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Article

Effect of polycaprolactone nanocapsules loaded with essential oils on biofilm formation by Staphylococcus aureus strains isolated from bovine mastitis cases

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Bovine infectious mastitis is largely resistant to antibacterial treatment, mainly due to mechanisms of bacterial resistance in the biofilms formed by *Staphylococcus aureus.* Melaleuca (MEO) and citronella essential oils (CEO) are promising agents for reducing or eliminating biofilms. Free melaleuca oil presented a medium Minimum Inhibitory Concentration (MIC) of 0.625% and a Minimum Bactericidal Concentration (MBC) of 1.250%, while free citronella oil showed medium MIC and MBC of 0.313%. Thus, free CEO and MEO demonstrate bacteriostatic and bactericidal potential. We generated polymeric nanocapsules containing MEO or CEO and evaluated their efficacy at reducing biofilms formed by *S. aureus*. Glass and polypropylene spheres were used as test surfaces. To compare the responses of free and encapsulated oils, strains were submitted to 10 different procedures, using free and nanoencapsulated essential oils (EOs) *in vitro.* We observed no biofilm reduction by MEO, free or nanoencapsulated. However, CEO nanocapsules reduced biofilm formation on glass ($p = 0.03$) and showed a tendency to diminish biofilms on polypropylene ($p = 0.051$). Despite nanoencapsulated CEO reducing biofilms *in vitro*, the formulation could be improved to modify the CEO component polarity and, including MEO, to obtain more interactions with surfaces and the biofilm matrix.

Keywords: Nanoencapsulation. Citronella. Melaleuca. Bovine mastitis. Biofilm. Bacteria. Essential oil.

INTRODUCTION

Bovine mastitis is the inflammation of the mammary glands/udder (intramammary inflammation, IMI) in cows (Sharun *et al*., 2021). To treat bovine mastitis, antibiotics have been widely used. However, their extensive and uncontrolled use has led to the emergence of multi-antibiotic-resistant strains (Feng *et al*., 2023). Mastitis increases the cost per animal due to expenses for treatment and prophylaxis programs as well as loss of animal productivity and milk quality (Acosta *et al*., 2016; Varela-Ortiz *et al*., 2018; Yuan, Peng, Gurunathan, 2017). The main etiological agent involved in mastitis is the gram-positive bacterium *Staphylococcus aureus*; this opportunistic pathogen may infect the surface and interior of the mammary gland, promoting mild to severe destruction of the

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glandular epithelium (Acosta *et al*., 2016; Budri *et al*., 2015; Varela-Ortiz *et al*., 2018).

Structurally, *S. aureus* has a cell framework that confers resistance to macrophage-mediated phagocytosis and constitutes a bacterial reserve within these cells (Rigby, DeLeo, 2012; Yuan, Peng, Gurunathan, 2017). The cell wall, rich in peptidoglycans and teichoic and lipoteichoic acids, forms a network that promotes protection of the internal phospholipidic membrane, which suffers a thickening of its lipid content if bacteria are exposed to external agents. Thus, this structure itself and other intrinsic resistance mechanisms, such as the production of toxins, enzymes, and biofilms, may lead to selection of strains of *S. aureus* resistant to conventional antibiotics (Ernst, Ejsing, Antonny, 2016; Perez-Lopez *et al*., 2019). As a result, the infection persists and amplifies, requiring increased doses of different antimicrobials. The formation of biofilms, one of the main resistance mechanisms of *S. aureus*, is associated with excessive doses, and treatment may require doses much higher than usual, resulting in toxic effects (Budri *et al*., 2015; Yuan, Peng, Gurunathan, 2017). Also, there is a high potential for contagion between humans and animals, as well as the possibility of biofilms adhering to surfaces such as glass and polypropylene, materials commonly found in cattle milking, which may constitute important sources of bacterial contamination (Marques *et al*., 2007; Yuan, Peng, Gurunathan, 2017; Notcovich *et al*., 2018; Varela-Ortiz *et al*., 2018).

Biofilms are composed of a polysaccharide matrix, DNA, and proteins, the so-called "extracellular polymeric substance" (EPS). Biofilm formation starts with a planktonic (solitary) cell that attaches to a surface. Bacterial pillars are formed to ensure nutrient supply, avoiding the toxic waste developed by the colony itself. Bacterial adhesion can become an irreversible condition, hampering the elimination of the colony and causing frequent reinfections (Duncan *et al*., 2015; Buldain *et al*., 2018). Thus, biofilms can confer loss of bacterial sensitivity to virtually all classes of antibiotics (Notcovich *et al*., 2018; Varela-Ortiz *et al*., 2018; Perez-Lopez *et al*., 2019).

