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Potential use of carvacrol and citral to inactivate biofilm cells and eliminate biofouling

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ABSTRACT

Biofouling (i.e., accumulation of microorganisms on wetted surfaces) represents a major problem in the food industries, since bacterial biofilms are common sources of persistent infections due to their resilience to cleaning and disinfection treatments. Therefore, alternative treatments based on the use of essential oils or their individual compounds against this bacterial adaptation phenomenon are currently being studied. This work presents a quantitative comparison of the disinfectant potential of 500 −2000 μL/L of carvacrol or citral against mature biofilms of *Staphylococcus aureus* SC-01, *Listeria monocytogenes* EGD-e or *Escherichia coli* MG1655. Treatments with 1000 ppm of carvacrol or citral at 45 °C for 60 min were capable of reducing more than 5 logarithmic cycles of the sessile cells forming part of mature biofilms of all the three species. Furthermore, the synergism observed between carvacrol and heat allowed for the physical removal of biofilms by treatments simulating *in situ* wash conditions (80 °C/60 s). These results demonstrate the great potential of the essential oils' constituents citral and carvacrol in the eradication of biofilms formed by foodborne pathogenic microorganisms.

1. Introduction

Most bacteria can grow not only in planktonic form (freely existing in bulk solution), but also in sessile form (attached to a surface in the confines of a biofilm). Certain environmental signals, such as low availability of nutrients, can induce the formation of biofilms through a cyclical process that begins with the attachment of planktonic cells to a surface. Afterwards, during a subsequent maturation phase, attached cells transform into sessile cells and produce a matrix of extracellular polymeric substances (EPS). A final dispersal phase occurs when the biofilm reaches a nutrient-deprived critical mass or as a response to changing environmental conditions (O'Toole, Kaplan, & Kolter, 2000). This causes the detachment of sessile cells from the outermost layers of the biofilm, which become planktonic single individuals (O'Toole et al., 2000; Srey, Jahid, & Ha, 2013). In comparison with planktonic cells, their sessile counterparts display distinct phenotypic attributes, like loss of mobility appendages and slower growth rate (Puga, Orgaz, Muñoz, & SanJose, 2015). These phenotypic characteristics, in combination with their protective matrix of EPS and their cell-to-cell communication through quorum sensing (Mah & O'Toole, 2001), can contribute to the observed high resistance of bacterial biofilms against antibiotics, disinfectants, and dynamic or hostile environments (Garrett, Bhakoo, & Zhang, 2008).

Given the advantages that biofilms provide for bacterial survival, it is not surprising the serious risks they can pose to the food industry. In fact, biofilms formed on food-contact surfaces can act as reservoirs of persistent contaminations, cross-contaminations, and post-processing contaminations in food products (Srey et al., 2013). In this regard, some bacterial pathogens should be especially considered due to their common presence in food industries, their involvement in foodborne outbreaks, or their strong biofilm-forming abilities, such as *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* (Lira et al., 2016; Perez-Conesa, Cao, Chen, McLandsborough, & Weiss, 2011).

Abbreviations: EO, Essential oil; EPS, Extracellular polymeric substances; IC, Individual constituent; MIC, Minimum inhibitory concentration.

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Among the measures to avoid biofilm production by these and other microorganisms, priority has been given to the implementation of efficient cleaning and disinfection regimes in food industries (Chmielewski & Frank, 2003; Srey et al., 2013). Cleaning and disinfection procedures, which aim at the removal of food debris and the inactivation of remaining biofilm cells, are indispensable to prevent food contamination (Chmielewski & Frank, 2003). Biocides such as quaternary ammonium compounds, acids, peroxygens, and chlorine (and other halogens) are thoroughly used for disinfection in food industries. However, their current use is being gradually constrained due to their high toxicity, their corrosive effect on surfaces, and the acquisition of resistance from bacterial biofilms to some of these chemicals (Akbas, 2015; Neyret, Herry, Meylheuc, & Dubois-Brissonnet, 2014). Besides, some disinfectants in use at present will probably be banned during the next few years because of regulatory changes (European Parliament, 2006). Consequently, there is a growing interest towards plant-derived compounds which might provide a safe and environmentally-friendly alternative to traditional biocides (Akbas, 2015; Saising et al., 2012).

Regarding natural antimicrobials, numerous studies have demonstrated the capacity of some plant essential oils (EOs) and their individual constituents (ICs) to inhibit bacterial biofilm production (Simoes, Bennett, & Rosa, 2009; Szabó et al., 2010). However, these compounds are generally less effective against mature biofilms (Aiemsaard, Aiumlamai, Aromdee, Taweechaisupapong, & Khunkitti, 2011; Budzyńska, Więckowska-Szakiel, Sadowska, Kalemba & Rózalska, 2011; Burt, Ojo-Fakunle, Woertman, & Veldhuizen, 2014; Jadhav, Shah, Bhave, & Palombo, 2013; Sandasi, Leonard, & Viljoen, 2010). For example, the efficacy of the IC carvacrol in inhibiting the biofilm development of *S. aureus*, *L. monocytogenes* or *E. coli* has been widely demonstrated (Burt et al., 2014; Espina, Pagán, López, & García-Gonzalo, 2015; García-Heredia, García, Merino-Mascorro, Feng, & Heredia, 2016; Knowles & Roller, 2001; Neyret et al., 2014; Nostro et al., 2007; Perez-Conesa, McLandsborough, & Weiss, 2006) and treatments with high carvacrol concentrations have been seen to disrupt pre-existing biofilms of *S. aureus* (Nostro et al., 2009). However, these findings are in contrast with studies which highlight the relative inability of carvacrol to eradicate biofilms (Burt et al., 2014; Knowles & Roller, 2001; Perez-Conesa et al., 2006). Similar inconclusive results have been obtained when evaluating the efficacy to disrupt *S. aureus*, *L. monocytogenes* or *E. coli* biofilms with the EOs lemongrass, lemon balm, and their major IC citral (Aiemsaard et al., 2011; Budzyńska et al., 2011; García-Heredia et al., 2016; Leonard, Virijevic, Regnier, & Combrinck, 2010).

