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Resveratrol inclusion complexes: Antibacterial and anti-biofilm activity against *Campylobacter* spp. and *Arcobacter butzleri*

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ABSTRACT

Worldwide, the consumption or handling of contaminated food has been described as one of the major causes of foodborne illness with campylobacteriosis being the most commonly reported zoonosis. *Campylobacter jejuni* and *Campylobacter coli* are considered the major cause of bacterial gastroenteritis, while *Arcobacter* spp. are also known to be human and animal pathogens. Furthermore, these bacteria are able to form biofilms which have become a relevant issue in a wide range of food industries since they are more resistant to disinfectants and so, more difficult to eliminate. This question gives rise to the research on the use of alternative substances that can effectively prevent biofilm formation or eradicate the biofilm already formed. Given this, the aim of this study was to evaluate the antimicrobial and anti-biofilm activity of resveratrol-hydroxypropyl-γ-cyclodextrin inclusion complexes (IC) against *C. jejuni*, *C. coli* and *A. butzleri* as well as their quorum sensing (QS) inhibition activity. Besides improving resveratrol solubility, the ICs showed anti-*Campylobacter* and anti-*Arcobacter* activity, inhibited biofilm formation and promoted the biofilm dispersion even at sub-MIC concentrations for both genera. It was also demonstrated the anti-QS activity of the ICs through the inhibition of violacein production by *Chromobacterium violaceum*. In conclusion, since the use of natural compounds can improve the safety and security of foods, our results showed that this IC could be developed as a new anti-biofilm agent and QS inhibitor to enhance the shelf-life and safety of foods.

1. Introduction

Despite the recent improvements in food safety, foodborne illness remains frequent in Europe and the United States (EFSA, (European Food Safety Authority), & ECDC, (European Centre for Disease Prevention & Control), 2015; Painter et al., 2013). It is estimated that, each year, approximately 300,000 persons in the European Union suffer from foodborne illness caused by a major pathogen (EFSA, (European Food Safety Authority), & ECDC, (European Centre for Disease Prevention & Control), 2015). According to the latest EFSA report, campylobacteriosis was the most commonly reported zoonosis in 2013, being *Campylobacter jejuni* and *Campylobacter coli* the major cause of bacterial gastroenteritis in humans worldwide (Duarte et al., 2014; EFSA, (European Food Safety Authority), & ECDC, (European Centre for Disease Prevention & Control), 2015). One of the major causes of foodborne illness continues to be the consumption of raw or undercooked chicken or the cross-contamination of other foods during raw chicken handling; which is mostly due to the high microbial load of fresh poultry products (Painter et al., 2013). This elevated microbial load present in chickens and other avian species is mainly a consequence of the fact that these animals serve as natural reservoir hosts for *Campylobacter* spp. which are able to colonize their intestinal tract (Silva et al., 2011b). Other pathogens also commonly found in poultry meat and products are *Arcobacter* spp., although the source of poultry contamination is still not clear (Ferreira, Fraqueza, Queiroz, Domingues, & Oleastro, 2013; Wesley & Miller, 2010). Within the *Arcobacter* genus, the species *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are known to be human and animal pathogens, with *A. butzleri* being included in the list of microbes considered to be a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (Collado & Figueras, 2011). Since poultry meat is a highly perishable and very popular food commodity, with a growing increased consumption world (Daniel, Cross, Koebnick, & Sinha, 2011), the microbiological safety of poultry products is of extreme importance. Therefore, the development of new antimicrobial strategies to control and/or eliminate *Campylobacter* and *Arcobacter* contamination in poultry and poultry products are in demand, not only to deal with the economic burden that represents bacterial foodborne illness but also to extend the shelf-life of these products. These newly developed strategies must also be effective in controlling and/or eliminating bacterial biofilms since it has been described that *Campylobacter* and *Arcobacter* could develop biofilms (Ferreira et al., 2013; Gunther & Chen, 2009) that are more resistant to disinfectants

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and become a major issue within the food industry, mainly the ones dedicated to poultry meat processing (Srey, Jahid, & Ha, 2013). Bacterial biofilm formation and other physiological activities, such as antibiotic resistance and motility can be related to the intercellular communication mechanisms like quorum sensing (QS) (Deep, Chaudhary, & Gupta, 2011) which has also been implied in bacterial proliferation in foods and food spoilage.

Keeping this into consideration, the inhibition of cell-to-cell communication underlying the QS could be considered a viable strategy to ensure food safety and quality (Alvarez et al., 2014; Zhang et al., 2014).

Resveratrol (3,5,4′-trihydroxystilbene) is a stilbene naturally present in foodstuffs with described antimicrobial activity against several pathogens (Ferreira, Silva, Queiroz, Oleastro, & Domingues, 2014b; Paulo, Ferreira, Gallardo, Queiroz, & Domingues, 2010; Paulo, Oleastro, Gallardo, Queiroz, & Domingues, 2011). However, this compound's bioactivity still remains an issue due to its low bioavailability stemming from its poor stability and solubility in aqueous media (Chen et al., 2007; Delmas et al., 2011). To overcome these challenges, in recent years, several encapsulation strategies have been proposed such as resveratrol inclusion in liposomes/niosomes, yeast cells, biopolymer particles such as proteins, chitosan and cyclodextrins (Augustin, Sanguansri, & Lockett, 2013; Davidov-Pardo & McClements, 2014). Cyclodextrins are the most commonly used encapsulating agents to form inclusion complexes (IC) in the food industry and are generally recognized as safe by the United States Food Drug Administration (Davidov-Pardo & McClements, 2014). Results have shown that, besides being able to significantly increase resveratrol aqueous solubility, cyclodextrins also improved or maintained the antioxidant activity of this compound (Das, Lin, Ho, & Ng, 2008; Davidov-Pardo & McClements, 2014; Lu, Cheng, Hu, Zhang, & Zou, 2009), while being capable of protecting resveratrol from the external environmental factors, such as temperature, light and pH, by entrapping it inside their cavities (Pinho, Grootveld, Soares, & Henriques, 2014a).

