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Antibiofilm effect of plant derived antimicrobials on *Listeria monocytogenes*

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ABSTRACT

The present study investigated the efficacy of sub-inhibitory concentrations (SICs, concentrations not inhibiting bacterial growth) and bactericidal concentrations (MBCs) of four, generally recognized as safe (GRAS), plant-derived antimicrobials (PDAs) in inhibiting *Listeria monocytogenes* (LM) biofilm formation and inactivating mature LM biofilms, at 37, 25 and 4 °C on polystyrene plates and stainless-steel coupons. In addition, the effect of SICs of PDAs on the expression of LM genes critical for biofilm synthesis was determined by real-time quantitative PCR. The PDAs and their SICs used for inhibition of biofilm were *trans*-cinnamaldehyde (TC 0.50, 0.75 mM), carvacrol (CR 0.50, 0.65 mM), thymol (TY 0.33, 0.50 mM), and eugenol (EG 1.8, 2.5 mM), whereas the PDA concentrations used for inactivating mature biofilms were 5.0 and 10.0 mM (TC, CR), 3.3 and 5.0 mM (TY), 18.5 and 25.0 mM (EG). All PDAs inhibited biofilm synthesis and inactivated fully formed LM biofilms on both matrices at three temperatures tested ($P < 0.05$). Real-time quantitative PCR data revealed that all PDAs down-regulated critical LM biofilm-associated genes ($P < 0.05$). Results suggest that TC, CR, TY, and EG could potentially be used to control LM biofilms in food processing environments, although further studies under commercial settings are necessary.

1. Introduction

Listeria monocytogenes (LM) is a major foodborne pathogen responsible for ~19% of all deaths resulting from foodborne illnesses in the United States (Scallan et al., 2011). Due to its ubiquitous distribution in the environment, coupled with the ability to tolerate a variety of harsh conditions such as desiccation, low temperature, low pH and high osmolarity, LM is a frequent contaminant of food processing and packaging environment (Chaturongakul et al., 2008; Donnelly, 2001; Farber and Peterkin, 1991; Jay, 2000; Seeliger and Jones, 1986). In food processing facilities, LM is able to attach and form biofilms on a variety of food processing equipment surfaces, including polystyrene, stainless steel, polymers, plastic, teflon and rubber (Blackman and Frank, 1996; Borucki et al., 2003; Chavant et al., 2002; Lunden et al., 2002; Meyer, 2003; Moretro and Langsrud, 2004). Once LM establishes biofilm, it can survive in food processing environments for extended periods of time (Fatemi and Frank, 1999; Frank and Koffi, 1990). LM cells in biofilms are surrounded by a self-generated matrix of hydrated extracellular polymeric substances (EPS) that constitute their immediate environment. The EPS accounts for a majority of the dry mass of the biofilm, and mainly consist of polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender, 2010). Because of the presence of EPS, cells in biofilms are more resistant to desiccation and disinfectants than planktonic cells (Chmielewski and Frank, 2003; Flemming and Wingender, 2010; Keskinen et al., 2008).

The persistence of LM in food processing facilities constitutes a significant food safety hazard, since biofilms protect the underlying bacteria from action of antimicrobials and sanitizers (Borucki et al., 2003; Folsom and Frank, 2007), and serve as a continuous source for contamination of food products. There are several reports of listeriosis outbreaks due to consumption of food products contaminated with persistent LM strains present on food processing surfaces (Gottlieb et al., 2006; Kathariou, 2002; Krysinski et al., 1992; Kumar and Anand, 1998; Pan et al., 2006; Tompkin, 2002). Therefore, controlling LM biofilms in food processing facilities is critical for improving food safety and reducing listeriosis in humans.

Most bacterial biofilms develop in a five-step process comprising of initial attachment, irreversible attachment, microcolony formation, biofilm maturation, and cell dispersion (Davey and O'Toole, 2000; Mclandsborough et al., 2006; Stoodley et al., 2002). Flagella-mediated initial cell attachment has been

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reported to be crucial for biofilm formation in LM (Lemon et al., 2007; O'Neil and Marquis, 2006; Todhanakasem and Young, 2008; Vatanyoopaisarn et al., 2000). The major genes involved in LM attachment include those associated with flagellar synthesis and motility (*flaA*, *fliP*, *fliG*, *flgE*, *motA*, *motB*, and *mogR*) (Chang et al., 2012; Grundling et al., 2004). Once the bacterial cells are attached, further maturation of biofilm occurs by means of complex cellular mechanisms involving quorum sensing. Two component systems have been found to be involved in the regulation of quorum sensing and biofilm formation in bacteria (Gueriri et al., 2008). A pleiotropic response regulator, DegU has been shown to coordinate biofilm formation in *Bacillus subtilis* and LM (Kobayashi, 2007; Murray et al., 2009). Similarly, the Agr-dependent quorum sensing system also plays a critical role in LM biofilm development (Rieu et al., 2007). Biofilm formation was significantly decreased in an *agrD* mutant of LM (Riedel et al., 2009). In addition, DnaK, a class I heat-shock response protein involved in stress resistance in LM, has been shown to contribute to biofilm formation and tolerance to disinfectants (Van der Veen and Abee, 2010). Moreover, the key transcriptional activator of virulence genes, PrfA, plays a major role in biofilm formation in LM, and mutants lacking *prfA* were found to be defective in biofilm synthesis (Lemon et al., 2010).

