

Anti-biofilm property of essential oils from *Cymbopogon* sp. against pathogenic bacteria in single-culture and co-culture

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ABSTRACT

In this study, we evaluated whether the essential oils (EOs) from *Cymbopogon flexuosus* and *Cymbopogon martinii* can prevent production of biofilms either in single or combined culture of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Biofilm formation was assessed by microtiter-plate test with further quantification of viable cells and biofilm biomass. The evaluated EOs at 0.78 % significantly ($P < 0.05$) reduced only the viable cells of *S. aureus* that inhabited biofilm. However, in single-and co-culture assays, both oils significantly ($P > 0.05$) decreased the amount of biofilm biomass. Biofilm reductions between 52-83% and 60-93% were achieved for the treatments with EOs from *C. flexuosus* and *C. martinii*, respectively. Although the biomass reductions of single and co-cultivated biofilms were significant, the same was not true for viable cells, except for *S. aureus*. Considering that the remaining colony forming units can reconstitute the EPS matrix, studies with higher concentrations than those used in this research are suggested in order to obtain greater logarithmic reductions of viable bacterial cells.

Keywords: Biofilm; *Staphylococcus aureus*; *Pseudomonas aeruginosa*

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1 INTRODUCTION

Biofilm is an assemblage of bacteria enclosed in a self-produced polymeric matrix that adheres onto a surface. By living into the biofilms, bacteria share their genetic material more easily, while remaining protected from adverse environmental conditions (WATNICK AND KOLTER, 2000). In biofilms, bacterial cells are embedded in an extracellular matrix of organic polymers such as polysaccharides, peptides, and extracellular DNA that are synthesized and released by the microbes themselves (LEWIS, 2001; ATSHAN et al., 2015; FAGERLUND et al., 2016). The matrix drastically reduces the susceptibility to different outer stress factors (DONLAN, 2002) indicated by up to 1000-fold higher tolerance to antimicrobials of the biofilm embedded cells compared to their planktonic counterparts (COSGROVE et al., 2002; SANCHEZ-VIZUETE et al., 2015).

Staphylococcus aureus is a remarkably versatile organism. It is adaptable, flexible and multifaceted in its interactions with its surroundings. It can exist comfortably in inanimate sites as well as in various niches in the animal host. It can exist harmlessly as a commensal, inhabiting the skin or mucous membranes, and it can survive in the blood or in a variety of tissue sites where it is responsible for disease states ranging from minor skin infections to toxins and systemic, life-threatening illnesses (NOVICK, 2003). *Pseudomonas aeruginosa* is the most common Gram-negative bacterium found in nosocomial infections (HARTMANN et al, 2012). Its pathogenicity is strongly related to the expression of an unusually large number of virulence factors, which cause tissue damage, delay airway epithelium wound repair, and suppress innate immune response (DUBERN AND DIGGLE, 2008). Drug and antimicrobial therapy are hindered by biofilms. The sessile cell communities are embedded in a matrix of extracellular polymeric substances and show a reduced growth rate and altered gene transcription (DONLAN AND COSTERTON, 2001). These factors prevent antimicrobial agents from penetrating and eradicating the bacteria. The regulation of virulence factor expression as well as biofilm formation is based on quorum sensing (QS), a cell-density

dependent intercellular communication system that uses small molecules (HARTMANN et al, 2012).

Biofilms attached on contact surfaces in the food industry increase the risk of persistent spread of pathogenic bacteria. For instance, surfaces in meat and dairy processing plants are characterized by the high incidence of *S. aureus* and *P. aeruginosa* (GUTIÉRREZ et al., 2012), pathogens whose ability to form biofilms enables them to resist the action of antimicrobials and sanitizers (MARQUES et al., 2007; NEIDIG et al., 2013). In this context, essential oils (EOs) have attracted interest for the purpose of controlling biofilms not only because they fit the concept of "Generally Known as Safe", but also because they have a broad spectrum of antibacterial activity (BURT, 2004). EOs are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. As typical lipophiles, they pass through the bacterial cell wall and cytoplasmic membrane, disrupt the structure of the different layers of polysaccharides, fatty acids and phospholipids and permeabilize them (BAKKALI et al., 2008).