Envisioning the application of resveratrol IC in the food industry, the goal of this study was to evaluate the antimicrobial activity of resveratrol-hydroxypropyl-γ-cyclodextrin ICs against *C. jejuni*, *C. coli* and *A. butzleri* while unveiling its mode of action in these bacterial cells. The ability of these IC in the inhibition of biofilm formation and in the dispersion of established biofilms was also evaluated, as well as its QS inhibition activity.

2. Material and methods

2.1. Inclusion complex formation

trans-Resveratrol was obtained from TCI Europe N.V. and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) from Sigma-Aldrich. The inclusion complex (IC) of resveratrol with HP-γ-CD was prepared as described by Silva, Figueiras, Gallardo, Nerín, and Domingues (2014) and resveratrol concentration in the IC was quantified by HPLC–DAD (Silva et al., 2014).

2.2. Bacterial strains

In this study, two *Campylobacter* spp. and two *Arcobacter butzleri* isolates were used. *C. coli* 873 was isolated from a faecal sample of a patient with acute gastroenteritis and *C. jejuni* 225421 was isolated from fresh poultry meat (Duarte et al., 2014). *A. butzleri* AB36/11 was isolated from poultry caecum and the strain INSA776 was isolated from a faecal sample of a patient with diarrhoea and abdominal pain (Ferreira, Queiroz, Oleastro, & Domingues, 2014a). All the strains were stored in Brain Heart Infusion (BHI) broth with 20% (v/v) glycerol at −80 °C and prior to antimicrobial susceptibility assays, each strain was inoculated on blood agar plates supplemented with 5% defibrinated horse blood (Oxoid, England) to ensure optimal growth.

2.3. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) for the IC and resveratrol were determined by the microdilution method according to the previously described methods to *Campylobacter* and *Arcobacter* spp. (Ferreira et al., 2014b; McDermott, Bodeis-Jones, Fritsche, Jones, & Walker, 2005). Briefly, serial two-fold dilutions of IC, ranging from 2048 to 16 μg/mL, and resveratrol from 400 to 3.125 μg/mL were prepared in a 96-well plate (50 μL per well) in Mueller Hinton broth (MHB, LiofilChem, Italy). A maximum of 1.5% of dimethyl sulfoxide (DMSO) was used to increase resveratrol aqueous solubility. The bacterial suspensions, with a turbidity of 0.5 McFarland, were prepared from overnight cultures on blood agar plates and then diluted and added to each well to yield a final concentration of about 10^6 colony-forming units (CFU)/mL. The plates were incubated at 37 °C for 48 h under microaerobic conditions and, after incubation, growth was visually assessed and confirmed spectrophotometrically at 590 nm. At least three independent assays were performed and the modal MIC values were selected.

2.4. Time–kill curves

Bacterial suspensions from exponentially-growing cultures obtained after 6 h of incubation in MHB at 37 °C and 100 rpm under microaerobic conditions were exposed to the IC and resveratrol at final concentrations of 1×, 2× and 4 × MIC and a final cell concentration of about 10^6 CFU/mL. HP-γ-CD, DMSO and culture medium were used as growth controls. Tubes were incubated at 37 °C under microaerobic (*Campylobacter* strains) or aerobic (*A. butzleri*) conditions and after 0, 2, 4, 6, 8, 10 and 24 h of incubation a sample of 20 μL was removed to assess cell concentration (CFU/mL) by the drop plate method (Chen, Nace, & Irwin, 2003). Data was obtained from three independent experiments. Bactericidal activity was defined as a reduction of 99.9% of the total number of CFU/mL and bacteriostatic activity as the maintenance or reduction of less than 99.9% of the original inoculum.

2.5. Flow cytometry assay

2.5.1. Exposure of bacteria to the inclusion complex

Bacterial suspensions for flow cytometry assays were prepared as described for time–kill curves. After 6 h of incubation, samples were centrifuged at 5000 rpm for 10 min and washed with PBS buffer. The resulting suspensions were used for fluorescent staining.

2.5.2. Staining procedure

To evaluate membrane depolarization, the previously prepared bacterial suspensions were incubated with 2.5 μg/mL of bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX; Molecular Probes®, Carlsbad, CA) in PBS buffer supplemented with 4 mM EDTA (pH 7.4) for 15 min in the dark at room temperature. In order to assess respiratory activity, the suspensions were incubated with 5 mM of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Inc., Warrington, PA) in BHI broth for 2 h at 37 °C and 100 rpm under microaerobic conditions. After the incubation with BOX or CTC, cells suspensions were centrifuged at 10,000 rpm, for 5 min and resuspended in PBS buffer with 4 mM EDTA for total cell staining with 10 μM of SYTO® 40 (Molecular Probes®, Carlsbad, CA). SYTO® 40 has the capacity to penetrate the cell, binding to nucleic acids, allowing to distinguish cells from possible residues or cellular remains. The final suspensions were incubated in the dark for 15 min at room temperature, washed, resuspended in PBS and analysed in the flow cytometer.

2.5.3. Flow cytometry acquisition data

Samples were acquired on a CyAn ADP (Beckman Coulter, USA) flow cytometer and analysis was performed using Summit 4.3 (Beckman Coulter, USA) software. Fluorescence signals were collected by FL1