



The impact of antimicrobial effect of chestnut inner shell extracts against *Campylobacter jejuni* in chicken meat



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ABSTRACT

The antimicrobial effect of chestnut inner shell extract (CISE) characterized against *Campylobacter jejuni* strains by total polyphenol and flavonoid contents, minimum inhibitory concentrations (MIC), auto-aggregation, hydrophobicity, and application in laboratory medium and chicken meat. Total polyphenol and flavonoid contents of CISE were 532.96 ± 3.75 mg gallic acid equivalents/100 g and 12.28 ± 0.03 mg quercetin equivalents/100 g, respectively. The MIC of CISE was 1–5 mg/mL as *C. jejuni* strains. CISE reduced auto-aggregation and increased hydrophobicity against *C. jejuni*, and these characteristics showed the inhibition of adhesion to the intestine. In laboratory media, *C. jejuni* was completely inhibited at 3 and 5 log CFU/mL of inoculum at 4 and 42 °C in the addition of 1 and 2 mg/mL of CISE. In chicken meat, the addition of 1 and 2 mg/g of CISE decreased the number of 2–8 log CFU/g *C. jejuni* cells compared to without CISE at 42 °C. Moreover, *C. jejuni* was not detected at 1 mg/g CISE with 3 log CFU/g of inoculum after 4 days at 4 °C. Therefore, CISE could be used as a natural antimicrobial for the reduction of *Campylobacter* in the poultry meat supply.

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1. Introduction

Campylobacteriosis is the world's leading bacterial foodborne illness, and *Campylobacter* is the most frequently reported contaminant in the foodservice system (Piskernik, Klančnik, Riedle, Brøndsted, & Smole Možina, 2011). The incidence of *Campylobacter jejuni* in raw chicken meat was shown to be as high as 90–100% in retail markets (Andrzejewska, Szczepańska, Śpica, & Klawe, 2015). *C. jejuni* was also reported to survive in foods such as meat, eggs, and milk (Guévremont, Lamoureux, Ward, & Villeneuve, 2015; Kärenlampi & Hänninen, 2004). Attempts to reduce the incidence of human *Campylobacter* infection have been made to isolate anti-*Campylobacter* substances from plant extracts for use as natural alternatives to chemical additives (Burt, 2004; Piskernik et al., 2011). Rosemary (*Rosmarinus officinalis* L.) (Klančnik, Guzej, et al., 2009; Klančnik, Piskernik, Lipoglavšek, Smole Možina, 2009), roselle calyx (*H. sabdaariffa* L.) (Yin & Cao, 2008), and berry pomace (Salaheen, Nguyen, Hewes, & Biswas, 2014), anti-*Campylobacter*

maternal antibody (Sahin, Zhang, Meitzler, & Harr, 2001), isothiocyanate (Dufour et al., 2012), and probiotics (Wang et al., 2014) have been reported.

Castanea crenata (Japanese chestnut) belongs to the *Castanea* family and is a woody plant native to Japan and South Korea. Chestnut shells contain an abundance of phenols and hydrolysable tannins (Hwang, Hwang, & Park, 2001; Pasch & Pizzi, 2002; Vázquez et al., 2008). The chestnut peeling process generates waste products, which are ~10% of the total weight, that are used as fuel (Vázquez et al., 2008). The chestnut (*C. crenata*) inner shell has been used for a long time in Korea as a medicinal herb to improve digestion and as cosmetic material for its anti-aging and anti-wrinkle properties (Kim et al., 2012; Noh et al., 2010; Son et al., 2005).

Chestnut (*Castanea sativa*) extracts have been reported to have antimicrobial activity against several species, including *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella* Typhimurium (Živković et al., 2010). However, the antimicrobial effect of chestnut (*C. crenata*) inner shell against *C. jejuni* has not been reported. Therefore, this study aimed to determine the antimicrobial effect of chestnut inner shell extract (CISE) against *C. jejuni* by total polyphenol and flavonoid contents, minimum inhibitory concentrations

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(MIC), auto-aggregation, and hydrophobicity. The antimicrobial effects of CISE were investigated at 3 different inoculum densities (3, 5, and 7 log CFU per mL or per g) and 2 temperatures (4 and 42 °C) in liquid medium and chicken meat.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *C. jejuni* strains (32-1, 32-2, 35-1, 36-2, 44-1, 44-2, 73-1, and 74) used in this study were obtained from the Korea Food Research Institute (Seongnam, Gyeonggi-do, Korea) and were grown in Mueller-Hinton agar (MH agar; Difco Laboratories, USA) supplemented with 5% defibrinated sheep blood at 42 °C in 10% CO₂. The *C. jejuni* strains were stored in MH media containing 20% glycerol at –80 °C. To enumerate *C. jejuni*, the bacteria were incubated in blood agar (Kisanbio, Seoul, Korea) at 42 °C in 10% CO₂. *Listeria monocytogenes* (ATCC 15313 and Scott A), *S. aureus* KCCM 40511, *Staphylococcus hylococcus* 54, *B. cereus* (KCCM 40935 and KFRI 181), and *Escherichia coli* (O157:H7 FRIK 125 and O157 ATCC 43985) were incubated in Tryptic Soy agar (TSA, Difco) at 35 °C and tested for antimicrobial effect.

2.2. Preparation of CISE

Chestnut inner skin was purchased from traditional medicinal markets in Seoul, Korea. Dried and ground chestnut inner skin (100 g) was dissolved in 1 L of ethanol and incubated at 60 °C for 24 h. The extracts were filtered using Whatman No. 2 filter paper, and the filtrates were concentrated under reduced pressure at less than 50 °C to obtain a crude extract.