Cymbopogon martinii (Roxb.) Will. Watson (*Poaceae*), commonly known as palmarosa and Indian geranium, is a lemongrass native to South and Southeast Asia, especially India and Pakistan, and it is often cultivated for its oil (COPE, 1982). Scientific studies on *C. martinii* demonstrated anthelmintic (KUMARAN et al, 2003; KAITI et al., 2011) antiseptic, antifungal, antibacterial (Prashar et al., 2003, Prasad et al., 2010, MILLEZI et al, 2016) and insect-repellent activities (TYAGI et al, 1998). It inhibits activity in a competitive manner (GACCHE et al., 2011) and has wound healing properties (TAMULI et al, 2012). *Cymbopogon martinii* has been reported to exert a-glucosidase inhibitory activity and helps in the management of postprandial glucose level (GHADYALE et al., 2012).

The EOs from plants of *Cymbopogon* genus (e.g. *C. flexuosus* and *C. martinii*) seem to be a reasonable option as anti-biofilm agents, once they are active against bacteria as *S. aureus* and *P. aeruginosa* (SCHERER et al., 2009; ADUKWU et al., 2012). *Cymbopogon flexuosus* is herbaceous plant of the *Poaceae* family, native to India. The EO of *C. flexuosus* is widely used in alternative medicine because of its antimicrobial,

insecticides, larvicides, antitumoral and cytotoxic properties (SHARMA et al., 2008). Z-citral (β -citral), geraniol and β -geranial (α -citral) are the major components EO of *C. flexuosus*, where citral is outstanding as responsible for the antimicrobial potential oil (ADUKWU, et al. 2016).

Several studies demonstrate the inhibitory effect of such EOs on planktonic cells or only disk-diffusion assay, which may not translate into satisfactory responses when bacteria grow encased by biofilms. Therefore, the aim of this study was to evaluate whether the EOs from *C. flexuosus* and *C. martinii* can prevent production of biofilms either in single or combined culture of *S. aureus* and *P. aeruginosa*.

2. MATERIALS AND METHODS

2.1 Essential oils (EOs)

Essential oils extracted by hydro-distillation from leaves of *Cymbopogon flexuosus* and *Cymbopogon martinii* were purchased from Ferquima Indústria Comércio Ltda. (Vargem Grande, São Paulo, Brazil). For CG analysis, a solution was prepared by dissolving 100 mg of EO in 10 ml of CH_2Cl_2 .

2.2 Gas chromatography with flame ionization detection (GC-FID)

The EOs were analyzed using an Agilent 7820A gas chromatograph system (Agilent Technologies, Inc., Shanghai, China) equipped with a split/splitless injector, a flame ionization detector (FID), and an Agilent 7693A autoinjector. The sample solution (1 μl) was injected in the split mode at a ratio of 1:10. Analyses were performed with an Agilent J&W HP-5 capillary column (30 m x 0.25 mm i.d., 1 μm film thickness, and stationary phase consisting of 5% diphenyl/95% dimethylpolysiloxane). The oven temperature program was as follows: held at 50 °C for 2 minutes, increased from 50 to 220 °C at a rate of 2 °C/min, held at 220 °C for 3 minutes.

Nitrogen was used as carrier gas at a flow rate of 1.2 ml/min. The injector and FID temperatures were fixed to 240 °C and 280 °C, respectively. The flow rates of air, H_2 , and N_2 in the FID were 300, 30, and 30 ml/min, respectively. The OpenLAB CDS

software was used for equipment management and data processing. The compositions of the EOs were expressed as percentage of normalized area.(EL-SAYED, 2018).