The need to overcome bacterial resistance mechanisms drives the search for new antimicrobials (Buldain *et al*., 2018; Varela-Ortiz *et al*., 2018). In this age of proliferation of microbial resistance to antimicrobials, it is imperative to source alternative medicines for the management and prevention of bovine mastitis (Ajose *et al*., 2022). In this sense, essential oils (EOs), as products of the secondary metabolism of plants, are natural and sustainable alternatives to conventional antibacterial treatment. They contain several synergistically acting components, such as phenylpropanoids and terpenoids, that contribute to a reduction in bacterial resistance via various mechanisms (Budri *et al*., 2015; Scazzocchio *et al*., 2016; Buldain *et al*., 2018; Saporito *et al*., 2018). The synergism of EO components is evident since each oil component presents lower activity than the total essential oil (Araujo, Longo, 2016; Scazzocchio *et al*., 2016; Buldain *et al*., 2018).

Melaleuca (*Melaleuca alternifolia*) essential oil (MEO) is mainly composed of the monoterpenoid terpinen-4-ol, a lipophilic compound which interferes with the permeability of the bacterial cell membrane, causing potassium leakage (Scazzocchio *et al*., 2016; Oliva *et al*., 2018). This mechanism supports the use of MEO as an anti-inflammatory (action on neutrophils) and an antiseptic (Carson, Mee, Riley, 2002; Oliva *et al*., 2018). Additionally, lipophilic terpinen-4-ol may combine with other nonpolar substances, such as biofilms. Thus, the antimicrobial property of MEO emerges as an alternative for treating infections caused by biofilm-forming bacteria (Araujo, Longo, 2016; Souza *et al*., 2017).

Citronella (*Cymbopogon winterianus*) essential oil (CEO) has antimicrobial action attributed to cinnamic acid molecules with the major aldehyde-terminal clusters (aldehyde compounds) cuminal and β-citronellal (Deletre *et al*., 2015; Scazzocchio *et al*., 2016). In addition, CEO is a source of terpenoids with antifungal, antimicrobial, and antibiofilm activity against a broad spectrum of bacteria, including *S. aureus* (Deletre *et al*., 2015; Singh, Fatima, Hameed, 2016).

Despite their antibacterial activity, EOs have not been widely applied due to their high volatility and compound lability, making them degradable and oxidizable by enzymes, light, heat, and pathogens. These issues could be solved with EO nanoencapsulation (Miladi *et al*., 2016; Saporito *et al*., 2018; Kokina *et al*., 2019).

Nanoprecipitation is one of the most used techniques to obtain nanocapsules. It is based on the interfacial deposition of biodegradable polymers, followed by deposition of a

water-miscible semi-polar solvent from a lipophilic solution (Fessi *et al*., 1989). The formed nanocapsules exhibit an oil core in which the lipophilic substance is confined and protected by a polymeric membrane. Thereby, besides promoting protection from early degradation, nanocapsules promote prolonged release of the compound load (Miladi *et al*., 2016; Melo *et al*., 2018; Kokina *et al*., 2019).

In the present study, we developed nanocapsules containing MEO or CEO and evaluated their efficacy in reducing biofilms formed by *S. aureus* isolated from cattle mastitis cases, *in vitro*.

MATERIAL AND METHODS

Material

Acetone was purchased from Dinâmica® Química Contemporânea (Indaiatuba, SP, Brazil). Acetonitrile HPLC-grade and methanol were purchased from J. T. Baker® (Phillipsburg, NJ, USA). Resazurin (cod. R7017), sorbitan monostearate (Span® 60), and polycaprolactone (PCL), average molecular weight ~80,000, were acquired from Sigma-Aldrich® (St Louis, MO, USA). Polysorbate 80 (Tween® 80) was purchased from Delaware® Importadora Química (Porto Alegre, RS, Brazil). Water was purified using a Milli-Q Plus system (Millipore) with a conductivity of 18 MΩ.

Melaleuca (*Melaleuca alternifolia*) and citronella (*Cymbopogon winterianus*) EOs were purchased from Laszlo Aromaterapia® (Belo Horizonte, MG, Brazil). The composition of the EOs was provided by the manufacturer (CG-MS) and was in accordance to ISO-4730 (melaleuca) and ISO-3848 (citronella).

The biofilm-forming strain *Staphylococcus aureus* ATCC 6539 was purchased from Newprov® (Pinhais, PR, Brazil). Tryptone soy broth (TSB), Mueller-Hinton broth (MHB), Mueller-Hinton agar (MHA), and Brain Heart Infusion broth (BHI) were obtained from Hi-media® Laboratories LLC (Pennsylvania, USA).

Bacterial strains

For this study, 27 strains of *S. aureus*, isolated from cases of persistent mastitis were donated by the Laboratório de Zoonoses Bacterianas do Departamento de Medicina Veterinária Preventiva e Saúde Animal da Universidade de São Paulo (LZB/FMV/USP-SP). The use of *S. aureus* strains was registered in SISGEN (protocol number AF210ED).

As soon as the clinical strains arrived at Federal University of Fronteira Sul (Realeza-PR), they were inoculated in 0.8 mL of sterile MHB and incubated at 37°C for 24 h. Then, 20% of sterile glycerin was added to the microtubes containing strains. These microtubes (mother-inoculums) were frozen at –80 °C until further utilization.

