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# Control of *Staphylococcus aureus* biofilms by the application of single and combined treatments based in plant essential oils

Daniel Vázquez-Sánchez*, Juliana Antunes Galvão, Marina Rodrigues Mazine, Eduardo Micotti Gloria, Marília Oetterer

*Department of Agri-Food Industry, Food and Nutrition, "Luiz de Queiroz" College of Agriculture (ESALQ), University of São Paulo (USP), Brazil*

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ABSTRACT

Effective and environmentally-friendly alternatives to traditional disinfectants are necessary to reduce the pollution and the emergence of antimicrobial-resistant bacterial strains in food-related environments. In the present study, treatments based in single and combined applications of plant essential oils (EOs) were evaluated for control *Staphylococcus aureus* biofilms. EOs of *Lippia sidoides*, *Thymus vulgaris* and *Pimenta pseudochariophyllus* showed a higher efficacy than peracetic acid and sodium hypochlorite against *S. aureus* planktonic cells and 24-h-old biofilms formed on polystyrene and stainless steel under food-related conditions. High concentrations of thymol and chavibetol were detected in these EOs, as well as the presence of other antimicrobial compounds such as carvacrol, eugenol, p-cymene, limonene, α-pinene, α-terpineol, terpinen-4-oil and linalool. *L. sidoides* oil were particularly effective against *S. aureus*, but doses higher than 2.75% (v/v) were required to completely eradicate 24-h-old biofilms. Binary combinations of *L. sidoides*, *T. vulgaris* and *P. pseudochariophyllus* allowed decrease significantly doses required to reduce 99.99% the number of biofilm cells. Furthermore, peracetic acid increased its efficacy against *S. aureus* biofilms by the combined application with these EOs. The most effective treatments against *S. aureus* biofilms were those combining *L. sidoides* with *T. vulgaris* or peracetic acid. Therefore, these EO-based treatments can be considered as an effective and environmentally-friendly alternative to control *S. aureus* biofilms in food-contact surfaces.

## 1. Introduction

One of the major concerns of the food industry is foodborne intoxications caused by the consumption of foodstuffs contaminated with staphylococcal enterotoxins (EFSA, 2016; Hennekinne et al., 2012; Kadariya et al., 2014). Most of control strategies applied were focused in the food handlers, as *Staphylococcus aureus* is part of the normal microbiome associated with human skin, throat and nose (DeVita et al., 2007; Sattar et al., 2001; Simon and Sanjeev, 2007; Wertheim et al., 2005). Nevertheless, food-contact surfaces can be also colonized by *S. aureus* through the development of biofilms, a community of cells embedded in a self-produced matrix of extracellular polymeric substances mainly composed by proteins, polysaccharides and extracellular DNA (Flemming and Wingender, 2010; Lister and Horswill, 2014). This matrix can highly reduce the efficacy of antimicrobial agents by slowing down their infiltration and neutralizing, binding and effectively diffusing them to sub-lethal concentrations before they can reach cell targets (Bridier et al., 2011; Srey et al., 2013; Vázquez-Sánchez and Rodríguez-López, 2018). Moreover, biofilm cells can counteract the effect of antimicrobials by activating efflux pumps, increasing or reducing the expression of the antimicrobial targets, liberating DNA that promotes the synthesis of biofilm matrix, and generating physiological changes between cells growing in the different interfaces of the biofilm (Anderson and O'Toole, 2008; Bridier et al., 2011; Vázquez-Sánchez and Rodríguez-López, 2018). Nearby biofilm cells can also exchange mobile genetic elements containing new antimicrobial resistance (Madsen et al., 2012).

The emergence of biocide resistances and the high environmental impact of disinfectants currently applied in the food industry have led to the search of novel antimicrobial compounds and develop innovative sanitizing procedures to control undesirable microorganisms such as *S. aureus* (Langsrud et al., 2003; Morente et al., 2013; Stanga, 2010; Zabala et al., 2011). A promising alternative to maintain the food safety and preservation are plant essential oils (EOs) with antimicrobial properties (Calo et al., 2015; Hyldgaard et al., 2012). The versatile composition of EOs can generate that several bacterial cell targets are

* Corresponding author at: Laboratório de Qualidade e Inovação Tecnológica de Pescado, Departamento de Agroindústria, Alimentos e Nutrição, Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Universidade de São Paulo (USP), Av. Pádua Dias 11, CEP: 13418-900 Piracicaba, SP, Brazil.
*E-mail addresses:* danielvazquezsanchez@gmail.com, danielvs@usp.br (D. Vázquez-Sánchez).