2.3 Gas chromatography coupled to mass spectrometry (GC-MS)

The EOs were analyzed using a Shimadzu GCMS-QP2010 gas chromatograph mass spectrometer (Shimadzu Corporation Technologies, Inc., Tokyo, Japan) equipped with a split/splitless injector. The sample solution (1 µl) was injected in the split mode at a ratio of 1:10. Analyses were performed with a Supelco Equity-5 capillary column (30 m x 0.2 mm i.d., 0.2 µm film thickness, and stationary phase consisting of 5% diphenyl/95% dimethylpolysiloxane). The oven temperature program was as follows: held at 50 °C for 2 minutes, increased from 50 to 220 °C at a rate of 2 °C/min, and held at 220 °C for 3 minutes.

Helium was used as carrier gas at a flow rate of 1.2 ml/min (constant linear velocity of 39.2 cm/sec). The injector temperature was set at 240 °C. The interface and ion source temperatures were fixed to 220 and 200 °C, respectively. Quadrupole mass spectrometer was operated in electronic impact mode at 70 eV, scanning the range m/z 35-350 in cycles of 0.5 s. The GCMS solution software was used for equipment management and data processing. Compounds were identified by searching the NIST 05 mass spectral library, and by comparison of their retention indices relative to C₇-C₃₀ n-alkane series with those values found in an online database (EL-SAYED, 2018).

2.4 Bacterial strains and standardization

Staphylococcus aureus (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 27853) strains were obtained from the Oswaldo Cruz Foundation (FIOCRUZ). Bacteria were incubated in Tryptic Soy Both (TSB) (Sigma, India) medium overnight at 37 °C. The standardization of the number of cells was determined by a growth curve at 630 nm, and CFU mL⁻¹ were measured until approximately 10⁸ CFU mL⁻¹.

2.5 Agar disk-diffusion assay

The detection of inhibitory effect of the EOs on the tested bacteria was carried out by agar disk-diffusion method based on the document M2-A8 of CLSI (2003). Sterile paper discs (9mm in diameter and 250g m⁻²) were impregnated with 25µL EO and placed on plates inoculated with suspensions of each culture, which were then incubated at 36°C for 18-24h. The diameter of inhibition zones, including the disc diameter, was measured in millimeters, and inhibition was scored as weak (10-13,9mm), moderate (14-18mm), or strong (>18mm) according to Carovic-Stanko (2010).

2.6 Bacterial biofilms

For assays involving biofilm formation, a 0.78% EO solution was prepared following the procedure adapted from Oliveira et al. (2010). First, the oil sample was dissolved in DMSO (2%) followed by dilution with saline solution (0.85%, m/v) containing Tween 80 at 0.5% (v/v).

Biofilm formation was assessed by microtiter-plate test according to (STEPANOVIĆ et al., 2000, MILLEZI et al., 2016). In 96-well polypropylene microplates, bacterial inoculum and EO solution were mixed into the wells at a ratio of 1: 1 (v/v). The plates were incubated aerobically at 37 °C for 18 h under orbital shaking (80 rpm) (Tecnal, Brazil). For the positive control, wells were filled with the bacterial inoculum and the TSB broth in the ratio of 1: 1 (v/v). In the negative controls, wells were filled with the solution of OE and TSB broth in the ratio of 1:1 (v/v) or only with the TSB broth.

2.7 Quantification of viable cells in biofilm

For quantification of viable cells after incubation, the wells were washed with sterile water to remove planktonic cells. The biofilm viable cells were removed by sonication for 5 minutes at 40 KHz (Sonders, Brazil). Then, 100 µl of each sample was pipetted into 900 µl of sterile distilled water, resulting in a solution that has been serially diluted up to 10⁻⁶.

Samples were plated on Tryptic Soy Agar medium (TSA) (Oxoid, England) according to Herigstad et al. (2001). The plates were incubated aerobically at 37 °C for 18 h prior to enumeration. The number of viable bacterial cells was further determined and expressed as Log CFU/cm² (sessile cells).

2.8 Quantification of biofilm biomass

In addition, biofilm biomass was quantified according to Stepanović *et al.*, (2000), with minor modifications (absorbance was measured at 570). In each of the wells, the attached biofilm was fixed with 200 µl of methanol. After 15 minutes, the methanol was removed, and the plates were dried out at room temperature. Then 200 µl of 1% violet crystal dye was added to each well for staining the cells attached to the walls.

