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Antimicrobial activity of gallic acid against thermophilic *Campylobacter* is strain specific and associated with a loss of calcium ions

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ABSTRACT

Gallic acid has been suggested as a potential antimicrobial for the control of *Campylobacter* but its effectiveness is poorly studied. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of gallic acid against *Campylobacter jejuni* ($n = 8$) and *Campylobacter coli* ($n = 4$) strains was determined. Gallic acid inhibited the growth of five *C. jejuni* strains and three *C. coli* strains (MIC: 15.63–250 μg mL^{-1}). Gallic acid was only bactericidal to two *C. coli* strains (MBC: 125 and 62.5 μg mL^{-1}). The mechanism of the bactericidal effect against these two strains (and selected non-susceptible controls) was investigated by determining decimal reduction times and by monitoring the loss of cellular content and calcium ions, and changes in cell morphology. Gallic acid did not result in a loss of cellular content or morphological changes in the susceptible strains as compared to the controls. Gallic acid resulted in a loss of calcium ions (0.58–1.53 μg mL^{-1} and 0.54–1.17 μg mL^{-1}, respectively, over a 180 min period) from the susceptible strains but not the controls. Gallic acid is unlikely to be an effective antimicrobial against *Campylobacter* in a practical sense unless further interventions to ensure an effective bactericidal mode of action against all strains are developed.

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1. Introduction

Thermophilic *Campylobacter jejuni* and *Campylobacter coli* are leading causes of acute bacterial gastrointestinal infections worldwide (Pearson and Healing, 1992; Skirrow, 1998) with more than 95% of cases of human campylobacteriosis attributed to these two species (Park, 2002). A number of sources and vehicles of *Campylobacter* infection have been identified with the majority of these being warm-blooded animals (Tang et al., 2009; Silván et al., 2013). Poultry is regarded as the most important of these with respect to human health with suggestions that 80% of food products derived from poultry are contaminated with *Campylobacter* worldwide (Xie et al., 2011).

The incidence of *Campylobacter* infections caused by antibiotic-resistant strains has increased in recent years and this has been attributed to the widespread use of antibiotics in animal husbandry for non-therapeutic purposes and to treat campylobacteriosis in humans (Zhang and Plummer, 2008). This issue has raised concerns in many countries (Silván et al., 2013) with, for example, a high prevalence of *Campylobacter* resistant to a range of antibiotics, including fluoroquinolones and macrolides which are often applied in cases of campylobacteriosis, reported in Malaysia (Wieczorek et al., 2013).

The development of new antimicrobial agents as alternatives to antibiotics to control *Campylobacter*, including in non-clinical settings such as on farms or in food products, is important. There is a growing interest in using "natural" bioactive compounds from plants for this purpose (Silván et al., 2013). The determination of the efficacy of these antimicrobials is an important issue and may be measured in a number of ways. The minimum inhibitory concentration (MIC) is widely used and is defined as the lowest concentration of an antimicrobial agent that inhibits bacterial growth. The minimum bactericidal concentration (MBC) as generally defined and used for food microbiology (and in this paper) is the lowest concentration of an antimicrobial agent that effectively reduces (kills) numbers of bacteria in the inoculum by ~5 log (Upadhyay et al., 2013).

Gallic acid (3,4,5-trihydroxybenzoic acid) is a bioactive phytochemical that commonly occurs in a wide range of land plants (Aruoma et al., 1993). Gallic acid and its derivatives are often present in the human diet and can be reasonably regarded as "safe"

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and "natural" in the context of the food production system (Aruoma et al., 1993). Gallic acid has been shown to have antimicrobial activity against *Salmonella* Typhimurium (Nohynek et al., 2006), *Escherichia coli* and *Staphylococcus aureus* (Chanwitheesuk et al., 2007) and *C. jejuni* (Friedman et al., 2003; Nohynek et al., 2006; Ganan et al., 2008; Alkan et al., 2011). In all the above studies on the effect of gallic acid on *C. jejuni* only one or at most two strains of the species were tested in each study and results extrapolated to the species as a whole. To our knowledge no reports of the effect of gallic acid on *C. coli* have been published. Furthermore, studies suggesting the potential application of gallic acid for control of *C. jejuni* on food (Alkan et al., 2011) only tested bacteriostatic activity under conditions conducive to growth of this pathogen. Thermophilic *Campylobacter* are unlikely to grow on foods due to a fastidious requirement for microaerobic conditions and temperatures above 30 °C (Hazeleger et al., 1998). For this reason only a bactericidal activity of gallic acid is relevant for its application directly on food. In previous studies it has been found that gallic acid killed *Salmonella* strains by permeabilizing the outer membrane through chelation of divalent cations which led to subsequent cell lysis (Nohynek et al., 2006). The mode of action of gallic acid against *Campylobacter* has not been established.

