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Carvacrol efficacy in reducing microbial biofilms on stainless steel and in limiting re-growth of injured cells

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ABSTRACT

Microorganisms organized in biofilm have demonstrated a decreasing susceptibility to classical disinfectants and the search of new active compounds, such as the natural ones, is still a challenge for the researchers. In this study, the effect of carvacrol against biofilms of *Escherichia coli* O157:H7 ATCC 35150, *Staphylococcus aureus* ATCC 43387, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 14053 developed on stainless steel coupons for 4 days at room temperature was assessed. After 30 s, 5 and 15 min of treatment with carvacrol 1%, the number of viable cells was quantified by plate count agar and the effect of carvacrol on microorganisms was observed in epifluorescence after orange acridine staining. In addition, carvacrol treated coupons were re-incubated for another 24 h at room temperature to determine the re-growth of the injured cells. Carvacrol induced a logarithmic reduction equal to or greater than 3 against all the tested biofilms and the epifluorescence observation revealed mostly green cells, indicating microorganisms with no active RNA transcription, contrarily to the active red ones observed in the controls. The treatment for 15 min was able to inhibit the re-growth of *E. coli* O157:H7, *S. aureus* and *P. aeruginosa* and to reduce the growth of *E. faecalis* and *C. albicans*.

Since in food processing environments new strategies to overcome bacterial persistence are necessary, carvacrol showed to possess anti-biofilm activity against all the tested microorganisms and to drastically reduce the re-growth of the injured cells after antimicrobial treatment.

1. Introduction

The adhesion of microorganisms to food contact surfaces is an important concern in food industries because the attachment can promote not only cell survival but also biofilm formation, that can lead to cross-contamination, reduced product shelf life and food-borne diseases (Bridier et al., 2015; Shi & Zhu, 2009). Biofilm, a three-dimensional structure of microbes embedded in a slimy matrix composed of extracellular polymeric substances (EPS) (Hall-Stoodley, Costerton, & Stoodley, 2004), can develop on abiotic and biotic surfaces and as its peculiar complexity confers to microbial cells a high level of antimicrobial resistance (Lee, Bae, Lee, & Lee, 2015; Srey, Jahid, & Ha, 2013). Some conditions in food industry can promote the bacterial attachment and subsequent biofilm development, such as flowing water, raw materials, organic load or suitable surfaces. In particular, the surfaces of equipment used for food handling, storage, or processing are recognized as the major source of microbial contamination because bacteria have the ability to attach to different types of materials, such as polystyrene, hydroxyapatite, glass, rubber, and stainless steel (Bae, Baek, & Lee, 2012). For these reasons, the prevention of biofilm formation requires regularly cleaning and disinfection procedures of surfaces for impeding bacterial attachment and biofilm development (Simões, Simões, & Vieira, 2010; Srey et al., 2013). Indeed, in literature is reported as bacteria embedded in biofilm can survive to disinfectant treatments (Bae et al., 2012; Cooper, White, Mahenthiralingam, & Hanlon, 2008; Weese & Rousseau, 2006), evidencing a decreasing susceptibility of mature biofilms to liquid chemical disinfectants such as sodium hypochlorite, quaternary ammonium salts, phenolic disinfectants, hydrogen peroxide, and silver ions (Song, Wu, & Xi, 2012). The increased resistance of biofilms to disinfectants and sanitizing agents can contribute to the in-efficacy of cleaning-in-place systems and, for this, higher sanitizer concentrations and/or prolonged the time of contact were necessary to

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obtain a well-done cleaning of the surface (Królasik, Żakowska, Krępska,; Klimek, 2011; Cabeça, Pizzolitto, & Pizzolitto, 2012). On the other hand, it's difficult that in food processing these "modified procedures" are really applied if no specific indications were given by the manufactures of the sanitizer products.

In recent years, several articles describing the antimicrobial action of crude essential oils (EOs) and/or their active components against biofilm embedded bacteria have been published (Nostro et al., 2007; Bridier et al., 2015; Campana et al., 2017; Engel, Heckler, Tondo, Daroit, & da Silva Malheiros, 2017; Pedonese et al., 2017). EOs are composed by complex mixtures of low molecular weight molecules, whose major typical components depend on the plant source. Among these, carvacrol, a monoterpene phenol (2-meth-yl-5-(1-methylethyl) phenol) present in the volatile oils of *Thymus vulgaris*, *Carum copticumand oreganum* species (Nabavi et al., 2015), is classified as Generally Recognized As Safe (GRAS) substances by the Food and Drug Administration (FDA, 2016) and registered by the European Commission as healthy with no risk to the consumers (Burt, Vlielander, Haagsman, & Veldhuizen, 2005).

Several studies have reported that carvacrol has different biological activities such as antioxidant, antimutagenic, antigenotoxic and antimicrobial (Friedman, 2014). In particular way, its antimicrobial activity has a wide spectrum extended to pathogenic bacteria (Baser, 2008; Nostro & Papalia, 2012), fungi and yeast (Nostro & Papalia, 2012), including drug-resistant and biofilm forming microorganisms (Knowles & Roller, 2001; Raei et al., 2017; Tapia-Rodriguez, Hernandez-Mendoza, Gonzalez-Aguilar, Martinez-Tellez, Martins, & Ayala-Zavala, 2017). For this reason, the aim of this study was to investigate the effect of carvacrol against five pathogens represented by *Escherichia coli* O157:H7 ATCC 35150, *Staphylococcus aureus* ATCC 43387, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 14053 organized in biofilm. The experimental design was divided in two different phases in order to verify: i) the ability of carvacrol to reduce microbial biofilms developed on stainless steel surface after different times of contact, and ii) the efficacy of the antimicrobial treatment on the re-growth ability of injured cells.