2.3. Determination of total phenolic and flavonoid contents

The total phenolic content of CISE was determined by the Folin-Ciocalteu method (Yoon et al., 2014). The CISE powder was dissolved in distilled water at a concentration of 1 mg/mL. A 100-μL aliquot was mixed with 2 mL of 2% sodium carbonate and incubated at room temperature for 3 min. Then, 0.1 mL of 50% Folin–Ciocalteu reagent (Sigma–Aldrich Co., Steinheim, Germany) was added, and the mixture was incubated for 30 min at room temperature. The absorbance of the reaction mixture was measured at 750 nm using a spectrophotometer (Optizen 2120 UV; Mecasys Co., Ltd., Daejeon, Korea). Total phenolic content was calculated based on a standard curve generated using gallic acid (0–250 mg/L; Sigma–Aldrich), and the results are expressed as mg of gallic acid equivalents per 100 g of CISE (mg GAE/100 g).

The total flavonoid content of the CISE was measured with an aluminum chloride assay (Lee et al., 2013). A 100-μL aliquot of the CISE was mixed with 1.5 mL of ethanol, 100 μL of 10% ammonium chloride, 100 μL of 1 M potassium acetate, and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the mixture was measured at 415 nm. Total flavonoid content was calculated based on a standard curve generated using quercetin (Sigma–Aldrich Co., St. Louis, MO, USA), and the results are expressed as mg quercetin equivalents per 100 g sample (mg QE/100 g).

2.4. Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) of the CISE was determined using the broth micro-dilution method (Klančnik, Piskernik, et al., 2009; Nkanwen, Gatsing, Ngamga, Fodouop, & Tane, 2009). A control was prepared with culture medium and a bacterial suspension containing approximately 1×10^5 CFU/mL.

CISE at concentrations ranging from 0.5 to 2 mg/mL was added to MH broth in plates containing approximately 1×10^5 CFU/mL *C. jejuni*. These plates were incubated at each incubation temperature for 24 h, and the optical densities of the cultures were measured at 570 nm using a microplate reader. All MIC measurements were determined at the lowest concentration of CISE to identify the inhibition of bacterial growth lower than 0.05 or with no visible growth. Gentamicin (AMRESCO Inc., Solon, Ohio, USA) was used as a positive control. All experiments were performed in triplicate.

2.5. Auto-aggregation and hydrophobicity of *C. jejuni* treated with CISE

Auto-aggregation and hydrophobicity were determined using a modification of the method described by Salameen et al. (2014). The *C. jejuni* bacterial cells were incubated at 42 °C for 24 h in either MH broth only (control) or MH broth supplemented with various concentrations of CISE to assess auto-aggregation. The cells were centrifuged at 3000×g for 10 min, and the supernatant was decanted. Cells were resuspended in 3 mL of PBS (pH 7.2) and the OD at 570 nm (OD₅₇₀) was adjusted to 0.5 (A₀). Then, the cell suspensions were incubated at 37 °C for 2 h. The supernatants were separated, and the absorbance was measured at 570 nm (A₂) using a microplate reader. Aggregation was determined by using the following equation: Auto-aggregation (%) = $(1 - A_2/A_0) \times 100$.

To determine the hydrophobicity, the bacterial cells were suspended in 2 mL of phosphate buffer saline (PBS, pH 7.2), and the OD₅₇₀ was adjusted to 0.5 (H₀). The solution was mixed with 1 mL of n-hexadecane and incubated for 5 min at room temperature. The absorbance of the aqueous phase was measured at 570 nm (H₁) using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The hydrophobicity was calculated using the following equation: Hydrophobicity (%) = $(1 - H_1/H_0) \times 100$.

2.6. Inhibition of cell growth in laboratory media and chicken meat

The kinetics of growth inhibition was evaluated using the broth microdilution method (Piskernik et al., 2011). A 10 mL of medium (MH broth) was inoculated with *C. jejuni* strain 74 at 3 different initial densities (3, 5, and 7 log CFU/mL), and 0, 0.5, 1, and 2 mg/mL of CISE were added. The samples were stored at 4 or 42 °C, diluted with 1 mL of 0.1% peptone water, and then 100-μL aliquots were plated onto blood agar and incubated microaerobically at 42 °C.

Chicken meat was purchased from major retail outlets in Seoul, Korea and transported to the lab within 30 min. Purchased chicken meat was cut into 10-g portions. The surface of the chicken meat was inoculated with *C. jejuni* strain 74 at 3 different initial densities (3, 5, and 7 log CFU/g) in a volume of 100 μL. Inoculated chicken meat samples were placed into sterile filter bags (Inter Science, Weymouth, MA, USA). Then, 0, 0.5, 1, and 2 mg/g of CISE dissolved in 90 mL of 0.1% peptone solution was added to the chicken samples, and these samples were stored at 4 or 42 °C. The entire samples were sampled and mixed with a stomacher (IUL instruments, Barcelona, Spain). The samples diluted with 1 mL of 0.1% peptone water, and then 100-μL aliquots were plated onto blood agar and incubated microaerobically at 42 °C.

2.7. Statistical analysis

C. jejuni cultures were assigned to the various treatments in triplicate, and analysis of variance (ANOVA), Duncan's multiple range test, and Pearson correlation analysis of experimental data were performed using SPSS 18 software (SPSS Inc., Chicago, IL, USA).

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