Routine plant hygiene and sanitation are critical for controlling the persistence of LM in food processing facilities (Tompkin et al., 1999). Although a variety of FDA-approved disinfectants, including quaternary ammonium compounds and hypochlorite, have been evaluated for plant sanitation (Krysinski et al., 1992), they were found to be not very effective in controlling LM biofilms, especially in the presence of organic matter and low temperatures (Pan et al., 2006; Heir et al., 2004; Holah et al., 2002; Romanova et al., 2002). Moreover, the presence of chemical residues and potential formation of organochlorine compounds from chlorine is of concern due to associated health risks (Donato and Zani, 2010; Parish et al., 2003). Thus, there is an increasing interest in identifying safe and effective antimicrobials for controlling LM biofilms in food processing plants.

Since ancient times, herbs and spices have been used to preserve foods as well as enhance food flavor. The antimicrobial activity of several plant-derived compounds has been previously demonstrated (Burt, 2004; Holley and Patel, 2005), and various active components of these oils have been identified. *Trans*-cinnamaldehyde (TC) is an aldehyde present in extract of cinnamon barks (*Cinnamomum zeylandicum*). Carvacrol (CR) and thymol (TY) are antimicrobial compounds in oregano oil obtained from *Origanum glandulosum*. Similarly, eugenol (EG) is an active ingredient in the oil of clove (*Eugenia caryophyllus*). All the aforementioned plant derived antimicrobials (PDAs) are classified as generally recognized as safe by the U.S. Food and Drug Administration (Adams et al., 2004, 2005; Kabara, 1991; Knowles et al., 2005).

This study was undertaken to investigate the efficacy of TC, CR, TY and EG in inhibiting biofilm formation and inactivating mature biofilms of LM at 37, 25, and 4 °C on two different matrices, namely polystyrene and stainless steel. Moreover, the effect of PDAs on exopolysaccharide synthesis and biofilm architecture was investigated. In addition, the effect of PDAs on LM genes critical for biofilm formation was determined. For determining ability of PDAs in preventing biofilm synthesis from planktonic cells, reducing exopolysaccharide production, and down-regulating biofilm-associated genes, sub-inhibitory concentrations (SICs, concentrations below MIC that do not inhibit bacterial growth) of TC, CR, TY, and EG were used, whereas the inactivation of preformed biofilms of LM was investigated using minimum bactericidal concentration (MBC) and a concentration above the MBC of the PDAs.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All bacteriological media were purchased from Difco (Becton Dickinson, Sparks, MD). Three strains of LM, including ATCC 19115, Scott A and Presque-598 were used in this study. In order to study the growth pattern of LM, each strain was cultured separately in 10 ml of sterile tryptic soy broth containing 0.6% yeast extract (TSBYE) in 30 ml screw-cap tubes and incubated at 37 °C for 18 h. Following incubation, the cultures were sedimented by centrifugation (3600 × *g* for 15 min) at 4 °C. The pellet was washed twice, resuspended in 10 ml of sterile phosphate buffered saline (PBS, pH 7.0) and serial ten-fold dilutions were cultured on duplicate tryptic soy agar (TSA) and oxford agar plates, followed by incubation at 37 °C for 24 h.

2.2. Plant compounds and determination of SIC and MBC

The SIC and MBC of each PDA were determined as previously described (Johny et al., 2010). Sterile 24-well polystyrene tissue culture plates (Costar, Corning Incorporated, Corning, NY) containing TSBYE (1 ml/well) were inoculated separately with ~6.0 log CFU of LM, followed by the addition of 1–10 μl of TC, CR, TY or EG (Sigma–Aldrich) with an increment of 0.5 μl. The plates were incubated at 37 °C for 24 h, and bacterial growth was determined by culturing on duplicate TSA and oxford agar plates. The two highest concentrations of each PDA below its MIC that did not inhibit bacterial growth after 24 h of incubation as compared to control samples were selected as its SICs for this study, whereas the lowest concentration of PDAs that reduced LM population in TSBYE by ~5.0 log CFU/ml after incubation at 37 °C for 24 h was taken as the MBC. Duplicate samples were included and the experiment was repeated three times.

2.3. Biofilm inhibition and inactivation assay on polystyrene microtiter plates

The effect of TC, CR, TY and EG in inhibiting LM biofilm formation on microtiter plates was determined according to a previously published method (Ayebah et al., 2006). LM strains, grown separately in TSBYE at 37 °C for 24 h were centrifuged (3600 × *g*, 15 min, 4 °C), washed in PBS, appropriately diluted and resuspended in TSBYE. Two hundred microliters of the washed culture (~6.0 log CFU) were used to inoculated sterile 96-well polystyrene tissue culture plates (Costar), followed by the addition of SICs of TC, CR, TY or EG. The biofilms of *L. monocytogenes* were developed at three temperatures (37, 25 and 4 °C). For developing biofilms at 37 and 25 °C, the tissue culture plates were incubated for a period of 96 h, whereas for developing biofilms at 4 °C the plates were incubated for 7 days. The LM population associated with the biofilm was enumerated at 24 h interval. The wells were gently rinsed with PBS three times and the number of planktonic population recovered in the PBS was enumerated. Subsequently, the wells were scraped with sterile pipet tip for 5 min/well and the detached cells from the biofilm were plated on duplicate TSA and Oxford agar plates (Amalaradjou et al., 2010; Ayebah et al., 2006). The plates were incubated at 37 °C for 48 h before counting bacterial colonies. The inactivation of LM mature biofilms by TC, CR, TY, and EG was determined by a microtiter assay, as described by Djordjevic et al. (2002). Sterile 96-well polystyrene tissue culture plates were inoculated with 200 μl of the bacterial inoculum (~6.0 log CFU) and incubated at 37, or 25 °C for 96 h, or at 4 °C for 7 days. After biofilm formation, the effect of respective MBC and a concentration greater than the MBC of TC, CR, TY, and EG were tested with a