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attacked simultaneously through diverse modes of action (Calo et al., 2015; Hyldgaard et al., 2012; Nazzaro et al., 2013), which could avoid (or at least reduce) the appearance of biocide resistances. EOs mostly composed by aldehydes, phenols and terpene alcohols have showed a high antimicrobial activity (Bassolé and Juliani, 2012; Perricone et al., 2015). However, interactions between the major and the minor constituents of EOs can also produce synergistic, additive and antagonistic effects that affect to the antimicrobial activity (Bassolé and Juliani, 2012). The activity of EOs mainly affect the integrity of bacterial cell wall and membrane, causing damage in membrane proteins and the disruption of the proton motive force, which lead to release of cell contents and cell lysis (Lopez-Romero et al., 2015; Saad et al., 2013). In addition, the effectiveness of EOs against bacterial biofilms seems to depend on the reactivity, hydrophobicity and diffusion rate of EOs into the biofilm matrix, as well as the composition and architecture of the biofilms (Vázquez-Sánchez et al., 2015).

The present study thus aimed to develop EO-based treatments highly effective against *S. aureus* biofilms formed on stainless steel and polystyrene, two materials usually found in the food industry. With this aim, EOs with high antimicrobial activity against *S. aureus* planktonic cells were selected and characterized chemically. *S. aureus* biofilms were then exposed to selected EOs in single applications, binary combinations and combinations with peracetic acid to determine the most effective EO-based treatment.

## 2. Material and methods

### 2.1. Bacterial strains and culture conditions

Two *S. aureus* strains (S8 and S10) isolated from tilapia-processing facilities were investigated. They have been previously identified as *S. aureus* by specific biochemical (catalase and coagulase) and genetic tests (*23s* rDNA), and subsequently genotyped as different strains by RAPD-PCR (Vázquez-Sánchez et al., 2017). S8 was characterized as an enterotoxin *seb* gene carrier, whereas S10 carried enterotoxin *sea*, *seh* and *sei* genes. In addition, these strains showed a higher biofilm-forming ability on stainless steel (S8) and polystyrene surfaces (S10) than *S. aureus* ATCC 6538, a reference Gram-positive strain in United States and European bactericidal standard tests.

Bacterial stocks of each strain were maintained at −80 °C in tryptic soy broth (TSB) (Kasvi, Brazil) containing 20% glycerol (v/v). Strains were thawed and sub-cultured twice in TSB at 37 °C for 24 h under static conditions prior to each experiment.

### 2.2. Antimicrobial assays

#### 2.2.1. Antimicrobial agents

Eleven pure essential oils (EOs) previously extracted by Ambrosio et al. (2017) from leaves and branches were tested (Table 1). They were diluted in ultrapure water with 0.15% (w/v) bacteriological agar (Kasvi) as stabilizing agent (Remmal et al., 1993). Peracetic acid (39% v/v) and sodium hypochlorite provided by Sigma-Aldrich (Brazil) were also evaluated as examples of industrial disinfectants, being diluted in ultrapure water. Working concentrations of all antimicrobial agents were prepared prior to each assay.

