating sharp angle connections where biofilms are more likely to form.

In conclusion, PLA was effective in inactivating both *L. monocytogenes* planktonic cells and listeria cells in biofilms, and 3% PLA was more effective than 3% LA, or 3% LVA plus 2% SDS in reducing listeria populations in more resistant biofilms of older maturity.

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1

TABLE 1. Inactivation of *L. monocytogenes* ATCC 5779 planktonic cells by different sanitizers at 21±2 °C

[illegible]

2

^a-, not detectable by direct plating and an enrichment procedure.

3

^b<1.7, detectable by an enrichment procedure but not by direct plating.

4

PLA = 3-Phenyllactic acid, LA = lactic acid, LVA = levulinic acid, SDS = sodium dodecyl sulfate.

1 **Fig. 1.** Inactivation of *L. monocytogenes* ATCC 5779 in biofilms grown at 37°C for 3 d (A) and 7 d (B) after treatment with different
 2 concentrations of PLA for 5, 10, 30, and 60 min. The bars represent mean values $\pm$ standard deviations (n=6). A different capital letter at each
 3 point indicates a significant difference between different treatments for the same treatment time ($P<0.05$). A different lower case letter at each
 4 point indicates a significant difference between the same treatment for a different treatment time ($P<0.05$). PLA = 3-Phenyllactic acid, LA =
 5 lactic acid, LVA = levulinic acid, SDS = sodium dodecyl sulfate.

6 **Fig. 2.** Inactivation of *L. monocytogenes* ATCC 5779 in biofilms grown at 15°C for 4 d (A) and 7 d (B) after treatment with different
 7 concentrations of PLA and other sanitizers for 5, 10, 30, and 60 min. The bars represent mean values $\pm$ standard deviations (n=6). A different
 8 capital letter at each point indicates a significant difference between different treatments for the same treatment time ($P<0.05$). A different lower
 9 case letter at each point indicates a significant difference between the same treatment for a different treatment time ($P<0.05$). PLA =
 10 3-Phenyllactic acid, LA = lactic acid, LVA = levulinic acid, SDS = sodium dodecyl sulfate.

11 **Fig. 3.** Representative CLSM images of *L. monocytogenes* ATCC 5779 biofilms. The biofilms were grown in BHI at 37°C for 3 days (A-1 and
 12 A-2), and treated for 5 min with 0.5% PLA (B-1), 1% PLA (C-1), 3% PLA (D-1), 3%LVA+2%SDS (E-1), and 60 min with 0.5% PLA (B-2), 1%
 13 PLA (C-2), 3 %PLA (D-2), 3%LVA+2%SDS (E-2). The scale bar is 10 μ m. PLA = 3-Phenyllactic acid, LA = lactic acid, LVA = levulinic acid,
 14 SDS = sodium dodecyl sulfate.

15 **Fig. 4.** Principal component analysis (PCA) diagrams revealing the discrimination of mean score factors for listeria inactivation in biofilms

16 which evaluates the influence of growth temperatures (37 °C/15 °C), growth time (3 d/4 d/7 d) and sanitizer exposure times (5/10/30/60 min) (A),
17 and the influence of different sanitizers on (B), early mature biofilms treated for 5 min and 10 min; (C), late mature biofilms treated for 5 min
18 and 10 min; (D), all biofilm samples treated for 30 min and 60 min;) on *L. monocytogenes* ATCC 5779 biofilms. PLA = 3-Phenyllactic acid, LA
19 = lactic acid, LVA = levulinic acid, SDS = sodium dodecyl sulfate.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.