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# Antimicrobial activity of free and liposome-encapsulated thymol and carvacrol against *Salmonella* and *Staphylococcus aureus* adhered to stainless steel

Juliana Both Engel[a], Caroline Heckler[a], Eduardo Cesar Tondo[a], Daniel Joner Daroit[b], Patrícia da Silva Malheiros[a,*]

[a] *Departamento de Ciências dos Alimentos, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul (ICTA-UFRGS), Porto Alegre, Rio Grande do Sul, Brazil*

[b] *Laboratório de Microbiologia, Universidade Federal da Fronteira Sul, Campus Cerro Largo, Rio Grande do Sul, Brazil*

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ABSTRACT

Antimicrobial activity of thymol, carvacrol and thymol/carvacrol liposomes (TCL) was evaluated against two bacterial pools, each one consisting of four strains of *Staphylococcus aureus* or *Salmonella enterica*. TCL were prepared using thin-film hydration, showing 270.20 nm average diameter (polydispersity index of 0.33) and zeta potential of + 39.99 mV. Minimum inhibitory concentration (MIC) of thymol, carvacrol and TCL against *S. aureus* pool was 0.662 mg/ml, while MIC for *Salmonella* pool was 0.331 mg/ml for thymol and carvacrol, and for TCL was 0.662 mg/ml. Bacterial pools (8.0 log CFU/ml), allowed in contact on stainless steel AISI 304 coupons in UHT skim milk for 15 min, resulted in adhered populations of 5.6–6.1 log CFU/cm$^2$. Adhered *S. aureus* ( ± 6.1 log CFU/cm$^2$) were inhibited after 1-min and 10-min treatments using thymol or carvacrol at MIC and 2.0 MIC. Reductions of 1.47–1.76 log CFU/cm$^2$ and 1.87–2.04 log CFU/cm$^2$ were obtained using 0.5 MIC of thymol and carvacrol, respectively. A 10-min contact with free (MIC and 2.0 MIC) and encapsulated (MIC) antimicrobials inhibited attached *Salmonella* ( ± 6.0 log CFU/cm$^2$); however, after 1-min of contact, 2.0 MIC of thymol and carvacrol were not able to inactivate adhered *Salmonella* MIC of TCL inactivated *S. aureus* and *Salmonella* after 10 min; however, after 1-min contact, adhered *S. aureus* and *Salmonella* populations were decreased in 1.62 log CFU/cm$^2$ and 2.01 log CFU/cm$^2$, respectively. Considering antimicrobial concentrations and contact times, thymol, carvacrol, and TCL could be employed in food-contact surfaces to prevent biofilm formation at early stages of bacterial attachment. Further investigations should be performed considering long-term antibacterial effects of TCL.

## 1. Introduction

Microbial adhesion to food contact surfaces is a serious concern for food industries and food services, because attachment can promote cell survival and biofilm formation, causing cross-contamination, reduced product shelf life and foodborne diseases. In fact, biofilm formation onto these surfaces is a major factor contributing to the occurrence of foodborne outbreaks (Shi and Zhu, 2009; Bridier et al., 2015). Surface properties have an important role in microbial attachment. Stainless steel, for instance, is widely used for the manufacture of equipment employed by the food industry due to its excellent physicochemical properties, ease of fabrication, relative low cost, mechanical strength and high resistance to corrosion. Nevertheless, this material has a high free surface energy, resulting in hydrophilic properties that usually favors bacterial attachment and biofilm formation (Chmielewski and Frank, 2003; Van Houdt and Michiels, 2010).

