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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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Running title: Effect of natural products on biofilm production

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

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Interpretative Summary

The production of biofilm by *S. aureus* is a problem in industry because this could facilitate the adhesion of bacteria to solid surfaces and contributes to the transmission of these bacteria during the food production. This work aims to investigate the effect of the essential oils of *Syzygium aromaticum* (clove) (EOSA) and *Cinnamomum zeylanicum* (cinnamon) (EOCZ) and their major components, eugenol and cinnamaldehyde, on *S. aureus* biofilm formation on different surfaces. The activity of this substances on stainless steel and polystyrene indicates their potential for an alternative sanitizing spray for surface cleaning in the food processing industry.

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 *S. 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 *S. 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%, $p>0.05$). 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 at 0.106 $mg.mL^{-1}$ may be considered for applications such as sanitizers in the food industry.

Keywords: mastitis, antibiofilm *Staphylococcus aureus*, *Syzygium aromaticum* and *Cinnamomum zeylanicum*.

Chemical compounds studied in this article:

Eugenol (PubChem CID: 3314) and Cinnamaldehyde (PubChem CID 637511).

1. 1. 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).

S. 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 *S. aureus* biofilms promote colonization of the mammary epithelium which precedes the establishment of a persistent infection. (Penadés and Lasa, 2006).

S. aureus polysaccharide production is mediated by the *ica* cluster (intercellular adhesin) which contains *icaA*, *ica*B, *ica*C 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 *S. aureus* is *bap* which encodes biofilm associated protein (Bap). Bap promotes primary binding to surfaces and intercellular adhesion (Lasa and Penadés, 2006), but its prevalence is reported to be relatively low (Seo et al, 2008).

Due to the high prevalence of biofilm production among *S. aureus* in mastitis there has been increased investment in industrial equipment disinfection programs that target biofilms (Gibson et al., 1999). Essential oils have antimicrobial and anti-biofilm activity against bacteria, parasites (Alexopoulos et al., 2011), fungi (Mari et al., 2003) and viruses (Bishop, 1995). More recently, the EOs of aromatic spices and medicinal plants have been tested for their activity against biofilms (Kwiecinski et. al. 2009). The EOs 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 *S. aureus* anti-biofilm activity may have applications in the beef-processing industry for enhanced surface or carcass cleaning.

The aim of this 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 *S. aureus* recovered from the milk of cows with subclinical mastitis.

2. 2. Material and methods

2.1 Samples and bacterial isolation

A collection of 64 isolates of *S. 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 polymerase chain reaction (PCR) amplification of the species-specific staphylococcal nuclease gene (*nuc*) using primers and PCR conditions as outlined in Table 1.

2.2 PCR to detect genes linked to biofilm production

DNA extraction was performed using the Minispin kit (GE Healthcare) according to the manufacturer's instructions. PCR reactions were performed with each primer pair in a final volume of 25 µl containing 2.5 µl of 10X PCR buffer, 2.0 mM magnesium chloride, 200 mM dNTPs, 1U Taq DNA polymerase (Fermentas), 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 Biosystem). PCR products were detected using 1.5% agarose gel in Tris - boric acid - EDTA (TBE) buffer and developed with Sybr Green (Invitrogen ®). Positive and negative controls for *ica*A and *icaD* were *S. aureus* ATCC 35983 and *S. epidermidis* ATCC 12.228. For *bap,* a positive and sequenced strain was used.

2.3. Extraction of EOSA and EOCZ and chemical analysis by gas chromatography-mass spectrometry (GC-MS)

The EOSA and EOCZ were extracted from *Syzygium aromaticum* and *Cinnamomum zeylanicume* by drag steam distiller (model MA480 - Marconi). 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, UK®).

Chemical characterization was determined by gas chromatography coupled to mass spectrometer (GC-MS) (model QP5050A - Shimazu) 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 essential oil analysed. EOSA and EOCZ were identified by matching their mass spectra to reference compounds in the National Institute of Standards and Technology (NIST), mass spectra library (Maryland, USA).

2.4. Determination of the MIC

Susceptibility tests were performed in triplicate for EOSA, EOCZ and their major components using the broth microdilution method and CLSI guidelines (CLSI, 2009). Briefly, inocula of *S. aureus* were prepared to the density of 0.5 McFarland using a densitometer (Densichek, BioMérieux) 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%, 0.80%, in final volumes of 200µl of Brain Heart Infusion broth (BHI, Oxoid) supplemented with 0.5% Tween 80. Positive growth controls and sterility controls were included.

