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Effect of essential oils of *Syzygium aromaticum* and *Cinnamomum zeylanicum* and their major components on biofilm production in *Staphylococcus aureus* strains isolated from milk of cows with mastitis

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ABSTRACT

Bovine mastitis is an inflammation of the mammary glands of cows and causes significant economic losses in dairy cattle. *Staphylococcus aureus* is one of the microorganisms most commonly isolated. Novel agents are required in agricultural industries to prevent the development of mastitis. The production of biofilm by *Staph. aureus* facilitates the adhesion of bacteria to solid surfaces and contributes to the transmission and maintenance of these bacteria. The effect of the essential oils of *Syzygium aromaticum* (clove; EOSA) and *Cinnamomum zeylanicum* (cinnamon; EOCZ) and their major components, eugenol and cinnamaldehyde, on *Staph. aureus* biofilm formation on different surfaces was investigated. The results showed a significant inhibition of biofilm production by EOSA on polystyrene and stainless steel surfaces (69.4 and 63.6%, respectively). However, its major component, eugenol, was less effective on polystyrene and stainless steel (52.8 and 19.6%, respectively). Both EOCZ and its major component, cinnamaldehyde, significantly reduced biofilm formation on polystyrene (74.7 and 69.6%, respectively) and on stainless steel surfaces (45.3 and 44.9%, respectively). These findings suggest that EOSA, EOCZ, and cinnamaldehyde may be considered for applications such as sanitization in the food industry.
Key words: mastitis, antibiofilm *Staphylococcus aureus*, *Syzygium aromaticum*, *Cinnamomum zeylanicum*

INTRODUCTION

Bovine mastitis is an inflammation of the mammary glands in dairy cattle, usually caused by bacteria. It leads to significant economic losses due to reduced milk production, increased use of drugs and animal morbidity and mortality (Melchior et al. 2006). *Staphylococcus aureus* is one of the most important causative agents of clinical, subclinical, or chronic mastitis (Vasudevan et al., 2003).

Staphylococcus aureus can produce biofilms, complex polysaccharide- or protein-bound bacterial structures that facilitate adhesion and multiplication of bacteria on environmental surfaces and on animal tissues. Bacteria in biofilms are resistant to phagocytosis, antimicrobial agents, and disinfectants due to the low diffusion through the matrix and altered cellular metabolism (Donlan and Costerton, 2002). These protective features of *Staph. aureus* biofilms promote colonization of the mammary epithelium which precedes the establishment of a persistent infection (Lasa and Penadés, 2006).

Staphylococcus aureus polysaccharide production is mediated by the *ica* cluster (intercellular adhesin), which contains *icaA*, *icaB*, *icaC*, and *icaD* (McKenney et al. 1998). Co-expression of *icaA* and *icaD* results in phenotypic expression of capsular polysaccharide (Arciola et al. 2001). Another important gene involved in biofilm production in *Staph. aureus* is *bap*, which encodes biofilm-associated protein. Biofilm-associated protein promotes primary binding to surfaces and intercellular adhesion (Lasa and Penades, 2006), but its prevalence is reported to be relatively low (Seo et al., 2008).

Due to the high prevalence of biofilm production among *Staph. aureus* in mastitis, increased investment in industrial equipment disinfection programs that target biofilms has been noted (Gibson et al., 1999). Essential oils (**EO**) have antimicrobial and antibiofilm activity against bacteria, parasites (Alexopoulos et al., 2011), fungi (Mari et al., 2003), and viruses (Bishop, 1995). More recently, EO of aromatic and medicinal

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plants have been tested for their activity against biofilms (Kwiecinski et. al. 2009). The EO target different cellular mechanisms, such as inhibition of peptidoglycan synthesis (Ogunlana et al., 1987), modification of bacterial membrane hydrophobicity (Cox et al., 2000), and modulation of quorum sensing (Gao et al., 2003). Sanitizers based on natural products, such as EO with specific *Staph. aureus* antibiofilm activity, may have applications in the beef-processing industry for enhanced surface or carcass cleaning. The aim of our study was to evaluate the effect of the essential oil of clove (*Syzygium aromaticum*; **EOSA**), cinnamon (*Cinnamomum zeylanicum*; **EOCZ**), and their major compounds, eugenol and cinnamaldehyde, on biofilm formation on stainless steel and polystyrene by isolates of *Staph. aureus* recovered from the milk of cows with subclinical mastitis.

