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Activity of essential oil-based microemulsions against *Staphylococcus aureus* biofilms developed on stainless steel surface in different culture media and growth conditions

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ABSTRACT

Food safety is a fundamental concern for both consumers and the food industry, especially as the numbers of reported cases of food-associated infections continue to increase. Industrial surfaces can provide a suitable substrate for the development and persistence of bacterial organized in biofilms that represent a potential source of food contamination. The negative consumer perception of chemical disinfectants has shifted the attention to natural substances, such as plant extracts.

The aim of this study was to investigate the possibility of using the essential oils (EOs) in the fight against *S. aureus* biofilms. First, the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Minimum Biofilm Inhibitory Concentration (MBIC), Minimum Biofilm Eradication Concentration (MBEC) of eleven EOs against *S. aureus* were determined. *Cinnamomum cassia* and *Salvia officinalis* EOs showed the greatest antibacterial properties with 1.25% MIC and MBC, 1.25% MBIC and 2.5% MBEC respectively. Gas Chromatography/Mass Spectrometry analysis revealed cinnamaldehyde (82.66%) and methoxy cinnamaldehyde (10.12%) as the most abundant substances of *C. cassia*, while cis-thujone (23.90%), camphor (19.22%) and 1.8-cineole (10.62%) of *S. officinalis*. Three different microemulsions, formulated with *C. cassia*, *S. officinalis* or both, were finally tested against *S. aureus* biofilms in different culture media and growth conditions, causing a >3 logarithmic reductions in *S. aureus* 24 h-old biofilms and desiccated biofilms, and up to 68% of biofilm removal after 90 min of exposure. The obtained data suggest the potential use of EOs, alone or in combination, for the formulation of sanitizers as alternative or in support in the disinfection of contaminated surfaces.

1. Introduction

Throughout the food chain, industrial surfaces can provide a suitable substrate for the development and persistence of spatially-organized systems called biofilms that represent potential sources of contamination and a significant concern to the food industry (Abdallah et al., 2014; Srey et al., 2013). Biofilm is defined as a microbial community where bacteria live together in an extracellular matrix (EPS) composed of polysaccharides, extracellular DNA and proteins, and is considered a key factor for bacterial survival in different environments and a way to colonize new sites (Hall-Stoodley et al., 2004). In the nature, biofilm can develop both on abiotic and biotic surfaces and as their complexity confers to microbial cells a high level of antimicrobial resistance (Lee et al., 2015; Srey et al., 2013). The prevention of biofilm formation requires regularly cleaning and disinfection of surfaces for impeding the cells to firmly attach to contact surfaces (Simões et al., 2010; Srey et al., 2013). Many chemical products can be applied in cleaning, such as surfactants or alkali products, to suspend and dissolve food debris by decreasing surface tension, emulsifying fats, and denaturing proteins (Wessel and Ingmer, 2013). Another goal of the cleaning should be to break-up or dissolve the EPS matrix associated with the biofilm to allow penetration of disinfectants inside the bacterial cells (Simões et al., 2006). However, effectiveness of the disinfectants is limited by organic material or environmental conditions, such as pH, temperature, water hardness, chemical inhibitors; concentration and contact time are also important factors influencing disinfectants' effectiveness (Bae et al., 2012; Bremer et al., 2002; Kuda et al., 2008).

Research for new substances in biofilm disinfection is an important area of focus. The recent growing negative consumer perception against synthetic chemicals has shifted this research effort toward natural alternatives (Calo et al., 2015; de Oliveira et al., 2012; Szczepanski and Lipski, 2014). In this context, the essential oils (EOs) emerge as feasible alternative natural disinfectants with the possibility to use their secondary metabolites or their constituents to control biofilm in food industry

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(Chorianopoulos et al., 2008; Li et al., 2011; Oliveira et al., 2010; Valeriano et al., 2012). EOs are complex natural compounds, exhibiting powerful antibacterial properties, characterized by a strong odor and formed by aromatic plants as secondary metabolites (Bakkali et al., 2008; Bozin et al., 2007). Previous studies have indicated that the EOs and extracts of edible and medicinal plants, herbs and spices constitute a class of very potent natural antibacterial agents (Burt, 2004; Ma et al., 2016; Vergis et al., 2015).

Staphylococcus aureus, one of the major responsible of food-borne diseases worldwide (Hennekinne et al., 2012; Kadariya et al., 2014), lives in a wide variety of environments. It is also able to attach to contact surfaces of food processing environment, develop biofilm and survive on these surfaces (Rode et al., 2007). The ingestion of foods contaminated with pre-formed enterotoxins produced by *S. aureus* (Argudín et al., 2010) is a consequence of cross-contamination from food handlers or from environment such as contaminated surfaces, industrial equipment or kitchen settings (Argudín et al., 2012).

For this, the attention of our study was focused on the possibility of using the EOs in the fight against *S. aureus* biofilms. The experimental design was subdivided in three distinct phases in order to: (i) evaluate the antimicrobial efficiency of different EOs against *S. aureus* in planktonic growth as well as organized in biofilm, (ii) select the most active natural compounds to formulate EOs-based microemulsions, (iii) test the efficacy of these EOs-based microemulsions against biofilms of *S. aureus* developed on the surface of stainless steel coupons in different culture media and growth conditions.

