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Resveratrol against *Arcobacter butzleri* and *Arcobacter cryaerophilus*: Activity and effect on cellular functions

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ABSTRACT

The frequent isolation of *Arcobacter butzleri* and *Arcobacter cryaerophilus* from food samples makes it imperative to search for potential compounds able to inhibit the development of these bacteria. Taking this into consideration, this study focuses on the antimicrobial activity of resveratrol and its mechanism of action against *A. butzleri* and *A. cryaerophilus*. The activity of resveratrol was assessed by a microdilution method and time–kill curves. Resveratrol effect on cellular functions was assessed by flow cytometry evaluating intracellular DNA content and metabolic activity. Ethidium bromide (EtBr) accumulation in the presence of resveratrol was also evaluated, as well as the susceptibility to resveratrol in the presence of phenylalanine-arginine β-naphthylamide (PAβN). Scanning electron microscopy (SEM) was used to further evaluate cell damage caused by resveratrol. Resveratrol presented MIC values of 100 and 50 μg/mL to *A. butzleri* and *A. cryaerophilus*, respectively. Based on the time–kill curves, resveratrol exhibited bactericidal activity, leading to a ≥ 3 $\log_{10}$ CFU/mL reduction of initial inoculums, for *A. butzleri* exponential phase cells incubated for 6 h with 1× MIC or with 2× MIC after 24 h for stationary phase cells. For *A. cryaerophilus* cells in exponential growth phase, 99.9% killing was achieved after 24 h incubation with 2× MIC, whereas, for stationary phase cells, bactericidal activity was only detected after incubation with 4× MIC. Incubation with resveratrol led to a decrease in both intracellular DNA content and metabolic activity. An increase in the accumulation of EtBr was observed in the presence of resveratrol, and the efflux pump inhibitor PAβN reduced the MIC of resveratrol. SEM analysis revealed disintegration of *A. butzleri* cells treated with resveratrol, whereas no morphological alteration was observed for *A. cryaerophilus* cells. Resveratrol has a good anti-*Arcobacter* activity, and the results obtained suggest that this compound could act through several different mechanisms in the inhibition of this microorganism. The results encourage the use of this compound for the development of potential strategies to control *Arcobacter* in food products.

1. Introduction

Arcobacter spp. belong to ε-subdivision of proteobacteria and together with the genera *Campylobacter* and *Sulfurospirillum* compose the Campylobacteraceae family. Comprising motile Gram negative small curved rods, including S-shaped or helical cells, this genus has been isolated from extremely diverse habitats (Vandamme et al., 2005). Currently, *Arcobacter* genus is composed of 18 species (Collado and Figueras, 2011; Figueras et al., 2011; Levican et al., 2012, 2013; Sasi Jyothsna et al., 2013) of which *Arcobacter butzleri* and *Arcobacter cryaerophilus* have been associated with gastrointestinal and extra-gastrointestinal disease, and are considered emerging enteropathogens and potential zoonotic agents (Collado and Figueras, 2011). *Arcobacter* have been found in human, animals, food such as poultry, cattle, milk, retail meat, shellfish, and ready-to-eat meals or water, with a frequent detection in products of animal origin (Collado and Figueras, 2011). Due to the frequent isolation of *Arcobacter* species from food and water, it has been suggested that these are the most probable transmission routes to humans and animals of this microorganism (Collado and Figueras, 2011). Additionally, *Arcobacter* spp. have shown to be resistant to common antimicrobials (Collado and Figueras, 2011), namely those relevant for treatment of Campylobacteraceae human infections, such as erythromycin and ciprofloxacin, and in addition, this pathogen is also able to survive several physical and chemical treatments (Cervenka, 2007). These resistance profiles may compromise this pathogen control and together with its high prevalence in the food chain make it important to find alternative control strategies, for which different physical and chemical treatments (Cervenka, 2007; Skrivanova et al., 2010), or natural products (Cervenka et al., 2006; Fisher et al., 2007; Nannapaneni et al., 2009) have been proposed.