Taking this into account, the present work targeted two different aspects in the elimination of biofouling: both the inactivation of sessile cells and the physical removal of the biofilms. For this purpose, the ICs carvacrol and citral were tested against biofilms formed by selected pathogens relevant to the food industry. Furthermore, several variable factors were considered, including the concentration of each IC, the pH, and the temperature of the disinfection treatments, as well as the maturation of the biofilms. The specific objectives of this study were (i) to determine the inactivation achieved in biofilm-contained sessile cells of *S. aureus* SC-01, *L. monocytogenes* EGD-e and *E. coli* MG1655 when treated at acidic or neutral pH with inhibitory concentrations of carvacrol or citral; (ii) to evaluate the effect of an increase in the treatment temperature on the biofilms' susceptibility to both ICs; (iii) to explore the possible role of the EPS matrix on the biofilms' resistance to both ICs; (iv) to investigate the effect of treatments on biofilms at different maturation stages, and (v) to test the ability of selected treatments to physically eliminate biofilms.

2. Material and methods

2.1. Antimicrobial compounds

Carvacrol (≥98%) and citral (95%) were purchased from Sigma Aldrich (Sigma-Aldrich Chemie, Steinheim, Germany). A vigorous shaking procedure described by Friedman, Henika, and Mandrell (2002) by vortex (Genius 3, Ika, Königswinter, Germany) agitation was used to prepare carvacrol or citral dispersions in broth media, avoiding the use of solvents for their possible detriment in the antibacterial activity of the compounds. The carvacrol or citral dispersions achieved with this methodology were stable for the duration of the experiments.

2.2. Microorganisms, growth conditions and biofilm formation

The strains used in this study were *Escherichia coli* MG1655 (obtained from the Collins Lab, MIT, Boston, MA, USA), *Listeria monocytogenes* EGD-e (Chatterjee et al., 2006) (kindly supplied by Dr. Chakraborty), and *Staphylococcus aureus* SC-01 (kindly supplied by Dr. López from the Kolter Lab, Harvard Medical School, Boston, MA, USA). The identity of the strains was verified by their suppliers, as published elsewhere (Bécavin et al., 2014; Koch et al., 2014; Kohanski, DePristo, & Collins, 2010), and confirmed in our lab.

During this investigation, cultures were maintained in cryovials at − 80 °C, from which plates of Tryptic Soy Agar (Oxoid, Basingstoke, Hampshire, England) (TSA) were prepared on a weekly basis.

Every single-species broth subculture was prepared by inoculating, with one single colony from a plate, a test tube containing 5 mL of sterile Tryptic Soy Broth (Oxoid) (TSB). After inoculation, the tubes were incubated overnight at 37 °C. 250 mL-flasks containing 50 mL of TSB were inoculated with 50 μL of the resulting subcultures. These flasks were incubated under agitation (130 rpm; Selecta, mod. Rotabit, Barcelona, Spain) at 37 °C until late-stationary growth phase was reached (24 h). Afterwards, these bacterial cultures were diluted 1:100 in TSB to attain an initial concentration of 10^7 cells/mL. Wells in twenty-four-well microtiter plates (Nunclon Delta Surface, Thermo Fisher Scientific, Roskilde, Denmark) were inoculated with 2 mL of this culture, and plates were incubated inside individual plastic bags at 37 °C in static conditions for 72 h.

Other growth times (24, 48 and 96 h) were added for further investigation on the influence of growth time on the biofilm mass. In additional experiments, the supernatant of each well was replaced by fresh TSB every 24 h. This was done in order to enhance biofilm growth by limiting the nutrient depletion imposed by static growth, without introducing the shear forces of a dynamic system (Garrett et al., 2008). The objective of this methodology was to make a preliminary observation on whether the resistance of bacterial cells to citral or carvacrol would decrease in less restrictive environmental conditions, as reduced growth rate in biofilm cells has been linked to a decreased susceptibility of biofilms to antimicrobials (Donlan, 2001).

2.3. Measurement of the inactivation of sessile cells from disintegrated biofilms, and of planktonic cells, after exposure to antimicrobials

First, the minimum inhibitory concentration (MIC) of each antimicrobial against planktonic cells was determined. This concentration was determined by inoculating 1 μL of bacterial preculture into capped test tubes containing 5 mL of TSB (initial concentration: 10^5 CFU/mL) with increasing concentrations of the corresponding antimicrobial compound (50, 100, 200, 500, 1000, 1500, 2000 or 5000 μL/L).