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Investigating the synergistic antimicrobial effect of carvacrol and zinc oxide nanoparticles against *Campylobacter jejuni*

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ABSTRACT

Campylobacter is a leading cause of foodborne gastroenteritis worldwide. *Campylobacter* contamination along the poultry production chain is believed to greatly contribute to human infections. Antibiotics are widely used in poultry industry to prevent infections as well as enhance animal growth, but the use of antibiotics is believed to be an important factor that promotes the emergence and dissemination of antibiotic-resistant *Campylobacter*. Alternative antimicrobial strategies will be beneficial to reduce the prevalence of this microbe in agri-foods and avoid the emergence of antibiotic resistance. Synergistic antimicrobial approach is to combine several antimicrobials of different mechanistic actions that can reduce the dosage of individual antimicrobial and expand the spectrum of antimicrobial activity. In this study, carvacrol and zinc oxide nanoparticles (ZnO NPs) were investigated regarding their synergistic antimicrobial effect against *Campylobacter jejuni*. The single treatment of either carvacrol or ZnO NPs at low concentration only generated a bacteriostatic effect. In contrast, the combination of carvacrol and ZnO NPs resulted in a bactericidal effect that significantly enhanced the antimicrobial efficacy. The mechanism of action of this synergism was investigated from both phenotypic and genotypic perspectives of *C. jejuni* using Raman spectroscopy and real-time quantitative polymerase chain reaction (RT-qPCR). Carvacrol was the inducing factor to damage the cell membrane and increase the susceptibility of *C. jejuni*, followed by the treatment of acting factor ZnO NPs to physically induce cell leakage. This study provides an alternative strategy to inactivate *C. jejuni* and demonstrates its potential to be applied in food industry against other foodborne pathogens.

1. Introduction

Bacterial contamination is one of the leading cause of foodborne illness worldwide. In 2010, *Campylobacter* spp. were responsible for 96 million cases of reported and diagnosed illnesses, ranked as the 2nd leading cause of foodborne infections (Kirk et al., 2015). Due to the low sensitivity of the current *Campylobacter* detection methods, the number of total cases caused by *Campylobac*ter spp. was believed to be underreported (Gibbons et al., 2014; Kaakoush, Castaño-Rodríguez, Mitchell, & Man, 2015). *Campylobacter* spp. is widely spread in the food processing environment and food production chain. Previous studies confirmed that poultry production was a major source of *Campylobacter* contamination (Altekruse, Stern, Fields, & Swerdlow, 1999). In 2014, almost half of the chicken breasts sampled in Canada were tested positive for *Campylobacter* (Canada, 2015). In order to control the contamination of pathogens as well as promote poultry growth, the use of antibiotics in poultry production was authorized (Malik et al., 2017; Murugesan, Syed, Haldar, & Pender, 2015). However, the indiscriminant use of antibiotics was likely to induce the emergence of antibiotic resistance of this microbe. The surveillance report indicated a strong correlation between the use of antibiotics in poultry feeds and the emergence of antibiotic-resistant isolates from poultry sources (Engberg, Aarestrup, Taylor, Gerner-Smidt, & Nachamkin, 2001; Witte, 1998). The prevalence of *Campylobacter* antibiotic-resistant strains is a serious challenge to not only the agri-food industry but also the public health. Alternative antimicrobials and strategies, such as synergistic

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antimicrobial treatment, are promising to reduce the prevalence of foodborne pathogens and avoid the emergence of antibiotic-resistant strains in agri-food systems.

Carvacrol ($C_{10}H_{14}O$), 5-isopropyl-2-methylphenol is a monoterpenoid phenolic compound and the major constituent of oregano (*Origanum vulgare*) (Friedman, Henika, & Mandrell, 2002) and thyme (*Thymus vulgaris*) essential oil (Fachini-Queiroz et al., 2012). The LD_{50} value (defined as the lethal dose at which 50% of the test population is killed in a given period of time) of carvacrol was 810 mg/kg body weight in rats, which was regarded as low toxicity potential (Jenner, Hagan, Taylor, Cook, & Fitzhugh, 1964). The cytotoxic effects were not observed in human cells at a maximum tested concentration of 90 mg/mL (Fachini-Queiroz et al., 2012). The use of carvacrol as a flavoring agent in foods was approved by the United States Food and Drug Administration (USFDA) under "Everything Added to Food in the United States (EAFUS)" status.

Zinc oxide nanoparticles (ZnO NPs) is an insoluble semiconductor that possesses electrochemical-coupling, pyroelectricity, and piezoelectricity properties (Wang, 2004). ZnO NPs have been identified as antimicrobial effective materials. Previous studies indicated that the size and shape of ZnO NPs had a closed relationship with its antimicrobial activity. ZnO NPs of small size can penetrate into the cell membrane and cause subsequent damage (Khan, Saeed, & Khan, 2017; Sirelkhatim et al., 2015). In addition, Tong and colleagues suggested that a large surface area of nanoparticles usually came along with the high antimicrobial activity. This was due to the increased exposure of the polar surface plane to the negatively charged bacterial cell membrane (Tong et al., 2013). With respect to agricultural applications, ZnO NPs have been considered as a "generally recognized as safe (GRAS) food additive" by the USFDA (Code of Federal Regulations: 21CFR182.8991). Good manufacturing practice of ZnO NPs in foods would not cause negative effects on human health as evaluated by the USFDA (Zhong & Shah, 2012).