2. Materials and methods

2.1. Bacterial strain, culture conditions and inoculum preparation

The reference strain *S. aureus* ATCC 43387 (American Type Culture Collection, Rockville, Maryland, USA) was routinely grown in Tryptic Soy Agar (TSA) (Oxoid, Milan, Italy) at 37 °C for 24 h and stored at −80 °C in Nutrient Broth no. 2 (Oxoid) with 15% of glycerol. To prepare the inoculums, *S. aureus* ATCC 43387 was inoculated in 50 mL of Tryptic Soy Broth (TSB) (Oxoid) and incubated overnight at 37 °C. Then, the number of cells necessary for each experiment, ranging between 10^6 and 10^7 CFU/mL, was determined by spectrophotometer ($OD_{610\ nm}$ about 0.13–0.15).

2.2. Essential oils (EOs)

Eleven pure EOs, kindly furnished by Farotti s.r.l. (Italy), were used in this study: *Cinnamomum cassia* leaf oil (100%), *Cupressus sempervirens* leaf oil (100%), *Cymbopogon nardus* oil (100%), *Cuminum cyminum* seed oil (100%), *Laurus nobilis* leaf oil (100%), *Cymbopogon citratus* leaf oil (Lemongrass) (100%), *Rosmarinus officinalis* leaf oil (100%), *Salvia officinalis* oil (100%), *Santalum album* oil (100%), *Melaleuca alternifolia* leaf oil (tee tree) (100%), *Thymus vulgaris* leaf oil (100%). The EOs were solubilized in dimethyl sulfoxide (Sigma, Italy) (DMSO)-water solution (1:1 v/v) to obtain the final concentration of 10%. The stock solutions were maintained in the dark at room temperature (RT) until use.

2.3. Preliminary screening of EOs antimicrobial activity

Initially, the susceptibility of *S. aureus* ATCC 43387 to EOs was determined using the agar well diffusion method (AWDM) according to Ramakrishnan et al. (2011), with some modifications. Briefly, 500 μL of *S. aureus* ATCC 43387 cell suspension (10^7 CFU/mL) were added to 25 mL of Nutrient Agar (Oxoid) maintained at 50 °C, poured into petri dishes and allowed to solidify for 20 min. At this point, wells of 6 mm in diameter were made on the agar with sterile stainless steel cylinders and 40 μL of each EO (10 and 1%, v/v) were dropped into the holes; in each plate, two holes, dropped with 40 μL of DMSO-water solution (1:1 v/v) and 40 μL of TSB, were used as controls. Plates were incubated for 24 h at 37 °C and the diameter of inhibition zone around each hole was measured. All the experiments were performed in duplicate.

2.4. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The EOs showing antibacterial activity in AWDM were subsequently tested for their inhibitory and bactericidal activities by microdilution method as described by Chamdit and Siripermpool (2012). Fifty microliters (about 10^6 CFU/mL) of *S. aureus* ATCC 43387 overnight culture in Mueller Hinton Broth (MHB) (Oxoid) were added in 96-well microtiter plates (Cellstar, Greiner bio-one). Then, 50 μL of each serial dilution of EOs (from 5 to 0.156%, v/v), prepared in Mueller Hinton Broth with 15% of Tween 80, were added in each well. At this concentration, Tween 80 did not affect the growth or viability of *S. aureus* ATCC 43387 (data not shown). Positive control (*S. aureus* ATCC 43387 in MHB) and negative control (MHB) were also included. The MIC was defined as the lowest concentration of EOs inhibiting visible growth after 24 h of incubation. Ten microliters from the invisible growth were inoculated in triplicate on TSA and incubated at 37 °C for 24 h; MBC was defined as the lowest concentration of EO that inhibited growth on TSA. All the experiments were performed in duplicate.

2.5. Determination of Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC)

The Minimum Biofilm Inhibitory Concentration (MBIC) and the Minimum Biofilm Eradication Concentration (MBEC) were determined as described by Chamdit and Siripermpool (2012) with some modifications. Briefly, after *S. aureus* ATCC 43387 biofilms formation in 24 well plates at 37 °C for 24 h, the medium was gently removed, the wells were washed with potassium phosphate buffer (PBS) and serial dilutions of EOs (from 10 to 1.25%, v/v) were added. Positive and negative controls represented by EOs-free wells and biofilm-free wells respectively were also included. MBIC was defined as the lowest concentration of EOs inhibiting the visible growth after 24 h incubation. Wells with no visible growth were PBS washed; then, adherent bacteria were mechanically removed and spread on TSA plates. The MBECs were determined as the lowest concentration of EOs at which no growth occurred on TSA. All tests were performed in duplicate.

2.6. Gas Chromatography/Mass Spectrometry (GC/MS) analysis of selected EOs

Quantitative and qualitative analysis of *C. cassia* and *S. officinalis* EOs, selected on the basis of their antimicrobial/anti-biofilm activities, were determined by Gas Chromatography/Mass Spectrometry (GC–MS) using Agilent 6890 N coupled to a mass spectrometer 5973 N and a non-polar capillary column HP-5MS (5% phenyl methyl siloxane, 30 m, 0.25 mm, 0.1 μm). It was set to the following temperature program of the oven: 60 °C for 5 min, then 4 °C/min up to 280 °C for 15 min, and finally 15 °C/min up to 300 °C. The total time of the chromatographic run was 65 min, with injector and detector temperature of 280 °C. The essential oils were diluted in n-hexane (1:100), then 2 μL were injected to the gas chromatograph in split mode (1:50), with a collection interval between 29 and 400 *m/z*. Mass spectra were acquired in electron impact mode (EI), with a voltage of 70 eV ionization. Identification of the volatile components was achieved by comparison of the mass spectrum and retention index of the peaks in the chromatogram with those of commercial databases (WILEY, NIST08, ADAMS, FFNSC2). Where possible, available commercial standards have been employed.