Plates were incubated at 35°C for 24h, after which time, 50µl of 0.01% resazurin was added to each well. The MIC was recorded as the lowest concentration of EO/EO components at which no growth was observed, as indicated by a change of colour from blue to pink.

2.5 Production of biofilm by *S. aureus* in the presence and absence of the EOs and their major compounds

Isolates were cultured in tryptone soy broth (TSB, Oxoid), at 37°C for 24h, and diluted to approximately 10^8 CFU/ml. In 200 µl of this dilution was added EO`s, eugenol and cinnamaldehyde separately to a final concentration of 0.106 $mg.mL^{-1}$ (sub-inhibitory concentration based on, MIC determination). The experiment was carried out in triplicate using 96 well microtiter plate. Control assays were prepared similarly but EO and major components were replaced with sterile TSB. Plates were incubated at 35ºC for 48h in a static incubator, washed three times with phosphate buffered saline (PBS) pH 7.4, dried at room temperature (RT) and stained with 1% gentian violet. After a further wash with distilled water, absorbance at 570nm was measured using an ELISA plate reader (Babsystems, Multiskan EX). *S. aureus* ATCC 35983 was used as positive control (biofilm producer) and *S. epidermidis* ATCC 12228, as a negative control for biofilm production (Vasudevan et al., 2003).

2.5.2 Effect of EOs and major components on biofilm formation on stainless steel

Sterile stainless steel coupons with a diameter of 1 cm were deposited at the bottom of a 24 well plate and *S. aureus* overnight cultures were diluted to 10^8

CFU/ml and 300µL aliquots were added to triplicate wells containing the coupons and incubated at 35°C for 48h, in the absence and presence of each EO and their major components (0.106 mg.mL^{-1}). The coupons were transferred to a new plate, washed three times with PBS, pH 7.4 and stained with 1% crystal violet for 15 min and washed a further three times. The biofilm was resuspended in 300µL of glacial acetic acid for 15 min and 200µl was transferred to a microplate and the absorbance measured at 570 nm.

2.6. Statistical Analysis

To compare the effect on biofilm production in the absence and presence of EOs and major components, analysis of variance was conducted in a randomized design followed by Tukey test means. *P* value <0.05 was considered significant.

3. Results

3.1. Genotypic analysis for the presence of *icaA*, *icaD* and *bap*

From 64 isolates of *S. aureus*, 26 (40.6%) were positive for all three biofilm genes investigated (*icaA*, *icaD* and *bap).* The *icaA* gene alone was detected in 85.9% and *icaD* in 84.3% of isolates. Representative amplicons from *bap*, *icaA* *icaD* PCRs were partially sequenced and confirmed in GenBank (GenBank accession numbers AY220730.1, CP006838.1 and JN226155.1 respectively).

3.2 Chemical analysis by gas chromatography-mass spectrometry (GC-MS)

Details of the physicochemical characteristics of EOSA, EOCZ, eugenol and cinnamaldehyde are shown in Table 2. The major component of EOSA was eugenol and of EOCZ, cinnamaldehyde (86.59%). MIC values towards *S. aureus* isolates are also shown.

3.3 Minimum inhibitory concentration of EOs and major compounds

The minimum inhibitory concentration of eugenol and EOSA were 0.392 $mg.mL^{-1}$ and 0.237 $mg.mL^{-1}$, respectively. The EOCZ and cinnamaldehyde showed MICs of 0.243 $mg.mL^{-1}$ and 0.199 $mg.mL^{-1}$.

3.4 Effect of EO's and major components on biofilm formation on polystyrene and stainless steel coupons.

The effect of EOs and their major components on *S. aureus* biofilm formation on polystyrene and stainless steel surfaces are summarised in Table 3.OD values (at 570 nm) obtained in the presence of natural compounds are shown in addition to the percentage remaining following exposure to the natural compounds. The values obtained in the absence of EO or their major components were 0.415 (100%) for polystyrene and 1.07 (100%) in stainless steel.