Before utilization of the strains in biofilm tests, a loop of each mother-inoculum was inoculated in 5 mL of sterile MHB tubes and incubated at 37°C for 24 h. Subsequently, a 0.9% NaCl solution was added to each strain tube until inoculum turbidity reached 0.5 McFarland (CLSI, 2018). Tests of biofilm formation were performed using the biofilm formator strain *S. aureus* ATCC 6538 (AOAC, 1990).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC values were determined by the broth microdilution technique (CLSI 2018). The initial concentration of each EO was 10% (20 µL EO in 180 µL MHB), and the final concentration in the microplate was approximately 0.009% of EO. To permit EO solubilization in broth, 1% polysorbate 80 was added. An amount of 10 µL of standardized inoculum at 0.5 McFarland was pipetted in triplicate to each well of a microplate, as well as negative control (MHB only), EO control (EO plus MHB), and positive control (inoculum plus MHB). After microplate incubation at 37°C for 24 h, MIC was determined by reading the $OD_{625\ nm}$ in an Elisa Multiskan FC (Thermo Scientific®) apparatus. The MIC values were interpreted as the well in which OD had the highest EO dilution, similar to the OD of the negative control.

The MIC was determined after 25 µL of 0.01% resazurin were pipetted in every well to determine microbial metabolism. After 1 h incubation at 37°C, a blue color indicated the absence of microbial metabolism,

and a pink color indicated its presence. The first blue well in a row corresponded to MIC (Coban, 2012).

After MIC determination, the well solutions were transferred to MHA plates and incubated at 37°C for 24 h. The MBC was considered the minimum concentration of EO capable of killing bacteria, revealed by the absence of bacterial growth.

The determined MIC indicated the amount of essential oil that is sufficient to inhibit bacterial growth; then, this specific volume of each oil was considered to develop the oil nanocapsules to perform biofilm tests on different surfaces.

Surface sterilization

Polypropylene and glass spheres with diameters of 4.0 mm were sanitized according to Marques *et al*. (2007). The spheres were soaked in 98% acetone for 10 min, rinsed with sterile distilled water, and submerged in neutral detergent. Subsequently, the spheres were rinsed again with sterile distilled water and immersed in 70% alcohol for 10 min. Finally, they were dried for 2 h at 60 °C and autoclaved at 121 °C for 15 min.

Preparation and characterization of *Citronella* or *Melaleuca* oil-loaded poli (ε-caprolactone) nanocapsules

The PCL nanocapsules containing either CEO or MEO were obtained by the nanoprecipitation technique according to Fessi *et al*. (1989), with some modifications. First, PCL (74 mg), sorbitan monoestearate (56 mg), and CEO or MEO (91 mg) were dissolved in acetone (25 mL). This phase was slowly poured (1 mL/min) into an aqueous phase containing 56 mg of polysorbate 80 and 50 mL of ultrapure water under constant stirring (2,000 rpm, 20°C) and kept in this condition until the organic solvent was removed. The particles were recovered by ultracentrifugation (15,000 rpm, 20 min, 24°C), washed with water to remove the tensoative agents, and centrifuged again. The supernatant was reserved, and the precipitate containing the nanocapsules was conditioned at room temperature until use.

Particle size and zeta potential analysis

Mean particle size and polydispersity index (PDI) were determined using dynamic light scattering (DLS) (ZS-90, Malvern®). Nanocapsules were diluted to 1:100 in distilled water and analyzed with a scattering angle of 90°, a temperature of 25 °C, and a wavelength of 659 nm. Zeta potential was assessed based on the electrophoretic mobility measured by Laser Doppler Anemometry (ZS-90, Malvern®). Samples were diluted to 1:100 in distilled water, packed in an electrophoretic cell at 25°C, and a potential of ± 150 mV was stablished. All measurements were performed in triplicate, and the results are shown as mean ± standard deviation.

Morphology

Nanocapsule morphology was investigated by scanning electron microscopy (SEM) (VEGA3, Tescan®) with an acceleration voltage of 20 kV. A drop of the nanocapsules was distributed in a metal support, and after drying, the sample was metallized with colloidal gold under a vacuum. Photomicrographs were taken with increases of 25 Kx.

Encapsulation efficiency

The percentages of MEO or CEO incorporated into PCL nanocapsules were determined by a direct method, with some modifications (Melo *et al*., 2018). A 100-µL aliquot of each nanocapsule was dissolved in acetone and vortexed for 20 min to extract the oil. The sample was diluted in methanol, filtered through a 0.45-µm pore-size filter, and analyzed by high-performance liquid chromatography (HPLC) with a PDA detector set at 203 nm (Waters® 2695 Alliance with PDA Photodiode Array detector 2998), equipped with an RP-C18 column (5 µm, 4 mm x 250 mm). The chromatographic conditions were acetonitrile: water (90:10, v/v), eluted under isocratic elution at a flow rate of 1.0 mL/min. Subsequently, the encapsulation efficiency (EE%) for each oil was calculated according to Equation (1):

$$\%EE = \frac{\text{Analytical amount of oil}}{\text{Theoretical amount of oil}} \times 100 \quad \text{(Equation 1)}$$

Biofilm formation and treatment

Maximum biofilm formation point

To guarantee that the treatment was applied until the point where *S. aureus* maximum biofilm formation occurs, a previous test was performed.

