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## Investigating the Potential of Plant-derived Antimicrobials for Controlling *Campylobacter jejuni* in Poultry and Poultry Products

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Investigating the Potential of Plant-derived Antimicrobials for Controlling *Campylobacter jejuni*  
in Poultry and Poultry Products

A dissertation submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy in Poultry Science

by

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This dissertation is approved for recommendation to the Graduate Council.

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## Abstract

*Campylobacter jejuni* infection in humans is strongly associated with the handling and consumption of contaminated poultry products. Interventions reducing *C. jejuni* contamination in poultry would reduce the risk of subsequent human infections. In the first study, the efficacy of a Generally Recognized as Safe (GRAS) compound, eugenol (EG; derived from cloves), as an antimicrobial dip treatment to reduce *C. jejuni* in postharvest poultry was evaluated. The antimicrobial efficacy of EG was studied in suspension, emulsion and nanoemulsion delivery systems. EG suspension reduced *C. jejuni* counts with the greatest reduction of >2.0 Log CFU/sample for the 2% dose of EG ( $P<0.05$ ). Eugenol emulsions or nanoemulsions did not provide any additional *Campylobacter* reduction when compared with suspension alone.

In the second study, the efficacy of pectin or chitosan coatings fortified with eugenol to reduce *C. jejuni* on chicken wingettes was investigated. Inoculated wingettes were randomly assigned to controls, eugenol (0.5, 1 or 2%), pectin (3%), chitosan (2%) or their combinations. Following 1 min of coating, wingettes were air dried (1 h) and sampled on d 0, 1, 3, 5, and 7. The incorporation of 0.5, 1 or 2% eugenol in the pectin improved coating efficacy against *C. jejuni* whereas the efficacy of chitosan coating was improved by 2% eugenol treatment ( $P<0.05$ ). Exposure of *C. jejuni* to eugenol, chitosan or combination significantly modulated select genes encoding for motility, quorum sensing and stress response.

In the third study, the efficacy of eugenol, *trans*-cinnamaldehyde and carvacrol in inhibiting *C. jejuni* biofilm formation and inactivating mature biofilm was evaluated. For the inhibition study, *C. jejuni* was grown either in the presence or absence of sub-inhibitory concentrations of phytochemicals and biofilm formation was quantified at 24 h intervals by enumeration. For the inactivation study, mature *C. jejuni* biofilms were exposed to the

phytochemicals (0, 0.25, 0.5, or 1%) for 1, 5, or 10 min, and surviving *C. jejuni* in the biofilms were enumerated. All phytochemicals reduced *C. jejuni* biofilm formation as well as inactivated mature biofilm at both temperatures ( $P < 0.05$ ). Moreover, scanning electron microscopy revealed disruption of biofilm architecture and loss of extracellular polymeric substances after treatment.

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## **Dedication**

This work is dedicated to my parents

Bhoj Raj Wagle

and

Nanda Kala Wagle

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### **List of Publications (Under Review)**

1. Wagle, B. R., A. Upadhyay, S. Shrestha, K. Arsi, I. Upadhyaya, A. M. Donoghue, and D. J. Donoghue. Pectin or chitosan coating fortified with eugenol reduces *Campylobacter jejuni* on chicken wingettes and modulates expression of critical survival genes. Poultry Science. (Chapter 3).

### **List of Publications (In Preparation)**

2. Wagle, B. R., K. Arsi, S. Shrestha, A. Upadhyay, I. Upadhyaya, K. Bhargava, A. M. Donoghue, and D. J. Donoghue. Eugenol as an antimicrobial dip treatment reduces *Campylobacter jejuni* in postharvest poultry. (Chapter 2).
3. Wagle, B. R., A. Upadhyay, I. Upadhyaya, S. Shrestha, K. Arsi, K. Venkitanarayanan, D. J. Donoghue, and A. M. Donoghue. The natural plant compounds, *trans*-cinnamaldehyde, eugenol or carvacrol reduce *Campylobacter jejuni* biofilms on poultry plant processing surfaces. (Chapter 4).