After 5 minutes, the excess of dye solution was removed, and the plates were gently washed in water. Further, 230 µl of acetic acid at 33 % (v/v) were added in each of the wells to solubilize the dye bounded to the adhered cells. The absorbance was measured at 570 nm using an Elisa spectrophotometer (Biotek, Winooski, VT, USA). The strains were classified according to their adhesion ability following the scale proposed by Stepanović et al. (2000).

2.9 Statistical analysis

Statistical analysis for antibiofilm activity was performed using Prism version 3.0 (GraphPad Software, Inc., La Jolla, USA). The assumptions for parametric test were checked prior to carry out the analysis. Data were analyzed by One-way ANOVA followed by Bonferroni test, where $P < 0.05$ was considered significant. The analyzes were performed in triplicate an assay were performed on three separate occasions.

3. RESULTS AND DISCUSSION

The main constituents of the studied EOs are presented in table 1. The EO from *C. flexuosus* showed a high percentage of citral that comprises the sum of isomers

geranial (41.8 %) and neral (33.2 %), geraniol represented only 5.4 % of *C. flexuosus* oil composition. According Da Silva, et al (2018) the major compounds in the *C. flexuosus* EO composition were β -geranial (45.74%), Z-citral (34.42%) and geraniol (6.01%). In the EO from *C. martinii*, geraniol was identified as the major constituent (79.7%), along with geranyl acetate (9.6%) and linalool (3.4%). The profile of marker compounds observed herein is similar to those earlier reported for *C. martinii* (RAINA et al., 2003, TSAI et al., 2014).

Table 1 - Chemical composition (%) of essential oils from *Cymbopogon flexuosus* and

Compound ^a	RI ^b	RIE ^c	<i>C. flexuosus</i> (%)	<i>C. martinii</i> (%)	Cy mb opo gon ma rtin ii.
α -Pinene	939	920	0,15	-	
Camphene	954	935	0,79	-	
6-Methyl-5-hepten-2-one	985	985	1,66	-	
β -Myrcene	990	989	0,37	0,22	
D-Limonene	1030	1025	0,27	0,14	
(Z)- β -Ocimene	1040	1036	0,19	0,37	
(E)- β -Ocimene	1050	1046	0,13	1,19	
4-Nonanone	1053	1072	0,59	-	
β -Linalool	1100	1101	1,54	3,38	
Isopulegol	1145	1143	-	-	
Citronellal	1153	1152	0,23	-	
4,5-Epoxy- <i>t</i> -carene		1182	1,04	-	
α -Terpeniol	1185	1189	0,61	-	
β -Citronellol	1228	1231	-	-	
Neral	1238	1240	33,25	-	
Geraniol	1253	1259	5,37	79,71	
Geranial	1267	1270	41,80	0,45	
Citronellyl acetate	1353	1352	-	-	
Geranyl acetate	1383	1382	4,23	9,62	
β -Elemene	1391	1386	-	-	
β -Caryophyllene	1419	1410	2,01	1,88	
γ -Muuroolene	1477	1505	1,07	-	
α -Muuroolene	1499	1494	-	-	
δ -Cadinene	1524	1516	0,42	-	
Elemol	1547	1544	-	-	
Geranyl butyrate	1562	1570	-	0,22	
Nerolidol	1564	1576	-	0,17	
Monoterpenes hydrocarbons			1,89	1,92	
Oxygenated monoterpenes			83,85	83,54	
Sesquiterpenes hydrocarbons			3,50	1,88	

Continuation...

Table 1 – Conclusion....

Compound ^a	RI ^b	RIE ^c	<i>C. flexuosus</i> (%)	<i>C. martinii</i> (%)
Oxygenated sesquiterpenes			-	0,17
Others			6,49	9,84
Total identified			95,72	97,34

^aCompounds are listed in order of their elution from a Supelco Equity-5 column (30 m, 0.2 mm, 0.2 μm).