For this, 5 mL tubes of TSB were previously inoculated with *S. aureus* ATCC 6538 (biofilm formator) and incubated at 37°C for 24 h. After incubation, TSB was added until inoculum turbidity reached 0.5 McFarland (CLSI, 2018). Then, 200 µL of standardized inoculum were distributed in polypropylene microtubes. Two microtubes of *S. aureus* ATCC 6538 were immediately submitted to biofilm resuspension, without incubation (zero point).

The remaining microtubes were incubated at 37 °C and kept under constant agitation. Two tubes (duplicate) of bacteria were withdrawn from incubation, and biofilm was resuspended and read at intervals of 4, 8, 24, 48, 72, 96, 120, and 144 h.

To resuspend the formed biofilm, all surfaces tested in this experiment were submitted to the process described by Stepanović *et al*. (2000), with some modifications. After incubation, each surface was individually washed three times with 200 µL of 0.9% NaCl saline, dried at room temperature, fixed for 15 min with 200 µL of methanol, and left to dry. Then, 200 µL of 1.0% crystal violet were added to the polypropylene microtubes for 10 min. After stain removal with distilled water, acetic acid was added to the microtubes, and the final solution was transferred to a new microplate to allow analysis of optical density $(OD)_{570\,nm}$ in an Elisa apparatus (Thermo Scientific®, Waltham, MA, EUA).

A graph with absorbances obtained demonstrated the moment of maximum biofilm formation; this maximum time point was used to analyze biofilm formation of the clinical strains of *S. aureus* on different surfaces.

Biofilm on surfaces

Clinical *S. aureus* strains were evaluated for their efficacy to form biofilms on polypropylene and glass surfaces. To simulate these surfaces, spheres of 4.0 mm in diameter of these materials were used.

To test biofilm formation on polypropylene and glass, after inoculation of clinical *S. aureus* strains in TSB at 37°C for 24 h, TSB was added to each strain tube until inoculum turbidity reached 0.5 McFarland (CLSI, 2018). Each standardized inoculum was pipetted in duplicate into a 96-well microplate. Polypropylene and glass spheres were inserted into each well.

In total, the clinical *S. aureus* strains were submitted to 10 different procedures. In two procedures, the strains were incubated with polypropylene or glass, without any treatment. Other procedures consisted of the application of different treatments to spheres of polypropylene or glass: free citronella (CEO) and melaleuca (MEO) oils, nanoencapsulated citronella oil (CNC), and nanoencapsulated melaleuca oil (MNC).

Free melaleuca and citronella oils were added in the same amount as used for MIC determination. To permit more precise pipetting of oils, MEO and CEO were diluted in water with 1% of polysorbate 80. Controls containing only a mixture of water and polysorbate 80 were used.

For nanoencapsulated citronella or melaleuca oil, the volume of solution to be added was calculated individually, considering each strain, according to Equation (2):

$$\text{Volume of Nanocapsules} = \frac{\text{MIC x Po x Vol}}{\text{5 x EE\% x 91}} \quad \text{(Equation 2)}$$

where MIC therm represents the amount of each oil pipetted in the MIC test as a percentage (%); Po therm represents the weight of 100 µL of EO (for citronella, 0.0860 g; for melaleuca, 0.0875 g); Vol therm represents the final volume of nanocapsules obtained after centrifugation (1.5 mL for citronella and 1.4 mL for melaleuca); EE% therm is the efficiency of encapsulation as a percentage (%).

After the treatments, microplates were incubated for 120 h at 37°C (point obtained in 6.2.1 session). Subsequently, the spheres were submitted to biofilm resuspension. To resuspend the formed biofilm, all surfaces tested in this experiment were submitted to a process described by Stepanović *et al*. (2000), with some modifications. After incubation, each surface was

individually washed three times with 200 µL of 0.9% NaCl saline, dried at room temperature, fixed for 15 min with 200 µL of methanol, and left to dry. Then, 200 µL of 1.0% crystal violet were added to the wells containing spheres for 10 min. The stain was removed with sterile distilled water, and the spheres were transferred to a new microplate and left to dry prior to biofilm resolubilization with 160 µL of 33% glacial acetic acid, in each well. The OD in each well was evaluated at 570 nm in an Elisa apparatus (Thermo Scientific®, Waltham, MA, EUA).

Statistical analysis

The MIC and MBC values and the interference with biofilm formation considering treatments and surfaces were analyzed by Shapiro-Wilk and D´Agostino normality tests. For MIC and MBC values, medians and percentiles were calculated by descriptive statistics. Combinations among all groups were performed through two-way ANOVA, and one-way ANOVA followed by Dunn´s test was performed to compare groups with the same surface. The differences between controls (polypropylene and glass), oils, and nanoencapsulated oils were analyzed directly in pairs, using the one-tailed t test. All statistical analyses were performed in GraphPad Prism 7.01 with significant different at $p < 0.05$.