2. Material and methods

2.1. Bacterial strains and growth conditions

Five reference human pathogens were used in this study, *E. coli* O157:H7 ATCC 35150, *S. aureus* ATCC 43387, *P. aeruginosa* ATCC 9027, *E. faecalis* ATCC 29212, *C. albicans* ATCC 14053. All the strains were routinely maintained in Tryptic Soy Agar (TSA) (Oxoid, Milan, Italy) at 37 °C, while stock cultures were keep at −80 °C in Nutrient broth (Oxoid) with 15% of glycerol.

2.2. Biofilm formation

Microbial biofilms were developed on stainless steel coupons type 304 (2 × 2x0.8 cm) (T.M.P, Pesaro, Italy), firstly cleaned with acetone, treated with HCl 5N for 15 min and washed with a detergent solution (ethanol 70%). After an additional double rinsed with distilled water, the coupons were air-dried and sterilized by autoclave for 15 min at 121 °C.

All the strains were grown in Tryptic Soy Broth (TSB), (Oxoid) at 37 °C for 24 h to obtain a microbial suspension at the end of the logarithmic phase. The density of each suspension was adjusted by spectrophotometer to OD_{610nm} of 0.15–0.18, corresponding to about 10^6–10^7 CFU/mL. The assembling of biofilms on stainless steel coupons was then carried out as described by Campana et al. (2017). Briefly, the coupons were placed in sterile Petri dishes, containing 2 mL of bacterial suspension and 18 mL of 1:10 water diluted TBS and then incubated at room temperature (RT). Every 24 h, the coupons were aseptically removed and washed in sterile phosphate saline buffer (PBS) to eliminate the unbound bacteria; finally fresh TSB medium (10 mL) was added and the incubation was prolonged at the same conditions described above. This procedure was repeated for 4 days to allow biofilm formation. At each time point, to assess biofilm formation in term of biomass production, the coupons were PBS washed, transferred in new dishes and covered with 5 mL of Crystal violet 0.1% (v/v) (CV, Sigma, Milan, Italy) for 15 min. The coupons were PBS washed again and air-dried. Finally, the remaining CV was dissolved in 85% ethanol (15 min at RT) and 200 μL from each dish was transferred to a 96-well plate for spectrophotometry at 570 nm using a Multiscan Ex Microplate Reader (Thermo Scientific, Italy). Each data point was averaged from at least 8 replicate wells. The experiments were performed in duplicate using independent cultures.

2.3. Carvacrol

Carvacrol (≤97.0% pure, v/v) was purchased from Sigma; the stock solution (10%, v/v) was prepared in dimethyl sulfoxide (DMSO) (Sigma), while the work solution (1%, v/v) in distilled sterile water. Previous experiments excluded a possible antimicrobial activity of DMSO (data not shown). All the solutions were maintained at RT in the dark.

2.4. Treatment of 4 days old biofilms with carvacrol

Biofilms were developed for 4 days on stainless steel coupons as described above. The experimental design for the treatment with carvacrol included, for each microorganism, two series of coupons subdivided as follows: three coupons for the contact with carvacrol solution (30 s, 5 and 15 min) and three control coupons for the contact with sterile water (30 s, 5 and 15 min).

Briefly, all the coupons were washed with 5 mL of sterile PBS and dipped in new dishes containing 5 mL of carvacrol solution (1%) for 30 s, 5 and 15 min. Parallel, several coupons were immersed in 5 mL of a control solution (NaClO 4.5% and KOH 10%, v/v) for the same times of exposure. At each time point, the coupons were transferred for 2 min into new dishes containing 5 mL of neutralizing solution (polysorbate 80, 3% v/v; saponin 3% w/v; lecithin 0.3% w/v) (Sigma). As negative controls, one coupon for each strain was treated with 5 mL of sterile distilled water instead of sanitizer. To enumerate the number of viable cells, the coupons were PBS washed and the adherent bacteria were removed from the upper side of each coupon by swabbing with sterile cotton swab. The swabs were transferred into sterile tube containing 1 mL of physiological saline solution and shaken for 2 min by vigorous vortexing. Finally, the bacterial suspensions were serially diluted in physiological saline solution and plated in triplicate 10 μL spots on TSA (Oxoid). The plates were then incubated at 37 °C for 24–48 h at the end of which the colony forming units (CFU) were enumerated.

The experiments were performed in duplicate using independent cultures and the averages of CFU/coupon counts were converted from units to log10 CFU/cm^2. The decrease of bacteria after antimicrobial treatment was derived from the formula [log (N/N_a)], where N is the count of CFU/cm^2 prior the treatment and N_a is the CFU/cm^2 after sanitizer treatment. In this study, we consider a logarithmic reduction (LR) greater than 3 (log CFU/cm^2) to be indicative of antibacterial activity.

2.5. Biofilm visualization in epifluorecence

Biofilms were visualized in epifluorecence after staining with Acridine Orange (AO, Sigma) (emission length between 520 and