#### 2.2.2. Minimal bactericidal concentration (MBC)

The efficacy of disinfectants against planktonic cells of *S. aureus* strains was assessed in terms of MBC (i.e. the lowest concentration at which no viable cells were detected under experimental conditions). An optimized method based in Mann and Markham (1998) was followed. Overnight bacterial cultures were adjusted to an absorbance value at 700 nm of 0.100 ± 0.01 with phosphate buffer saline (PBS, composed by 7.6 g/L NaCl, 0.2 g/L KCl, 0.245 g/L $Na_2HPO_4$ and 0.71 g/L $K_2HPO_4$ (Labsynth, Brazil)), which corresponds to $10^8$ CFU/mL approximately. Inoculum size was checked in all cases by plating on tryptic soy agar (TSA) (Kasvi). PBS-suspended cells were then serially diluted in TSB, and aliquots of 75 µL (containing approximately $7.5 \times 10^4$ CFU) were exposed to 75 µL of a specific concentration of each disinfectant in a sterilized 96-well U-bottom microtiter plate (Kasvi). Planktonic cells were exposed to final concentrations of 0.010%, 0.025%, 0.050%, 0.075%, 0.100%, 0.500%, 1%, 2%, 3% and 4% (v/v) of each disinfectant, being tested in triplicate in two independent experiments. A positive control with no disinfectant, a negative control with no inoculum and a blank with medium only were included in all assays. After 24 h of incubation at 37 °C under static conditions, wells were stained with 10 µL of 0.01% (w/v) resazurin sodium salt solution (Sigma-Aldrich, Brazil) and incubation continued for further 2 h. Color change from blue to pink indicated the presence of viable cells in cultures. Visually undetectable growth was also checked by plating 0.1 mL of cultures on TSA and incubating for 24 h at 37 °C.

**Table 1**
Origin of plant essential oils used in this study.

| Origin | Plant species | Common name |
|---|---|---|
| Campinas (SP/Brazil) 22°53′36.3″S 47°03′55.6″W | *Cordia verbenacea* | Black-sage |
| Itatinga (SP/Brazil) 22°59′34.8″S 48°41′14.4″W | *Corymbia citriodora* | Lemon-scented gum |
| | *Eucalyptus camaldulensis* | River red gum |
| | *Eucalyptus staigeriana* | Lemon ironbark |
| | *Eucalyptus urograndis* | *Eucalyptus urograndis* |
| Campinas (SP/Brazil) 22°47′42.5″S 47°06′40.4″W | *Cymbopogon winterianus* | Lemongrass |
| | *Lippia sidoides* | Pepper-rosmarin |
| | *Thymus vulgaris* | Thyme |
| Piracicaba (SP/Brazil) 22°43′31″S 47°38′57″O | *Melaleuca alternifolia* | Tea tree |
| | *Melaleuca leucadendron* | White paperbark |
| Registro (SP/Brazil) 24°29′16″S 47°50′38″W | *Pimenta pseudochariophyllus* | Craveiro |

**Table 2**
Coded and natural values of independent variables used in central composite rotatable experimental designs.

| Coded values | | Natural values (% v/v) | |
|---|---|---|---|
| Disinfectant 1 | Disinfectant 2 | Disinfectant 1 | Disinfectant 2 |
| 1 | 1 | 1.25 | 1.25 |
| 1 | −1 | 1.25 | 0.25 |
| −1 | 1 | 0.25 | 1.25 |
| −1 | −1 | 0.25 | 0.25 |
| 1.41 | 0 | 1.46 | 0.75 |
| −1.41 | 0 | 0.04 | 0.75 |
| 0 | 1.41 | 0.75 | 1.46 |
| 0 | −1.41 | 0.75 | 0.04 |
| 0 | 0 | 0.75 | 0.75 |

#### 2.2.3. Logarithmic reduction of viable biofilm cells (LR)

The efficacy of disinfectants against *S. aureus* biofilms was expressed in terms of LR, which was defined as the difference between the logarithm of the total number of viable cells in non-disinfectant-exposed biofilms and the logarithm of the number of surviving viable cells in disinfectant-exposed biofilms.

*2.2.3.1. Conditions for biofilm formation.* The flat bottom of 1.93 $cm^2$ of each well of 24-well polystyrene microtiter plates (Kasvi) and stainless steel coupons (AISI 304, 2B finish, laser cut) (Botam Oxicorte, Brazil) of 1 $cm^2$ (and 0.8 mm thickness) were used as experimental surfaces. Stainless steel coupons were previously soaked in 2 M NaOH to remove residues, rinsed several times with distilled water and dry-heat