RESULTS

Determination of MIC and MBC by essential oils

Both MEO and CEO demonstrated bacteriostatic and bactericidal effects against the 27 *S. aureus* strains tested. In this experiment, CEO demonstrated lower MIC and MBC values than those obtained with MEO. The descriptive statistics of these data are in Table I.

TABLE I - Descriptive statistics of MIC and MBC results obtained using melaleuca and citronella essential oils against clinical strains of *S. aureus* (n = 27)

	MIC Melaleuca (%)	MIC Citronella (%)	MBC Melaleuca (%)	MBC Citronella (%)
Minimum	0.078	0.078	0.313	0.078
25th Percentile	0.625	0.156	1.250	0.313
Median	0.625	0.313	1.250	0.313
75th Percentile	1.250	0.313	2.500	0.625
Maximum	2.500	0.625	10.000	2.500

MIC – Minimum Inhibitory Concentration; MBC – Minimum Bactericidal Concentration.

Although both oils showed inhibitory activity of 0.078%, the median achieved by MEO was double than that obtained with CEO. In addition, the 75th percentile indicates that 20 of the 27 strains were inhibited by up to 1.25% with MEO, and 20 strains were inhibited by up to 0.31% with CEO. Even though the strain that required the maximum concentration of MEO was not the same as that requiring the maximum CEO concentration, the maximum concentration of MEO was four-fold higher that required with CEO. This difference is observed even when mediums of MEO and CEO are compared. MEO presented a medium MIC of 0.981%, with 0.249% for CEO; for the MBC parameters, MEO presented a medium of 2.840%, with 0.503% for CEO. Thus, our data indicate that CEO has a higher bacteriostatic activity than MEO.

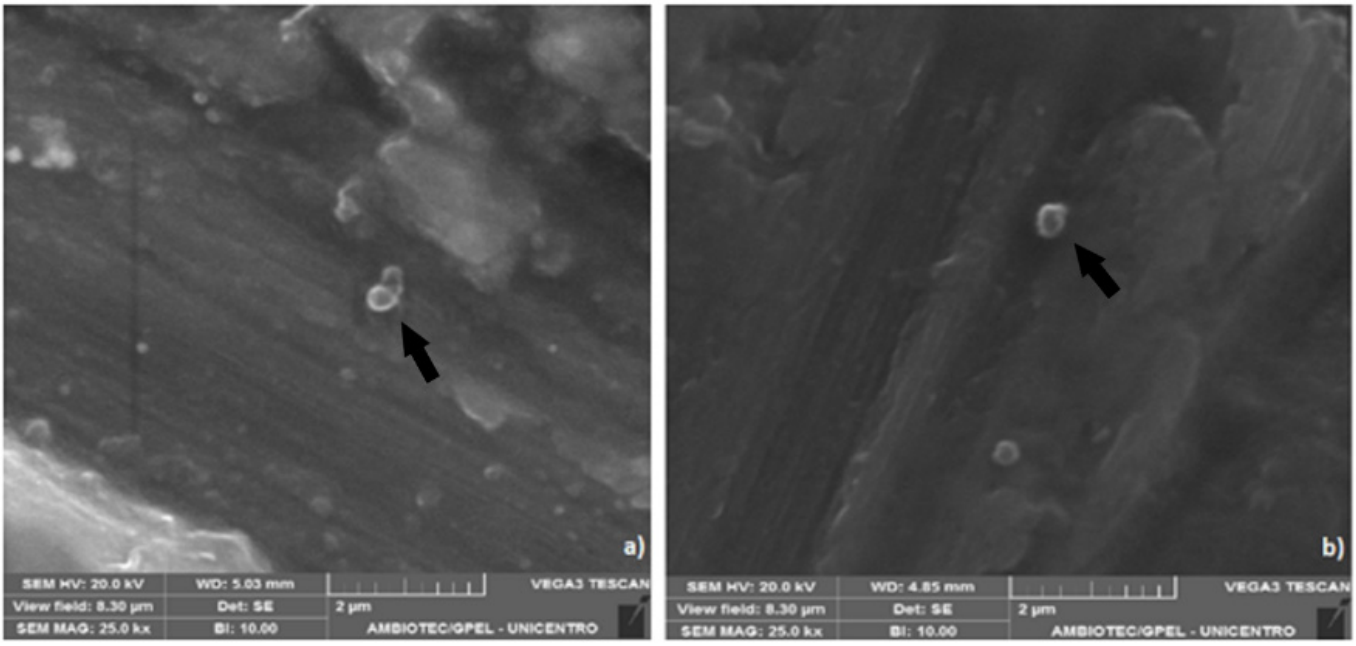
The *S. aureus* ATCC 6535 control strain also was tested and presented a medium MIC and MBC values of 0.65%.

