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Reduced *Campylobacter jejuni* colonization in poultry gut with bioactive phenolics

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ABSTRACT

This study was designed to determine the effects of bioactive phenolics (BPE) extracted from blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium corymbosum*) byproducts/pomaces on *Campylobacter jejuni* colonization in broiler cecum. We raised a total of 120 Cobb-500 broiler chicks in duplicate trials up to 3 weeks to determine the effect of BPE on the reduction of colonization using a *C. jejuni* RM1221 marker strain in broiler cecum. We observed that, as a water supplement, 1.0 g Gallic Acid Equivalent (GAE)/L of BPE reduced *C. jejuni* colonization level by 1 log in three weeks old broiler cecum compared to the control group. 1 g GAE/L of BPE also resulted a complete inhibition of the *C. jejuni* marker strain in drinking water with a potential for reduced horizontal transfer in poultry flocks. In a separate experiment, we also raised a total of 200 Cobb-500 broilers in duplicate trials up to 6 weeks to investigate natural colonization of *Campylobacter* in presence of BPE and observed that 1.0 g GAE/L of BPE reduced natural colonization level of *Campylobacter* by 2 logs in broiler cecum as a water supplement. Relative expression of several *C. jejuni* stress response genes, including rod shape determining protein (*mreB*) were down-regulated in the presence of sub-lethal concentration of BPE. Prolonged exposure of *C. jejuni* to BPE resulted the minimum inhibitory concentration (MIC) of BPE to increase from 0.5 to 1.0 mg GAE/mL; however, after single sub-culture in BPE-free broth, the MIC value revived to 0.5 mg GAE/mL. Findings from this study reveal the high potential of berry phenolics as green antimicrobials against enteric pathogen *Campylobacter* and application in the reduction of pre-harvest colonization level of *Campylobacter* in poultry gut.

1. Introduction

Campylobacter jejuni, a major enteric foodborne pathogen, is associated with 1.3 million illnesses and 76 deaths per year in the US and the most common bacterial cause of human gastroenteritis in the world (Kaakoush, Castaño-Rodríguez, Mitchell, & Man, 2015). According to a press release from the Centers for Disease Control and Prevention (CDC), the rate of campylobacteriosis in humans were increased by 13% compared to the rates observed in 2006–2008 (Crim et al., 2015). As poultry is considered the major source of human campylobacteriosis, increased popularity of white meat, especially chicken or turkey compared to red meat in recent years may be the key contributor of high campylobacteriosis rates in the US and many other developed countries (Lin, 2009). *Campylobacter* is optimally suited to the poultry niche, and is capable to grow at the increased body temperature of chicken of 42 °C (Williams, Fonseca, & Humphrey, 2016). Epidemiological and observational studies on the prevalence of *Campylobacter* in organic and conventional poultry farms and poultry carcasses revealed that antibiotic growth promoters were associated with reduced *Campylobacter* colonization in poultry while contributed in the development of antibiotic resistance (Luangtongkum et al., 2006; Salaheen et al., 2016). Consequently, alternative antimicrobials are essential to reduce *Campylobacter* colonization in poultry without the risk of antibiotic resistance development.

Bioactive phenolics from berries, especially blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium Corymbosum*) byproducts commonly known as pomace showed potential applicability due to their high abundance and antimicrobial effects against a wide variety of bacterial pathogens (Salaheen, Almario, & Biswas, 2014;

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Salaheen, Nguyen, Hewes, & Biswas, 2014; Salaheen, Nguyen, Mui, & Biswas, 2015; Yang, Hewes, Salaheen, Federman, & Biswas, 2014). Modes of actions of berry phenolics on bacterial pathogens include cell membrane damage (Lacombe, Tadepalli, Hwang, & Wu, 2013), inhibition of metabolic enzymes (Scalbert, 1991), deprivation of important growth substrates (Puupponen-Pimiä et al., 2005), and synergistic influence on the growth of probiotics for competitive exclusion of pathogenic bacteria (Salaheen et al., 2015). Berry pomace phenolics were also observed to reduce the natural colonization of *Salmonella* in poultry gut (Salaheen, Nguyen, et al., 2014). Clinical research reported that phenolics take up to 56 h to leave the gut as their absorption rate is slow which in turn come in contact with epithelial cells and gut microbiota (Seeram, Lee, Scheuller, & Heber, 2006). Therefore, exposure of chicken gut epithelial cells to berry phenolics should be investigated. In addition, the possibility of *Campylobacter* to develop resistance or stress response against berry phenolics should also be checked for persistent usability of these phenolics in chickens as water feed/water supplement.

In this study, we aim to determine the effect of bioactive phenolics from berry pomaces on *Campylobacter* stress response and its colonization in chicken cecum. The findings from this research will provide significant insight into the usability of berry phenolics from the byproducts to reduce pre-harvest colonization of *Campylobacter* in chickens.

2. Materials and methods

2.1. Bacterial strain and growth condition

In this study, we used *C. jejuni* RM1221 (ATCC BAA-1062™) for *in vitro* experiments and wild type strain to incorporate Kanamycin resistance (km^R) cassette into the chromosome. The bacterium was grown in Blood agar (Himedia, India) with 5% defibrinated sheep blood (Ward's Science, Rochester, NY) at 37 °C under microaerophilic (10% CO_2, 5% O_2, and 85% N_2) condition. *E. coli* DH5α was used as the host for cloning experiments, and pJET was used as the cloning vector (Labigne-Roussel, Harel, & Tompkins, 1987). *E. coli* β2155 and suicide vector pDS132 were used for conjugative transfer of km^R cassette in *C. jejuni* RM1221 genome.