^bRetention index obtained at <http://www.pherobase.com/>.

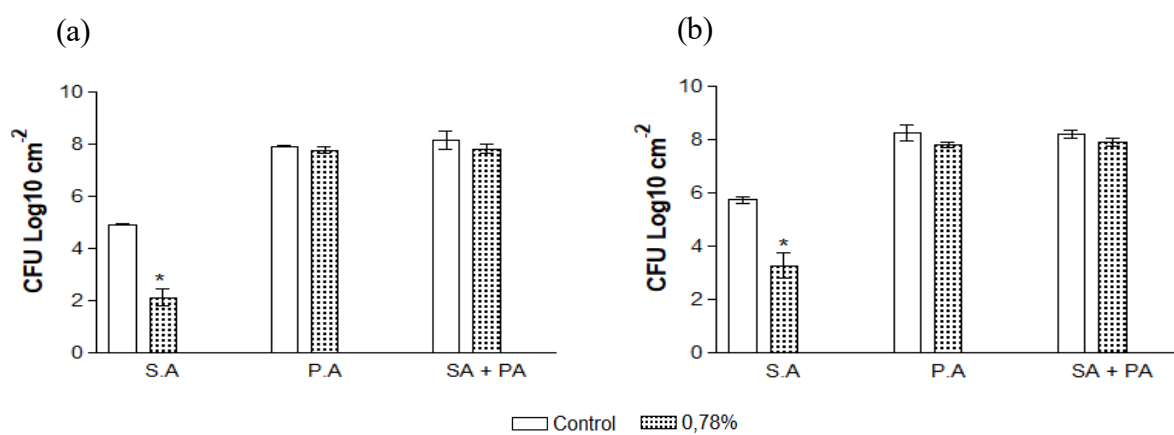
^cRetention index experimentally determined on a Supelco Equity-5 column, using a homologous series of C7-C30 alkanes.

In the agar disc-diffusion test, *C. flexuosus* EO strongly inhibited *S. aureus* and *C. martinii* moderately. *Pseudomonas aeruginosa* was not inhibited, De Silva et al (2018) reported also reported resistance of *P. aeruginosa* in similar test. The inhibition areas are like to those observed by Karkala and Gangerwala (2009) for EO *C. flexuosus* against *S. aureus*. The results of this study on the inhibition of *S. aureus* by *C. martinii* EO corroborate with those previously found by Millezi et al (2016) in disc-diffusion assay.

At the concentration of 0.78 %, the EOs from *C. flexuosus* and *C. martinii* significantly ($P < 0.05$) reduced viable *S. aureus* cells encased into the biofilm (Figure 1a). On average, we found 2.8 and 2.5 log₁₀ CFU/cm² reductions in cell viability of *S. aureus* by using EOs from *C. flexuosus* and *C. martinii*, respectively, possibly because geraniol, causes leakage of K⁺ and Mg²⁺ ions from microorganism cells through changes in the compositions of the cell membranes (PRASHAR et al., 2003). However, both oils were not effective in decreasing viable cells of *P. aeruginosa* that inhabited biofilm under the same conditions (Figure 1b). When the assay was performed in combined culture, the number of viable cells (including *S. aureus* and *P. aeruginosa*) in EO treatments showed no differences ($P > 0.05$) in comparison with the untreated controls. According Sanchez-Vizueté et al. (2015), it is becoming increasingly obvious that social behavior within a mixed community confers bacterial tolerance to environmental stresses, including the action of disinfectants that until now has been largely underestimated. A great number of studies showing an increased resistance to disinfectants in multispecies biofilms. For example, the association in a mixed biofilm of *Bacillus cereus* and *Pseudomonas fluorescens* two species frequently isolated on surfaces in food processing industries, led to a remarkable increase in their tolerance