There were significant differences in biofilm production between isolates grown in the absence and presence of EOs/major components. There was a statistically significant reduction in biofilm formation in the presence of EOSA and EOCZ ($p < 0.01$) on both polystyrene as stainless steel. Cinnamaldehyde and eugenol resulted in a statistically significant reduction in biofilm formation on polystyrene ($p < 0.001$) but on stainless steel, a significant reduction in biofilm was seen for cinnamaldehyde ($p<0.01$) but not eugenol.

Comparing the anti-biofilm activity of each EO to its major component, similar anti-biofilm activity was found for EOCZ and its major, cinnamaldehyde, on both test surfaces. However, EOSA was more effective in reducing biofilm on polystyrene and stainless steel, than was eugenol.

4. Discussion

Among the 64 isolates, 40.6% carried *icaA*, *icaD* and *bap*, simultaneously. *IcaD* and *icaA* are reported more frequently in *S. aureus* isolates from cows with mastitis than *bap* (Atshan and Shamsudin, 2011). However, the prevalence of *bap* among *S. aureus* isolates from bovine mastitis found here are significantly higher than previously reported by others (Vautor et al 2008, Seixas et al., 2014). Cucarella et al. (2001) found 5% positivity for *bap* in 350 strains tested and Vautor et al. (2008) could not detect *bap* among 262 *S. aureus* associated with different diseases and recovered from humans and animals. The relatively high *bap* carriage rate found here compared to other studies, suggests that *bap* acquisition by *S. aureus* may be a recent event or that its transmission by horizontal transfer remains limited despite its presence on a mobile transposon-like element, SaPIbov2. Transmission events involving this gene may be on the increase.

The chemical composition of EO's extracted from *Syzygium aromaticum* and *Cinnamomum zeylanicum* were similar to that found by other authors. The main components of the EOSA were eugenol (90.21%) and eugenol acetate (6.5%). Bauer (2001) observed the same components at concentrations of 75-85% and 8-15% respectively. The components of EOCZ were cinnamaldehyde (86.5%) and benzaldehyde (4.2%) similar to those reported by Unlu et al. (2010), 68.9% and

9.9% respectively. However, unlike the study of Unlu et al, small quantities of cinnamaldehyde acetate (7.4%), were found in the present study. As suggested by Burt et al. (2004), it is possible that intraspecies variations in composition may be due to genetic variation, seasonality, geographic location, harvest time and plant parts used in the preparation of oil.

The MIC values of OE's and their major components towards planktonic cells of *S. aureus* were similar to those observed in previous studies (Unlu, 2010.). From the values of MIC, sub-MIC dose of 0.106 $mg.mL^{-1}$ of EO and their component were used for biofilm studies. Furthermore, no corrosion of polystyrene plate surface, attributed to effects of OEs (which may have resulted in false-positives due to dye uptake) was observed at this concentration.

Biofilm formation on polystyrene surfaces decreased by 69.4% and 74% in the presence of EOSA and EOCZ. Cinnamaldehyde also reduced biofilm on polystyrene by 69.6% and eugenol treatment resulted in a 52.8% reduction. The greatest reduction in biofilm formation on stainless steel was found for EOSA (63.5%) followed by EOSZ (45.3) and cinnamaldehyde (44.9%). Eugenol had the least effect on biofilm on this surface (19.6%).

Our finding that the eugenol component of EOSA did not significantly contribute to the anti-biofilm activity of EOSA, suggests that other component within the oil may be responsible. According to Bassolé et al. (2010), the antibacterial activity of an essential oil, is mainly due to its major components, but antimicrobial synergy may result from interactions with other minor components. On the other hand, based on the similarity in biofilm disruption on two surface types, for EOCZ and its major component cinnamaldehyde, we suggest the efficiency of EOCZ is closely linked to its major component.

Several studies have investigated antibacterial activity of natural compounds and several mechanisms have been suggested including, membrane disruption, increased bacterial permeability and leakage of cellular contents and coagulation of cytoplasmic components (Lambert et al., 2001; Ultee and Smid, 2001). Inactivation of membrane proteins specifically may contribute to disruption of biofilm production in the early stages (Ultee et al., 1999). These effects would likely result in loss of adhesion and adsorption to the surface leading to areduction in biofilm.