It is known that plants produce a great number of small molecules that help them fight off infections successfully (Klancnik et al., 2012). Therefore it is worthwhile to investigate if natural antimicrobials can be valid alternatives to synthetic molecules. Resveratrol (3,4′,5

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trihydroxystilbene) is a naturally occurring phytoalexin synthesized by plants in response to phytopathogenic microorganisms or other injurious substances (Sadruddin and Arora, 2009). This naturally occurring molecule is considered one of the most promising ones for application in nutrition and medicine (Kiselev, 2011) as it exhibits several properties beneficial to human health, among which are antioxidant, anti-inflammatory, antiatherosclerotic, antidiabetic or cardioprotective activities (Sadruddin and Arora, 2009). Resveratrol also presents antimicrobial activity against bacteria, yeasts and fungi (Paulo et al., 2011a) and is even capable to inhibit relevant bacterial virulence factors, such as the urease activity of *Helicobacter pylori* (Paulo et al., 2011b; Wang et al., 2006), swarming, cell invasion ability and hemolysin activity (Wang et al., 2006). The capacity of resveratrol to inhibit cellular adhesion and cytokine production in intestinal epithelial cells as a response to *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* Scott A adhesion has also been described (Selma et al., 2012).

Although some studies refer to antimicrobial properties of resveratrol, its action mechanisms are still largely unknown. Thus, the aim of this study focuses on the antimicrobial properties of resveratrol as well as its potential cellular targets, by using *A. butzleri* and *A. cryaerophilus* as exemplary cases.

2. Materials and methods

2.1. Bacterial organisms and handling

Two reference strains, *A. butzleri* LMG 10828 and *A. cryaerophilus* LMG 10829 were obtained from BCCM/LMG Bacteria Collection (Belgian Co-ordinated Collections of Micro-organisms, Ghent University, Belgium). The bacterial strains were stored in Brain Heart Infusion (BHI) broth with 20% (v/v) glycerol at −80 °C. Prior to susceptibility testing, each strain was inoculated on blood agar (blood agar base (Oxoid, Hampshire, England) supplemented with 5% (v/v) defibrinated horse blood) to ensure optimal growth and purity.

2.2. Determination of minimum inhibitory concentration (MIC)

A. butzleri LMG 10828 and *A. cryaerophilus* LMG 10829 were grown on blood agar plates for 18 h at 30 °C in microaerobic conditions (6% O_2, ± 7.1% CO_2, 3.6% H_2, 83% N_2). The broth microdilution method was used for measuring the MIC for resveratrol. Serial two-fold dilutions of resveratrol (from 400 to 3.125 μg/mL) were prepared in a 96-well plate (50 μL per well) in Tryptic Soy Broth (TSB, Liofilchem, Italy) with a maximum dimethyl sulfoxide (DMSO) concentration of 1.5% that was used to increase the solubility. Inoculum suspensions were prepared from blood agar cultures, and a diluted bacterial suspension was added to each well, to give a final concentration of about 10^6 colony-forming units (CFU)/mL, confirmed by viable counts. The microtiter plate was incubated for 48 h at 30 °C in microaerobic conditions and growth was visually assessed. At least three independent assays in duplicated wells were performed and the modal MIC values were selected.

2.3. Time–kill curves

Bacterial strains were exposed to final resveratrol concentrations of 1, 2 and 4× MIC over time. An exponentially or stationary-growing culture of each species was obtained by incubation at 30 °C and 150 rpm, in TSB medium. The growth phase was determined by conducting growth curves prior to time–kill assays. *Arcobacter* inoculums were added to test tubes containing resveratrol, DMSO or culture medium to reach a final concentration of 10^6 CFU/mL in 1 mL final volume. Tubes were incubated at 30 °C and viable counts were performed after 0, 2, 4, 6, 8, and 24 h of incubation. At these time intervals, 20 μL was sampled, serially diluted with TSB and plated onto blood agar by drop plate method, with a lower limit of detection of 2 $\log_{10}$ CFU/mL.