Considering the bactericidal potential of the EOs against *S. aureus* strains, CEO showed a greater

effectiveness than MEO. In some cases, the bactericidal concentration was the inhibitory concentration itself, and when compared to MEO, four-fold less essential oil was required to promote bacterial death.

Preparation and characterization of PCL nanocapsules containing CEO or MEO

The PCL nanocapsules containing CEO or MEO could be obtained by the nanoprecipitation method and had an ovoid shape (Figure 1).



Note: scanning electron (SEM) images of citronella (a) and melaleuca (b) nanocapsules. Images are in 10,000 x magnification.

FIGURE 1 - SEM images of polycaprolactone nanocapsules loaded with melaleuca or citronella essential oil.

The mean diameter according to DLS analysis was about 280 nm. The IPD was low, demonstrating a monomodal size distribution. The zeta potential revealed that the nanocapsule surface presented a negative charge. For both oils, the EE% using PCL as polymer was greater than 90%. The main characteristics of the nanocapsules are shown in Table II.

TABLE II - Characteristics of polycaprolactone nanocapsules loaded with citronella or melaleuca essential oil (n = 3)

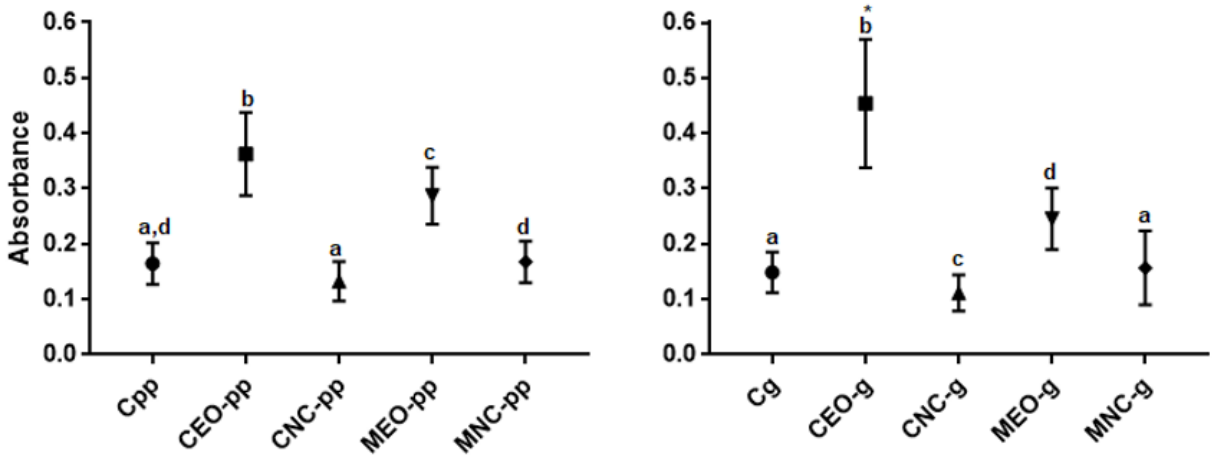
Formulation	Mean diameter ± SD (nm)	PDI ± SD	Zeta Potential ± SD (mV)	EE ± SD (%)
CNC	280.7 ± 3.7	0.102 ± 0.014	-31.6 ± 5.35	98.73 ± 1.14
MNC	281.5 ± 7.2	0.162 ± 0.016	-32.5 ± 4.99	97.13 ± 1.62

Citronella Nanocapsules (NCC); Melaleuca Nanocapsules (NCM); Polydispersity Index (PDI), adimensional; Efficiency of Encapsulation, in percentage (EE%) Mean diameter, in nanometers (nm), and Zeta Potential in millivolts (mV). Values were calculated based on triplicate measurements for each kind of nanocapsule.

Biofilm formation and treatment

The biofilm-forming bacterial strain *Staphylococcus aureus* ATCC 6538 was used to determine the moment of maximum biofilm formation. We selected this strain because of its characteristics shown in AOAC (1990) protocols regarding biofilm tests. The test revealed that *S. aureus* ATCC 6538 showed maximum biofilm formation at 120 h.

Clinical strains of *S. aureus* were prepared and biofilm formation on surfaces was analyzed. After 120 h of surface incubation (polypropylene or glass) in TSB and with standardized strains, and with different treatments (MEO, CEO, or their respective nanocapsules), biofilms were resuspended, and $OD_{570\,nm}$ was determined. Results are presented in Figure 2.



Note: C - Control: *S. aureus* without treatment; CEO: non-encapsulated Citronella oil; MEO: non-encapsulated Melaleuca oil; CNC: encapsulated Citronella oil; MNC: encapsulated Melaleuca oil. The "pp" indicates polypropylene surface (a) and "g", glass surface (b). Similar letters in graph bars of the same surface means no statistical differences ($p > 0.05$) among the treatments, while different letters indicate a significant difference ($p < 0.05$). Asterisks indicate statistical differences between polypropylene and glass, considering the same treatment.