Due to the lack of systematic studies on the antimicrobial activity of gallic acid against *Campylobacter* and the generality of the claims made regarding its potential use this study was undertaken to: (1) establish the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of gallic acid against a range of *C. jejuni* and *C. coli* strains that display resistance to antibiotics and (2) establish the mode of action of gallic acid as a bactericidal agent against *Campylobacter* killed by it. The results of the study provide insights into the limitations of this gallic acid to improve food safety with respect to *Campylobacter*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Seven strains of *C. jejuni* (2862, 2863, 2864, 2865, 2866, 2868 and 2869) and four strains of *C. coli* (2872, 2874, 2875 and 2876) isolated from poultry products from retail outlets in Malaysia were used in this study. These wild type strains have been characterised and reported to be resistant to a range of antibiotics in a previous study (Wieczorek et al., 2013). *C. jejuni* ATCC 33291, obtained from the American Type Culture Collection (Manassas, USA), was also used in this study. All strains were grown on Horse Blood Agar (HBA; Oxoid, UK) or in Mueller Hinton Broth (MHB; Oxoid, UK) with shaking at 150 rpm at 37 °C for 48 h under microaerobic conditions which was achieved by using gas producing sachets (CampyGen; Oxoid, UK) in anaerobic jars (Oxoid) according to the manufacturer's instructions unless otherwise stated.

2.2. Antimicrobial susceptibility tests

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of gallic acid against the 12 *Campylobacter* strains were determined using the micro-broth dilution method as previously described (Carson et al., 1995; Upadhyay et al. 2013). The concentrations of gallic acid (doubling dilution in MHB) used in the tests ranged from 7.8 to 1000 μg mL^{-1} and the microtiter plates (Jet Biofil, China) were incubated at 37 °C for 48 h under microaerobic conditions. Gentamicin (MP Biomedicals, France), which was reported to have strong antimicrobial activity against *Campylobacter* spp. (Goodman et al., 1984), was used as a positive control. The pH of the liquid medium (MHB and gallic acid) in the microtiter plate wells were also determined.

2.3. Preparation of bacterial suspensions for mode of actions studies

Bacterial suspensions were prepared by centrifuging 100 mL MHB cultures (grown 37 °C for 48 h under microaerobic conditions) at 12, 000 *g* for 12 min at 4 °C. The resultant pellets were washed gently with phosphate buffered saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 137 mM NaCl, pH 7.2; 1st BASE, Singapore) and resuspended in 50 mL PBS at a cell density of ~1×10^9 CFU mL^{-1}.

2.4. Decimal reduction time

The antimicrobial activity of the gallic acid against the *Campylobacter* strains were further evaluated by measuring the reduction in numbers (log CFU mL^{-1}) over 180 min as described previously (Carson et al., 2002) with modifications. A 9 mL bacterial suspension containing gallic acid at the MIC for the strain was incubated at 37 °C for 180 min under microaerobic conditions. Cells suspended in sterile deionised water at pH 6.99 containing the same concentration of gallic acid was used as a non-buffered control. Cells suspended in an inorganic acid, hydrochloric acid (HCl; R & M Chemicals, Malaysia), and an organic acid, formic acid (PC Laboratory Reagents, Malaysia), at the same concentration were used as controls for any concentration-dependent effects that may influence the survival of the cells. The pH of gallic acid in PBS, gallic acid in water, HCl and formic acid, all with bacterial cells suspended in them, were measured. A 1 mL sample was removed from the suspension at 45 min intervals and centrifuged at 12, 000 *g* for 5 min at 4 °C to remove the suspending liquid. The pellet was resuspended in 1 mL PBS, serially diluted, plated on Mueller Hinton Agar (MHA; Oxoid, UK) and incubated at 37 °C for 48 h under microaerobic conditions before enumeration.

2.5. Loss of cellular content

Any loss of bacterial cellular content associated with exposure to gallic acid was determined by measuring the change of absorbance of the suspending liquid at 260 nm (for nucleic acids) and at 280 nm (for proteins) over 180 min as described previously (Miksusanti et al., 2008) with modifications. A 15 mL bacterial suspension at a cell density of ~1×10^7 CFU mL^{-1} in PBS containing gallic acid at the MIC for the strain was incubated at 37 °C for 180 min under microaerobic conditions. A suspension without the addition of gallic acid was used as a control. A 3 mL sample was taken from the suspension at 45 min interval and filtered through a syringe driven hydrophilic filter (pore size: 0.2 μm; polyethersulfone (PES) membrane; Millipore, USA). The filtrate was diluted 100 folds and the absorbance was taken at 260 and 280 nm.

2.6. Loss of Ca^{2+}

Loss of Ca^{2+} after exposure to gallic acid was determined by measuring the Ca^{2+} concentration in the suspending liquid over 180 min using Atomic Absorption Spectroscopy (AAS) as described previously (Miksusanti et al., 2008) with modifications. A 1 L 48 h old MHB culture was centrifuged at 12, 000 *g* for 12 min at 4 °C using a large volume centrifuge (6930; Kubota, Japan). The pellet was resuspended in 500 mL PBS containing gallic acid at the MIC of the strain (or in PBS without gallic acid as a control) and incubated at 37 °C for 180 min under microaerobic conditions. A 50 mL sample was taken from the suspension at 45 min intervals, digested in an 8.8 M HNO_3/H_2O_2 solution (7:3; vol/vol) at 100 °C for 8 h and freeze dried for 48 h. The resultant powder was dissolved in 10 mL PBS. The Ca^{2+} concentration in the solution was determined using an AAS (200 Series AA; Agilent Technologies, USA). The results were

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