Therefore, cleaning and disinfection processes should be common practices in order to remove attached microorganisms and avoid biofilm formation. Particularly, disinfection is of utmost importance considering that cleaning is not designed to kill a sufficient amount of the bacterial population (Srey et al., 2013). Many commercial disinfectants are used in food industries, although chlorine is probably the most widely used due to its antimicrobial spectrum, ease of application and cost-effectiveness. Nevertheless, chlorine is readily inactivated by organic materials, its efficacy is pH-dependent, presents corrosive effect even towards stainless steel, and could combine with organic compounds, forming toxic by-products (Chmielewski and Frank, 2003; Guzel-Seydim et al., 2004; Van Houdt and Michiels, 2010). These

* Corresponding author at: ICTA-UFRGS, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, Brazil.
*E-mail address:* patricia.malheiros@ufrgs.br (P. da Silva Malheiros).

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issues, also shared by other disinfectants, coupled with the increasing consumer concerns regarding their own health and environmental awareness, drive research on new disinfectant and antimicrobials (Knowles et al., 2005; Desai et al., 2012).

Essential oils are aromatic and volatile liquids extracted from plants. These compounds can be composed by complex mixtures of low molecular weight molecules, whose major typical components depend on the plant source. Biological activities of essential oils and their components are extensively recognized, including potent antimicrobial properties towards bacteria, molds and yeasts (Burt, 2004; Hyldgaard et al., 2012; Calo et al., 2015; Reyes-Jurado et al., 2015). However, stability issues, volatility and poor water solubility may decrease their antimicrobial activity. Liposomes are colloidal structures with a spherical shape composed by phospholipid bilayer membranes and an internal aqueous compartment, which could be used for encapsulation and controlled release of essential oil constituents, increasing stability and contributing to their action as surface disinfectants (Desai et al., 2012; Sherry et al., 2013; El Asbahani et al., 2015; Cui et al., 2016a, 2016b).

Carvacrol and its isomer thymol are terpenoids found as major constituents of oregano and thyme essential oils (Hyldgaard et al., 2012), which are classified as Generally Recognized As Safe (GRAS) substances by the Food and Drug Administration (FDA, 2016). Considering that most foodborne illnesses in Brazil are caused by *Salmonella* spp. and *Staphylococcus aureus* (Brasil, 2016), and also the well-known capability of these bacterial species to adhere to diverse surfaces and to create biofilms (Chmielewski and Frank, 2003; Kostaki et al., 2012; Srey et al., 2013; Casarin et al., 2016), the aim of this study was to evaluate the antimicrobial effect of free and liposome-encapsulated thymol and carvacrol against pools of *Staphylococcus aureus* and *Salmonella* strains in suspension and adhered to stainless steel.

## 2. Materials and methods

### 2.1. Bacterial cultures

Two bacterial pools were used, each one composed by four different bacterial strains of *S. aureus* or *Salmonella* The four *S. aureus* strains used for pool composition were: *S. aureus* 4668/03, isolated from food involved in a foodborne outbreak; *S. aureus* S6, isolated from poultry slaughterhouse stainless steel surface before cleaning; *S. aureus* S8, isolated on a poultry meat cutting board before cleaning; and *S. aureus* ATCC 2998. The four *Salmonella* used for pool composition were: *S.* Enteritidis SE86, isolated from cabbage involved in a foodborne outbreak, exhibiting the same genetic profile of > 90% of *Salmonella* Enteritidis-related to outbreaks reported in Rio Grande do Sul State, Southern Brazil (Capalonga et al., 2014); *S.* Typhimurium, isolated from pork feces; *S.* Newport and *S.* Saint Paul, isolated from meat products.

All strains were grown separately on Brain Heart Infusion broth (BHI; Himedia, India) or UHT bovine skim milk (purchased in a local market), at 37 °C for 18–24 h. After incubation, 2.5 ml of BHI broth or UHT milk containing each strain were used to compose 10 ml of *S. aureus* pool and 10 ml of *Salmonella* pool, separately.

### 2.2. Preparation of the antimicrobial agents

Carvacrol (98% purity) and thymol (≥ 99.5% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the preparation of the sanitizing solution, 0.106 g of each compound were diluted in 10 ml of 20% (v/v) ethanol solution (Chorianopoulos et al., 2008). The antimicrobials concentration was determined from previous experiments. Before applying the solutions on stainless steel coupons, the sanitizing solutions were diluted with sterile distilled water in order to determine Minimum Inhibitory Concentration (MIC).