FIGURE 2 - Statistical differences between biofilm formation, in absorbance, considering polypropylene (a) or glass (b) as the test-surface.

The clinical strains of *S. aureus* were able to form biofilms at the same intensity on both tested surfaces when no treatment was applied (control groups). When both surfaces were treated with free CEO or MEO, biofilm formation increased in comparison to the control groups. Comparing the free oils, MEO resulted in lower biofilm formation than CEO on polypropylene ($p < 0.001$) and glass ($p = 0.001$).

Nanoencapsulated CEO significantly reduced biofilm formation on the glass surface ($p = 0.030$) and showed a tendency to diminish biofilm formation on the polypropylene surface ($p = 0.051$), when compared to controls for each surface. Nanoencapsulated MEO was not able to significantly reduce biofilm formation on both surfaces when compared with the control groups. Furthermore, comparatively, nanoencapsulated CEO was more effective than nanoencapsulated MEO at decreasing

biofilm formation on polypropylene ($p = 0.026$) and glass ($p = 0.002$) surfaces when compared to the respective controls.

DISCUSSION

The inhibitory and antimicrobial effects of citronella and melaleuca essential oils against 27 clinical strains of *S. aureus*, obtained from bovine persistent mastitis cases, were evaluated. Both essential oils mainly contain terpenoids as active compounds, originating from secondary plant metabolism, that protect the plants against predators, UV light, insects, fungi, and bacteria. Terpenoids have an amphipathic characteristic that allows interactions between them and cell membranes as well as other structures (Singh, Fatima, Hameed, 2016; Saporito *et al*., 2018; Zhang *et al*., 2018; Kokina *et al*., 2019).

Considering all strains tested, CEO demonstrated a medium MIC of 0.249% (v/v) and medium MBC of 0.503%, lower values when compared with the findings of Oussalah *et al*. (2007), who tested citronella EO (*Cymbopogon winterianus)* against *S. aureus* strains and obtained a medium MIC ≥ 0.8% (v/v) and an MBC ≤ 0.8% (v/v). Another citronella oil species tested in Oussalah *et al.*'s (2007) experiment, *Cymbopogon martinii*, revealed an MIC ≤ 0.40% (v/v) and an MBC ≤ 0.40% (v/v) against *S. aureus*; these values are more similar to our findings since only two strains showed an MIC higher than 0.6% (v/v) and an MBC ≥ 0.08% (data not shown). Differences in EO composition and techniques may explain these variations, where a higher amount of terpenoids could cause a decrease in MIC and MBC values, as indicated by Kim *et al*. (1995).

The MEO demonstrated a medium MIC of 0.981% (v/v) and a medium MBC of 2.840% for the clinical strains, while for the control strain *S. aureus* ATCC 6538, it showed MIC and MBC values of 0.65%. Carson, Mee, and Riley (2002) obtained a MIC of 0.5% for *S. aureus* ATCC 9144, while Oliva *et al*. (2018) obtained a MIC close to 0.5% using a methicillin-sensitive strain (MSSA) and a resistant strain (MRSA). In a study performed by Araujo and Longo (2016), clinical strains, compared with ATCC strains, showed different sensitivities against antimicrobials. Clinical strains tend to show the highest MIC values when exposed to external agents and, consequently, exhibit different patterns of resistance when compared to purified ATCC strains (Araujo, Longo, 2016). Moreover, adverse external conditions cause a thickening of the lipid content of the bacterial membrane, making it more resistant to bactericides.

In our study, the oils were loaded into PCL nanocapsules to verify their efficacy at inhibiting biofilm formation by *S. aureus* strains. Nanocapsules were obtained by the nanoprecipitation method, which consists of the deposition of the PCL polymer over the oil core. In this method, polymer precipitation occurs before solvent evaporation, resulting in solidified particles with an irregular shape. The size of the nanocapsules is according to the requirements for biological applications, and PDI showed a monodisperse size distribution, since values < 0.25 were obtained (Fessi *et al*., 1989; Miladi *et al*., 2016; Melo *et al*., 2018).

The zeta potential of the nanocapsules was negative, which could play a key role in the physical stability of the nanosuspension due to repulsion among particles, preventing aggregation (Miladi *et al*., 2016; Melo *et al*., 2018; Kokina *et al*., 2019). Encapsulation efficiency was high due to the entrapment of oil phase, represented by EOs in the core surrounded by the PCL covering, avoiding oil leakage into the aqueous phase (Miladi *et al*., 2016; Melo *et al*., 2018).

PCL is an anionic, biodegradable, biocompatible polyester often found in nanoformulations for a variety of biological applications, including treatments against bacterial biofilms (Budri *et al*., 2015; Duncan *et al*., 2015; Miladi *et al*., 2016; Saporito *et al*., 2018). One of the main characteristics of PCL nanocapsules concerns the release kinetics of the encapsulated substance. Many studies have demonstrated that surface erosion of PCL nanocapsules occurs within 120 days, a time considered superior to that of other polymers used to prepare nanocapsules (Costa *et al*., 2015; Miladi *et al*., 2016; Saporito *et al*., 2018). It is possible to infer that during the time in which the EO is contained in the core of the nanocapsule, it is protected from agents and is slowly released to the outside (Duncan *et al*., 2015; Kokina *et al*., 2019; Miladi *et al*., 2016). Due to the low glass transition temperature of PCL (between -60 and -70°C), this polymer presents itself as a soft rubber at room temperature; in nanostructures, it confers permeability to small particles (Costa *et al*., 2015).