2.2. Preparation of pomace extracts

Commercial blackberry and blueberry pomaces, kindly donated by Milne Fruit Products Inc., were stored at −20 °C and was used to extract phenolics according to the protocol previously described (Salaheen, Nguyen, et al., 2014; Salaheen, Almario, et al., 2014). Spectrophotometric method was used to determine the total phenolic contents in blackberry and blueberry pomace extracts and expressed as Gallic Acid Equivalent (GAE) (Singleton, Orthofer, & Lamuela-Raventos, 1999). Berry pomace extract (BPE) was comprised of blackberry and blueberry pomace extracts at 1:1 v/v ratio.

2.3. Culturing C. jejuni with sequential increase of BPE concentration in broth

The concentrations 0, 250, 500, 750, 1000, 1250, 1500, and 2000 μg GAE/mL of BPE were added to Bolton broth (Himedia) with 5% FBS to a final volume of 290 μL in 8 wells of 96-well tissue culture plates (Greiner Bio-One CellStar, Kremsmünster, Austria). *C. jejuni* RM1221 was cultured in Blood agar with 5% defibrinated sheep blood (Innovative Research) at 37 °C under microaerophilic for 24 h and bacterial suspension was prepared in PBS. 10 μL bacterial suspensions containing approximately 2×10^5 cfu/mL was added to each of the 8 wells of 96-wells plates. The Plates were incubated for 72 h at 37 °C under microaerophilic condition with shaking at 120 rpm. After 72 h, 10 μL bacterial suspensions from the well with highest concentration of BPE and with visible bacterial growth was transferred to new sets of 8 wells of 96-well tissue culture plates and this way we continued upto 24 days. After each 72 h time points, we determined the Minimum Inhibitory Concentration (MIC) of BPE on *C. jejuni* isolates from each of the 8 wells according to the protocol previously described (Salaheen, Almario, et al., 2014).

2.4. Evaluate the relative expression of stress related genes in C. jejuni with quantitative RT-PCR assay

C. jejuni RM1221 was grown in Bolton broth with 5% FBS in the absence (control) or presence (test) of sublethal (0.2 mg GAE/mL) concentration of BPE at 37 °C for 24 h under microaerophilic condition. Bacterial cells were harvested and RNA extraction was carried out, followed by cDNA synthesis and qRT-PCR was performed in EcoTM (Illumina, San Diego, CA) according to the protocol previously described (Salaheen, Almario, et al., 2014). PCR cycle was: 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec, 55 °C for 15 sec, and 72 °C for 10 sec. The custom-synthesized oligonucleotides (Erofins MWG Operon, Hanover, MD) were used as primers (Xie, He, Irwin, Jin, & Shi, 2011). The relative expression levels of genes were calculated by the comparative method (Livak & Schmittgen, 2001). The housekeeping gene, 16S rRNA, was used as the reference gene for normalization of target gene expression. Quantitative RT-PCR assay was carried out in triplicate.

2.5. Determine the role of BPE on cultured host cells

Human intestinal epithelial cells (INT407, ATCC® CCL-6™) and chicken fibroblast cells (DF1, ATCC® CRL-12203™) were cultured in Dulbecco's Modification of Eagle's Medium (DMEM, Corning) with 10% fetal bovine serum (FBS, Corning) at 37 °C under 5% CO_2 according to the protocol previously described (Salaheen, Almario, et al., 2014).

Viability of cultured human intestinal epithelial (INT407) and chicken fibroblast (DF1) cells in the presence of various concentration of BPE was measured following the method described previously with some modifications (Strober, 2001). Briefly, INT407 and DF1 cell monolayers were washed and 1 mL of fresh DMEM containing 10% FBS and various concentrations of BPE (0, 50, 100, 500, 1000, and 2000 μg GAE/mL) were placed in triplicate wells followed by incubation for 1 h at 37 °C and viability assay was carried out with 0.4% Trypan Blue solution (Sigma, St. Louis, MO).

2.6. Generation of the C. jejuni RM1221 marker strain by inserting kanamycin resistance cassette

C. jejuni RMKm marker strain was developed by incorporating kanamycin resistance (Km^R) cassette in place of cdtABC of the *C. jejuni* RM1221 genome. Km^R cassette was obtained from pJET-KM plasmid kindly donated by Dr. Fidelma Boyd (University of Delaware, DE) by restriction digestion with *Eco*RI and *Bam*HI (Thermo Fisher Scientific, Waltham, MA).

Genomic DNA from *C. jejuni* RM1221 was used as a template for PCR amplification of two fragments (AB from upstream and CD from downstream of cdtABC gene). AB fragment was PCR amplified using primer pair AB-F (TCTAGAGCCGCGATGATAATCCAAGC) and AB-R (GAATTC GGTACC GGATCCATAGCCCCTTGCACCCTAGAT). Primers AB-F and AB-R were included with *Xba*I and *Eco*RI, *Kpn*I and *Bam*HI, (underlined) respectively. CD fragment was PCR amplified using primer pair CD-F (GGATCCGGTACCGAATTCGCGAGGATTGATCCCATTTA) and CD-R (GAGCTCTTTTCCAAAGGGTCTTTCCA).