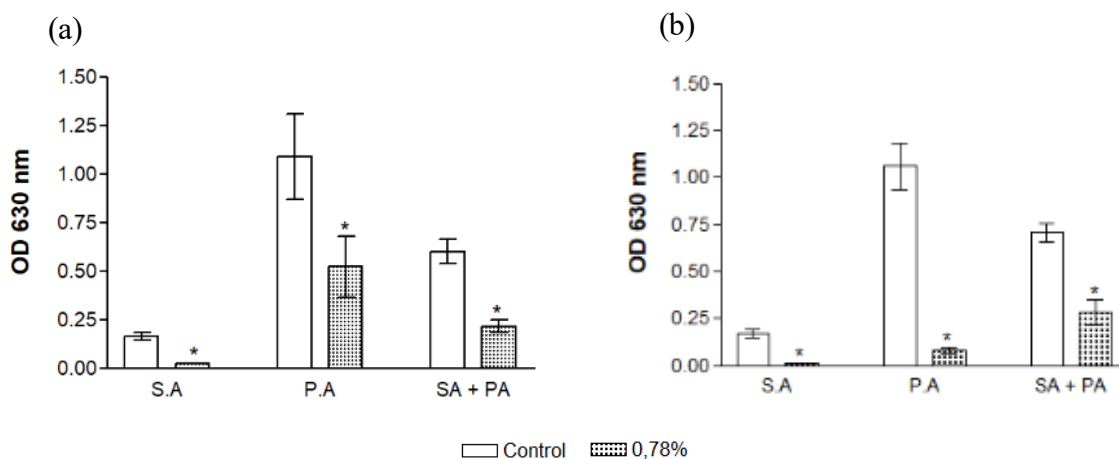
to two frequently used disinfectants, chloride dioxide and glutaraldehyde (LINDSAY et al., 2002; SIMÕES et al., 2009).

Figure 1- Viable cells in biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. SA: single culture of *S Staphylococcus aureus*; PA: single culture of *Pseudomonas aeruginosa*; SA + PA: co-culture of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. EO from *Cymbopogon flexuosus* (a), and *Cymbopogon martinii* (b). The values refer to the mean of three replicates and the bars indicate the standard deviation. Asterisk indicates a significant difference ($P < 0.05$).



Unlike *S. aureus* strain, *P. aeruginosa* was classified as strongly adherent according to the scale proposed by Stepanović et al. (2000) based on biomass results. Despite these circumstances, we detected in the single-culture assays that EOs from *C. flexuosus* and *C. martinii* significantly ($P > 0.05$) reduced biomass attached to the wall of wells in polystyrene plates (Figure 2a and 2b). The biomass-lowering effect was also confirmed when the bacteria were grown together. The biomass reductions were between 52-83% using the EO from *C. flexuosus* and 60 - 93% with that of *C. martinii*. Among the results, we highlight the action of *C. martinii* EO against *P. aeruginosa* biomass whose reduction was 93% (Figure 2b).

Figure 2- Biomass of biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. SA: single culture of *Staphylococcus aureus*; PA: single culture of *Pseudomonas aeruginosa*; SA + PA: co-culture of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. EO from *Cymbopogon flexuosus* (a), and *Cymbopogon martinii* (b). The values refer to the mean of three replicates and the bars indicate the standard deviation. Asterisk indicates a significant difference ($P < 0.05$).



Active substances in EOs, such as citral and geraniol, can inactivate bacteria even in the planktonic form, thus limiting the number of viable cells able to interact with the biofilm zone. According Nostro *et al.*, 2007, the reduction of biomass, suggesting that the EOs interact with the matrix of exopolysaccharide (EPS) that is disrupted. The mechanism of action of the monoterpenes (limonene, citronellal, citronellol, geraniol) involves mainly toxic effects on the structure and function of the cell membrane. As a result of their lipophilic character, the monoterpenes will preferably dislocate from the aqueous phase toward the membrane structures (SIKKEMA *et al.* 1995). Besides that, the active constituents likely affect the regular cycle of biofilm attachment by inhibiting the quorum-sensing, the signaling mechanism by which bacteria regulate their population behavior. Both reasons explain the EOs activity against *S. aureus* biofilm (WANG *et al.*, 2014; ESPINA *et al.*, 2015), while only the second is coherent for *P. aeruginosa* case (JARAMILLO-COLORADO *et al.*, 2012; MASÁK *et al.* 2014).