### 2.3. Liposome-encapsulation of thymol and carvacrol

Liposomes were prepared by the lipid film hydration technique (Malheiros et al., 2010a). First, 0.08 g of soy phosphatidylcholine (Lipoid S100; Lipoid GmbH, Germany) were weighed in a round bottom flask and then 20 ml of chloroform were added. The solvent was removed using a rotary evaporator (Laborota 4000; Heidolph Instruments GmbH & Co., Germany) and a film on the inner surface of the flask was formed. To ensure complete chloroform removal, the round bottom flask with the film was kept in a desiccator for 24 h. The film was rehydrated by adding 10 ml of a solution composed by 0.106 g of thymol and 0.106 g of carvacrol in 10% (v/v) dimethyl sulfoxide (DMSO; polar solvent). DMSO was used for dilution since previous experiments indicated that ethanol destabilized the liposome structure. The solution was homogenized in a water bath at 40 °C and mixed using a vortex stirrer. Finally, to reduce and homogenize the size of the liposomes already formed, an ultrasonic treatment (50 kHz; Sonics & Materials Inc. VCX 400, Danbury, CT, USA) was used for 3 cycles of 1 min each in an ice bath. This experiment was performed in duplicate.

The average diameter and polydispersity of the liposomes containing the natural antimicrobials were determined using a dynamic light scattering instrument (Model EMI 9863, Brookhaven Instruments Co., NY, USA), with three repetitions. The zeta potential was also determined, with three repetitions (ZetaPALS Model 31,450, Brookhaven Instruments Co., NY, USA). Before liposomes characterization, they were diluted with distilled water. The antimicrobial activity of encapsulated thymol/carvacrol liposomes was determined through MIC.

### 2.4. Minimum Inhibitory Concentration (MIC) determinations

MIC was determined for thymol, carvacrol and thymol/carvacrol liposomes using 96-well microdilution plates. Each bacterial suspension pool was adjusted to approximately $10^8$ CFU/ml, diluting each suspension with sterile BHI broth in order to obtain an absorbance ($OD_{630nm}$) of approximately 0.5, using a spectrophotometer (Ultrospec 3100 Pro, Amersham Biosciences, UK). For confirmation, plate counts were performed on BHI agar plates. Initially, 100 µl of BHI broth were added to each well. After that, 100 µl of disinfectant solution (free and encapsulated) were added to the first well, mixed and transferred to the second well, from the second to the third, and so on. Then, 100 µl of the respective bacterial pool were added to each well. Bacteria were not added to negative controls, while in positive controls, a 20% (v/v) ethanol solution without antimicrobials was used. The microdilution plates were incubated at 37 °C for 18–24 h, and turbidity of each well was observed.

### 2.5. Preparation of AISI 304 stainless steel coupons

Stainless steel AISI 304 coupons (2 cm × 2 cm × 1 mm thickness) were washed with a neutral detergent, properly rinsed with distilled water and dried at 60 °C. After drying, coupons were autoclaved (121 °C, 15 min) and again dried at 60 °C (Rossoni and Gaylarde, 2000). The coupons were kept in sterile vessels at room temperature until testing.

### 2.6. Microbial adhesion and antimicrobial action

Stainless steel AISI 304 coupons were immersed in 10 ml of bacterial pools of *S. aureus* or *Salmonella* ($10^8$ CFU/ml), for 15 min, at 37 °C, aiming at the adherence of bacterial cells to onto the surfaces. After this period, the coupons were rinsed with 10 ml of 0.1% peptone water in order to remove non-adhered cells. Each coupon with adhered cells was then immersed in 10 ml of free thymol, free carvacrol and thymol/carvacrol liposomes prepared according to the MIC determination results. Experiments were also conducted using disinfectant solutions corresponding to 0.5 and 2.0 MIC. Contact times between

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