In the current study, we developed a synergistic antimicrobial treatment by combining carvacrol and ZnO NPs against *C. jejuni*. The mechanism of action of this synergistic treatment was investigated on both chemical and transcriptional perspectives using Raman spectroscopy and RT-qPCR. The findings from this research will provide insight into the usability of nanoparticles and natural plant derivatives to reduce *Campylobacter* and other foodborne pathogens in the agri-food environment.

2. Materials and methods

2.1. Preparation of C. jejuni culture

A mixture of three *C. jejuni* isolates was used for antimicrobial tests, including a quality control strain *C. jejuni* ATCC 33560 and two clinical isolates from human (*C. jejuni* F38011 and *C. jejuni* human clinical 10). Each strain was resuscitated individually on Mueller-Hinton agar (Difco, New Jersey, USA) supplemented with 5% defibrinated sheep blood (Quad Five, Montana, USA) and incubated at 37 °C under a indiscriminant condition (10% CO2, 5% O2) for 48 h. One colony of each strain was then inoculated into 7 mL of Mueller-Hinton broth (MHB, Difco, New Jersey, USA) and incubated at 37 °C under a microaerobic condition with agitation for 18 h. Each culture was diluted to OD_{600nm} = ~0.3 (1×10^9 CFU/mL) with fresh MHB and mixed at equal volumes. The final density of the bacterial cocktail was adjusted to ~1×10^8 CFU/mL.

2.2. Susceptibility of C. jejuni to carvacrol and ZnO NPs

The microdilution broth method (Clinical Laboratory & Standard Institute) was employed with minor modifications (Jorgensen, Hindler, Reller, & Weinstein, 2007; Wiegand, Hilpert, & Hancock, 2008). Carvacrol (purity, ≥98%; Food Chemical Codex, Food Grade; Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (Fisher Bioreagents, purity > 99%) to an initial concentration of 10 mg/mL. Serial dilutions of carvacrol were prepared in MHB to achieve the final concentrations of 13.5 μg/mL, 18 μg/mL, 20.25 μg/mL, 27 μg/mL, 40.5 μg/mL, and 54 μg/mL in *C. jejuni* cocktail. Serial dilutions of ZnO NPs were prepared in MHB to achieve the final concentration of 12.5 μg/mL, 25 μg/mL and 50 μg/mL in *C. jejuni* cocktail. *C. jejuni* in MHB alone and MHB with 0.54% (v/v) DMSO were used as the control. Treatment and control samples were incubated at 37 °C under microaerophilic condition with agitation for 0, 4, 8, 12, and 24 h. At each time point, the sample was serially diluted in sterile phosphate buffered saline (PBS) (pH = 7.4) and plated on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. The viable cells were enumerated after 48 h of incubation at 37 °C under microaerobic condition.

2.3. Synergistic antimicrobial effects of carvacrol and zinc oxide nanoparticle against C. jejuni

The time-kill curve method was used to evaluate the synergistic antimicrobial effect of ZnO NPs and carvacrol against *C. jejuni* (Reller, Weinstein, Jorgensen, & Ferraro, 2009). Overnight *C. jejuni* culture was diluted in MHB to a concentration of 1×10^8 CFU/mL. The *C. jejuni* culture was then challenged with 12.5 μg/mL ZnO NPs and 18 μg/mL or 20.25 μg/mL carvacrol. The viable cells were enumerated at 0, 8, 12, and 24 h on MH agar supplemented with 5% defibrinated sheep blood.

2.4. Sample preparation for Raman spectral collection

C. jejuni strain F38011 treated with different antimicrobials (*i.e.,* untreated, carvacrol, ZnO NPs and combination) were collected after 6 h of treatment. A total of 1 mL of each suspension was spun in a centrifuge at 13,000 × *g* for 2 min. The supernatant was removed and cell pellets were washed twice with 500 μL sterile distilled deionized water to remove antimicrobial residues. Cell pellets were then re-suspended in 10 μL of water. Re-suspended samples (1 μL) were then transferred onto a gold-coated microarray chip (Thermo Scientific Inc., Waltham, MA) and dried in air for 10 min before the collection of Raman spectra.

2.5. Confocal micro-Raman spectroscopy

A confocal micro-Raman spectroscopic system (Leica Biosystems Inc., Germany) with a near-infrared laser (λ = 785 nm, 300-mW) was applied to characterize the chemical change of *C. jejuni* cells after antimicrobial treatment. Raman laser was introduced onto samples through a 50 × objective lens (Leica Biosystems, Wetzlar, Germany). Raman spectrometer was controlled via WiRE v.3.0 software (Renishaw, Gloucestershire, United Kingdom) for spectral acquisition. Raman spectra were collected at the wavenumber region of 400–3000 cm^{-1} with 10-s exposure time. Raman spectral collection was conducted from 8 random locations for each sample. The experiment was done in triplicate.

2.6. Raman spectral processing and chemometric analysis

Raman spectra were analyzed in the wavenumber region of 550–1650 cm^{-1}. The baseline of each spectrum was corrected with a fifth-order polynomial fitting, followed by a five-point boxcar smoothing using Vancouver Raman Algorithm software (The University of British Columbia). Processed Raman spectra were normalized on the basis of peak intensity at 1450 cm^{-1} using OMNIC v.8.2 Software (Thermo Scientific Inc., Waltham, MA). Chemometric analysis was employed. Specifically, a principal component analysis (PCA) model was constructed using MATLAB R2016a software (MathWorks, United States) with in-house programs and codes.

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