However, despite the efficiency of the EOs tested in inhibiting EPS production, it should be considered that there was no satisfactory reduction of colony forming units which may mean that these communities are able to reconstitute the polymer matrix.

The combination of viable cell inhibition and EPS is an important fact, which we did not achieve in this study. The genetic and molecular basis of biofilm formation in staphylococci is multifaceted, the ability to form a biofilm affords at least two properties: the adherence of cells to a surface and accumulation to form multilayered cell clusters. A trademark is the production of the slime substance PIA, a polysaccharide composed of β -1,6-linked N-acetylglucosamines with partly deacetylated residues, in which the cells are embedded and protected against the host's immune defense and antibiotic treatment (GÖTZ, 2002). According Atshan et al. (2015), extracellular proteins expressed *S. aureus* during planktonic growth and biofilm development are different, a significant variation in spot size intensities was observed in the production of extracellular proteins. This variation could possibly have contributed to the degree of virulence even within the same clonal genotype. Although both bacteria showed strong biofilm formation, *P. aeruginosa* was found to have 5 times more adherence than *S. aureus*. It is noteworthy that *P. aeruginosa* in this study may have dominated *S. aureus* in a competitive relationship and thus contributed to the protection of cells against the antibacterial action of OEs.

Biofilms of *P. aeruginosa* (and other microorganisms) are formed from individual planktonic cells in a complex and presumably highly regulated developmental process. Planktonic cells are thought to initiate interactions with a surface in response to various signals, including the nutritional status of the environment (WIMPENNY AND COLASANTI, 1997; O'TOOLE AND KOLTER, 1998; PRATT AND KOLTER, 1998). Brözel et al (2002), monitored changes in global gene expression patterns in attached *P. aeruginosa* cells and found more than 11 proteins whose levels were altered during various stages of attachment. The physiological changes in the transition from planktonic to attached cells are profound and undoubtedly complex. In addition, these results demonstrate that biofilm bacteria are physiologically different from planktonic bacteria.

Gündel, et al (2018) report that the use of free EO may be a limiting factor in antibacterial activity, in tests with *C. flexuosus* EO, the free oil showed no activity against *P. aeruginosa* at any of the tested concentrations, however, the nanoemulsion

showed potential bactericidal activity at concentrations above 11.33 mg/mL. Against *S. aureus* bacteria, free oil and nanoemulsion showed a MIC of 0.58 mg/mL. In this case, there was no improvement, but it can also be verified that the nanoencapsulation process did not negatively influence the activity against this microorganism.

Although several studies report the action of EOs against bacteria such as *S. aureus* and *P. aeruginosa*, most address antibacterial activity focusing on planktonic cells, disc diffusion, Minimum Inhibitory Concentration (MIC) testing methodologies, and against the formation of simple biofilms (KARKALA et al., 2009; CAROVIC-STANKO et al., 2010; MILLEZI et al, 2016). In this study, we studied, besides the effect of *C. martinii* and *C. flexuosus* EOs on the formation of individual biofilms, the perspective of the action of these oils against co-cultivation, the biofilms formed together of a Gram negative bacterial species (*P. aeruginosa*) and Gram positive (*S. aureus*) (NOSTRO et al, 2007, ADUKWU et al, 2012; MILLEZI et al. 2019).

In conclusion, there was a reduction of biomass in the treatments with *C. flexuosus* and *C. martinii* EOs in simple and mixed cultivation of *P. aeruginosa* and *S. aureus*, however, we cannot say that these EOs were efficient alternatives, despite the reduction of CFUs from *S. aureus*, viable cell growth, this suggests that EPS may again be produced by the remaining bacterial cells and reconstitute the matrix. Studies with higher concentrations than those used in this research are suggested in order to obtain greater logarithmic reductions of viable bacterial cells

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