A previous test of biofilm formation was performed before treating different surfaces with the PCL nanocapsules. Maximum biofilm formation was observed after 120 h (5 days), and these results are concordant with those obtained by Leite (2008), where SEM images also showed maximum biofilm formation after 120 h. This stage was performed prior to the surfaces tests to avoid false positives, since a biofilm reading after the moment of maximum biofilm formation could demonstrate lower results due to the potential accumulation of toxic components of the colony itself, falsifying the results. In addition, this amount of time was possibly necessary for *S. aureus* to obtain a minor distance between material and colony bacteria. An experiment conducted by Chaves (2004) revealed that when bacteria reduce the distance between external membrane and surface to 5-20 nm, adhesion of planktonic cells turns irreversible, and the biofilm can mature and become established (Araújo *et al*., 2010).

Nanoencapsulated or free CEO and free MEO were evaluated for their efficacy at inhibiting biofilm formation of *S. aureus* on different surfaces. Only CEO nanocapsules were effective against biofilm formation. Both free EOs presented the opposite effect, facilitating more biofilm formation, also when compared with the groups without any treatment. This most likely, this occurred because the free oils were readily consumed or because the concentration of the oil used in the experiment for biofilm formation on surfaces was based on MIC values (a bacteriostatic measure) and not on MBC values (bactericidal potential). Therefore, some remnant cells could restart the colony, forming biofilms to protect the bacteria. When no treatment was applied, the colony possibly reached stabilization and the population declined due to the accumulation of toxic products. In this way, biofilm formation was lower than with the addition of free oils.

In solution, free CEO is capable to modify the selective permeability of the membrane, thereby changing the proton motive force. This causes the bacteria to pump protons to the exterior for glucose uptake from the environment. When glucose is lacking, the bacteria cannot remain stable and die (Sikkema, Bont, Poolman, 1994; Singh, Fatima, Hameed, 2016). Similarly, free MEO causes potassium leakage, also promoting bacterial destabilization (Carson, Mee, Riley, 2002; Oliva *et al*., 2018). However, in our experiment, the non-elimination of bacteria possibly enabled the activation of defense mechanisms of the bacterial colony to protect the remaining cells. It is known that *S. aureus* modifies the surface charge as a response mechanism to external agents and forms biofilms to mitigate environmental stress (Rigby, DeLeo, 2012).

One explanation for the difference in the efficacy between free CEO and MEO and nanoencapsulated oils may be their different release profiles from nanocapsules (Duncan *et al*., 2015; Souza *et al*., 2017). The oil nanocapsule may have entered the biofilm structure, and due to their small size, they were effective even at MIC (Duncan *et al*., 2015; Miladi *et al*., 2016; Souza *et al*., 2017; Perez-Lopez *et al*., 2019).

Citronellal, due to its linear chemical structure and higher hydrophilia, may be released by diffusion faster than terpinen-4-ol, which presents a cyclic structure. Due to the increased size of carbon chains and higher cyclization in the terpene molecule, this compound has more pronounced lipophilic characteristics than those with small acyclic chains (Martins, Lopes, Andrade, 2013; Sikkema, Bont, Poolman, 1994). In this case, citronellal, a major linear aldehyde found in CEO, can establish more hydrogen bonds than terpinen-4-ol, the main terpenoid found in MEO. Consequently, besides being able to escape from the nanocapsule structure more easily than terpinen-4-ol, citronella oil can also act on hydrophilic surfaces, such as glass, with greater effectiveness. On polypropylene, the hydrophobicity presented on the surface interface with the external environment avoids adherence of hydrophilic substances (Araújo *et al*., 2010; Martins, Lopes, Andrade, 2013). Therefore, it is likely that non-encapsulated MEO was more effective than non-encapsulated CEO because of the solubility and polarity of its major compounds (Rigby, DeLeo, 2012).

CONCLUSION

Based on our results, CEO and MEO demonstrate bacteriostatical and bactericidal potential and can be used to develop a pharmaceutical form against *S. aureus*. Besides, when exploring the nanoencapsulated forms,

CEO interacted more significantly with hydrophilic substances, such as glass, hindering biofilm formation. Despite the polar characteristics of the main CEO compounds, nanoencapsulated CEO demonstrated a tendency of biofilm reduction on polypropylene. In contrast, nanoencapsulated MEO could not interact sufficiently with the biofilm matrix and surfaces. Further studies are necessary to enhance the antibacterial and antibiofilm properties of these oils and their respective nanoformulations to improve their efficiencies against bacterial colonies on various surfaces.

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