MATERIALS AND METHODS

Samples and Bacterial Isolation

A collection of 64 isolates of *Staph. aureus*, previously recovered from the milk of cows with subclinical mastitis, was tested. Presumptive identification was by Gram stain and further confirmation was made based on catalase, coagulase, and DNase production, as described by Koneman et al. (2008). Molecular confirmation was by PCR amplification of the species-specific staphylococcal nuclease gene (*nuc*) using primers and PCR conditions as outlined in Table 1.

PCR to Detect Genes Linked to Biofilm Production

The DNA extraction was performed using the Minispin kit (GE Healthcare, Little Chafont, Buckinghamshire, UK) according to the manufacturer's instructions. The PCR reactions were performed with each primer pair in a final volume of 25 μL containing 2.5 μL of 10× PCR buffer, 2.0 m*M* magnesium chloride, 200 m*M* deoxynucleotides, 1 U of Taq DNA polymerase (Fermentas, St. Leon Rot, Germany), 10 pmol of each primer, and 3 μL of the DNA template. The primers used for detection of *icaA*, *icaD*, and *bap* and their properties are listed in Table 1. The PCR conditions were those described in the references provided (Table 1) with reactions performed in a Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA). The PCR products were detected using 1.5% agarose gel in Tris-boric acid-EDTA buffer and developed with Sybr Green (Invitrogen, Grand Island, NY). Positive and negative controls for *ica*A and *icaD* were *Staph. aureus* ATCC 35983 and *Staphylococcus epidermidis* ATCC 12.228. For *bap*, a positive and sequenced strain was used.

Extraction of EOSA and EOCZ and Chemical Analysis by GC-MS

The EOSA and EOCZ were extracted from *Syzygium aromaticum* and *Cinnamomum zeylanicum* by drag steam distiller (model MA480, Marconi, Piracicaba, Brazil). Densities were calculated according to Fonseca and Librand (2008). The major compounds of the EOSA and EOCZ, eugenol and cinnamaldehyde, were sourced commercially (Sigma-Aldrich, St. Louis. MO).

Chemical characterization was determined by gas chromatography coupled to GC-MS (model QP5050A, Shimadzu, Kyoto, Japan) with the use of a CBP-5 capillary column with a 0.25 mm of internal diameter and 0.25 μm film thickness. The chromatographic conditions were set according to the EO analyzed. Both EOSA and EOCZ were identified by matching their mass spectra to reference compounds in the National Institute of Standards and Technology mass spectra library (Gaithersburg, MD).

Determination of the Minimum Inhibitory Concentration

Susceptibility tests were performed in triplicate for EOSA, EOCZ, and their major components using the broth microdilution method and CLSI (2009) guidelines. Briefly, inocula of *Staph. aureus* were prepared to the density of 0.5 McFarland using a densitometer (Densichek, BioMérieux, Durham, NC) in 0.85% saline solution. The inocula were further diluted to an approximate concentration of 10^5 cfu/mL and incubated with test compounds at concentrations of 0.025, 0.04, 0.06, 0.08, 0.10, 0.20, 0.40, and 0.80%, in final volumes of 200 μL of brain-heart infusion broth (Oxoid, Basingstoke, UK) supplemented with 0.5% Tween 80. Positive growth controls and sterility controls were included.

Plates were incubated at 35°C for 24 h, after which 50 μL of 0.01% resazurin was added to each well. The MIC was recorded as the lowest concentration of EO or EO components at which no growth was observed, as indicated by a change of color from blue to pink (Coban, 2012).

Production of Biofilm by Staph. aureus in the Presence and Absence of the EO and Their Major Compounds

Isolates were cultured in tryptone soy broth (Oxoid), at 37°C for 24 h, and diluted to approximately 10^8 cfu/mL. Eugenol and cinnamaldehyde was added to 200 μL of the tryptone soy broth dilution separately to a final concentration of 0.106 mg/mL (subinhibitory concen-