Other essential oils and their major components also exhibit inhibition of initial biofilm formation. Carvacrol, one of the main antibacterial components of oregano oil and other essential oils, was reported to inhibit biofilms of *S. aureus* and S. typhimurium in the initial growth phase and prevented the formation of mature biofilms (Knowles, et al., 2005). Nostro et al. (2007) reported low biofilm formation by various strains of *S. aureus* and *S. epidermidis* in the presence of sublethal concentrations of EO of oregano, carvacrol and thymol. Similar results were obtained for strains of S. typhimurium in the presence of sub-lethal concentrations of EO of thyme, oregano and carvacrol (Soni et al., 2013).

Strong anti-biofilm was observed here for EOSA, EOCZ and cinnamaldehyde against *S. aureus* recovered from cases of sub-clinical bovine mastitis. However, some studies have shown that low concentrations of EOs, like tea tree oil and cinnamaldehyde, can increase bacterial metabolic activity in relation to biofilm production due to environmental stress in *S. aureus* and *Pseudomonas aeruginosa*, respectively (Kwiecinski, 2009).

5. 5. Conclusion

Despite the low extraction yield of EOSA, EOCZ and cinnamaldehyde from *Syzygium aromaticum* and *Cinnamomum zeylanicum,* the strong anti-biofilm activity reported here on stainless steel and polystyrene, at low concentrations of these substances, indicates their potential for development. A specific application could be as an alternative sanitizing spray for surface cleaning in the food processing industry.

6. 6. Acknowledgements

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7. 7. References

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418
419

Table 1: Oligonucleotides used in the detection of biofilm genes and confirmation of identification of *Staphylococcus aureus* strains isolated from milk of cows with subclinical mastitis.

gene	*primer*	sequence	Amplicon size	T_m (ºC)	Reference
bap	bap-F	ccctatatcgaaggtgtagaattg	971 bp	65	Cucarella et al. (2001)
	bap-R	gctgttgaagttaatactgtacctgc			
icaA	*icaA*-F	cctaactaacgaaggtag	1315 bp	49.5	Vadesuvan et al. (2003)
	icaA-R	aagatatagcgataagtgc			
icaD	*icaD*-F	aaacgtaagagaggtgg	381 bp	50	Vadesuvan et al. (2003)
	icaD-R	ggcaatatgatcaagatac			
nuc	nuc-F	cgtaaatgcacttgcttcagg	257 bp	55	CRL (UE)
	nuc-R	tcagcaaatgcatcacaaacag			

T (ºC): annealing temperature, bp: base pairs

425

426 Table 2. Physicochemical analyses and MIC values towards *S. aureus* isolates of EOSA,
427 EOCZ and their major compounds.

Species or compound	Density ($mg.mL^{-1}$)	MIC[a] ($mg.mL^{-1}$)	Composition (%)
Syzygium aromaticum	1060	0.392	eugenol (90.2%), eugenol acetate (6.5%), β- caryophyllene (1.3%), others (1.9%)
eugenol	1050	0.237	eugenol (100%)
Cinnamomum zeylanicum	1075	0.243	cinnamaldehyde (86.5%), benzaldehyde (4.2%), cineole (1.7%), cinnamic acid (1.5%) (0.6%), eugenol (0.1%), Others (5.42%)
cinnamaldehyde	1080	0.199	cinnamaldehyde (100%)

428 [a] MIC values shown are the mean found for 26 *S. aureus* isolates; EOSA: clove essential oil; MIC:
429 minimum inhibitory concentration; EOCZ: cinnamon essential oil.

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Table 3. *S. aureus* biofilm formation on polystyrene and stainless steel in the presence of EOSA, EOCZ and its major components.

	Polystyrene OD_{570} (% reduction)	stainless steel OD_{570} (% reduction)	*p* value (pol./ss.)
Control	0.415 ±0,075 (0%) a	1.07 ±0,222 (0%) y	-
EOSA	0.127 ±0,047 (69.4%) b	0.390 ±0,080 (63.5%) z	p<0.01 e p<0.01
Eugenol	0.196 ±0,067 (52.8%) c	0.860 ±0,220 (19.2%) y	p<0,01 e p>0.05
EOCZ	0.105 ±0,040 (74.7%) b	0.585 ±0,158 (45.3%) z	p<0.01 e p<0.01
Cinnamaldehyde	0.126 ±0,078 (69.4%) b	0.589 ±0,100 (44.9%) z	p<0.01 e p<0.01

Values followed by same letter in the column do not differ; EOSA: clove essential oil; EOCZ: cinnamon essential oil; pol: polystyrene; ss: stainless steel