Bactericidal activity was defined as a reduction of 99.9% of the total number of CFU/mL in the original inoculum and bacteriostatic activity as the maintenance or reduction of less than 99.9% of the original inoculum concentration.

2.4. Flow cytometry

2.4.1. Exposure of Arcobacter to resveratrol

A. butzleri LMG 10828 and *A. cryaerophilus* LMG 10829 suspensions were obtained from an exponentially-growing culture of each strain on TSB at 30 °C and 150 rpm. This cell suspension was used to inoculate tubes containing resveratrol concentrations ranging from 50 to 200 μg/mL according to the MIC value obtained for each strain to obtain a final cell density of 1×10^6 CFU/mL. The tubes were incubated at 30 °C and 150 rpm for 2 h for 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining or 6 h in the case of deep red-fluorescing bisalkylaminoanthraquinone number five (DRAQ5) staining. The incubation time for DRAQ5 was longer to enable cell growth and DNA replication to a greater extent. At the end of each incubation period, samples were centrifuged at 10,000 *g* for 5 min at room temperature, washed and resuspended in sterile media for CTC staining. For DRAQ5 staining, cells were first fixed with 70% ethanol as previously described (Silva et al., 2010).

Control experiments were also carried out in parallel: cells were incubated in TSB with or without DMSO addition (solvent control) and handled under the same conditions.

2.4.2. Staining procedures for flow cytometry

In order to assess respiratory activity, cellular suspensions (1×10^6 CFU/mL) were incubated with 5 mM CTC (Polysciences, Inc., Warrington, PA) in TSB for 1 h at 30 °C and 150 rpm in the dark. Afterwards, cells were washed once with PBS and stained with 5 μM SYTO® 9 for total cell staining. The suspension was incubated in the dark for 15 min, washed once in PBS and resuspended in the same buffer for subsequent flow cytometric acquisition. Negative and positive CTC staining controls consisted of stationary-growing cells, heat-killed for 30 min at 70 °C and exponentially-growing cells, respectively.

For the analysis of intracellular DNA content, fixed cell suspensions (1×10^7 CFU/mL) were washed with PBS and incubated with 7.5 μM DRAQ5 (Biostatus Limited, Leicestershire, UK) for 30 min in the dark (Silva et al., 2011a). All experiments were conducted at least in duplicate.

2.4.3. Flow cytometry method

Bacterial samples were acquired on a CyAn ADP (Beckman Coulter Inc., Brea, CA) flow cytometer. Acquisition was performed with Summit Software (Beckman Coulter Inc., Brea, CA). The acquisition was based on light scatter and fluorescence signals resulting from 25 mW solid state laser illumination at 488 nm and 60 mW diode illumination at 642 nm. Fluorescence signals were collected by FL1 (530/40 nm, SYTO® 9), FL4 (680/30 nm, CTC) and FL8 (665/20 nm, DRAQ5) bandpass filters. Light scattering, SYTO® 9 and CTC fluorescence measurements were acquired logarithmically; whereas DRAQ5 fluorescence was acquired in a linear scale. Threshold was set on FL1 (SYTO® 9) (to exclude noise, other particles and debris). Cells were gated according to light scatter parameters. Sample acquisition was operated at a flow rate of no more than 300 events per second and a total of 10,000 and 20,000 events were acquired for each CTC or DRAQ5-stained sample, respectively. Data analysis was performed using FCS Express version 4 Plus Research Edition (De Novo Software).

2.5. Effect of resveratrol on the accumulation of ethidium bromide

To measure the level of ethidium bromide (EtBr) accumulation in *A. butzleri* and *A. cryaerophilus*, the bacteria were grown in TSB for 4 h, harvested, washed with PBS and resuspended to a final OD_{620}