**Chapter 1**  
**Introduction/Literature review**

## **CAMPYLOBACTER AND FOOD SAFETY CONCERNS**

*Campylobacter* is one of the leading causes of human bacterial gastroenteritis in the United States and across the world (Mangen et al., 2016; Marder et al., 2017). The majority of *Campylobacter* cases are caused by *C. jejuni* and accounts for 90% of the total human *Campylobacter* infections (Wagenaar et al., 2015). Campylobacteriosis accounts for 17.83 cases per 100,000 population in the United States and is one of the leading causes of bacterial foodborne infections (Marder et al., 2017). Campylobacteriosis symptoms include fever, nausea, vomiting and bloody diarrhea. In a small subset of individuals, immune mediated diseases such as Guillain-Barré syndrome and reactive arthritis are observed (Spiller, 2007; Gradel et al., 2009). In general, *Campylobacter* infections in humans are self-limiting, however, severe infections in children and immunocompromised people can occur requiring immediate treatment (Allos and Blaser, 1995; Allos, 2001). Studies have shown that health care expenses due to campylobacteriosis in the United States can be as high as \$1,800 per case leading to an overall economic burden of ~ \$1.7 billion per annum (Hoffmann et al., 2012; Scharff, 2012). In addition, the emergence of antibiotic resistant strains have further raised public concern for treating this disease (Zhao et al., 2010).

*Campylobacter jejuni* is part of the normal microflora in the gut of wild and domesticated animals, including poultry (Beery et al., 1988). *Campylobacter* rapidly colonizes the ceca with a very low dose (<50 CFU) and reaching as high as  $10^8$  CFU/g in the cecal contents without affecting the health and performance of birds (Stern et al., 1995; Achen et al., 1998). The high microbial load in the poultry gut leads to contamination of meat, processing equipment and the surrounding environment during poultry processing. Studies have shown that approximately 80% of the retail chicken meat are contaminated with *Campylobacter* (Zhao et al., 2001; EFSA, 2010) and presents a major risk factor for human infections (Friedman et al., 2004). Despite being nutritionally fastidious, *C. jejuni* survives well in the poultry processing environment and processed products

(Murphy et al., 2006; García-Sánchez et al., 2017; Castro et al., 2018). The ability of *C. jejuni* to tolerate common stressors including pH, temperature, osmolarity and low nutrients enables the pathogen to survive the transition from live birds to poultry meat (Lázaro et al., 1999; Murphy et al., 2006; Jackson et al., 2009; Feng et al., 2018). In such conditions, *C. jejuni* can form biofilms and/or transition into a viable but non-culturable form for survival in the environment (Jackson et al., 2009; Bronowski et al., 2014; Feng et al., 2018).

The environmental persistence of *C. jejuni* can be attributed (at least in part) to its ability to form biofilms (Joshua et al., 2006; Jackson et al., 2009; Feng et al., 2018). Bacterial biofilms are complex bacterial communities attached to the surfaces and are enclosed in extracellular polymeric substances (EPS) containing nucleic acids, proteins and polysaccharides (Donlan and Costerton, 2002). Biofilm allows *C. jejuni* to survive for a longer period of time (up to 24 days) as compared to planktonic cells under atmospheric conditions or submersed in water (Joshua et al., 2006; Lehtola et al., 2006). Moreover, biofilms are advantageous to bacteria because they provide increased resistance to disinfectants, antimicrobials and antibiotics (Reuter et al., 2010; Sofos and Geornaras, 2010). A recent study demonstrated that *C. jejuni* in a biofilm exhibits higher resistance (up to 32 fold) to antibiotics than in the corresponding planktonic forms (Malik et al., 2017). Bacterial communities may exhibit higher tolerance to environmental stress than single cells because of limited permeability of the extracellular polymeric substances, slower metabolism of bacteria and emergence of persister cells (Penesyan et al., 2015; Feng et al., 2018). In the laboratory setting, several strains of *C. jejuni* can form biofilms on a wide range of surfaces such as glass, stainless steel, polyvinyl chloride and nitrocellulose membranes (Trachoo et al., 2002; Kalmokoff et al., 2006; Reuter et al., 2010; Bronowski et al., 2014; Brown et al., 2014; Bronnec et al., 2016). Moreover, studies have demonstrated that *C. jejuni* biofilm formation increases in

the presence of atmospheric oxygen and chicken meat juice (Reuter et al., 2010; Brown et al., 2014). In the processing environment, *C. jejuni* can survive in established multi-species biofilms in addition to forming *de novo* biofilms (Teh et al., 2014). In the processing plant, researchers have detected *C. jejuni* on many food contact surfaces including conveyor belts and stainless steel tables and also demonstrated their ability to form biofilms (Lindsay and Geornaras, 1996; Zimmer et al., 2003; Peyrat et al., 2008). Continuous shedding of bacteria from the biofilms spreads the pathogens in the environment and subsequently contaminating the foods that they contact. Thus, biofilms could serve as a potential reservoir for *C. jejuni* and act as a source of contamination for poultry products in the processing plant. Besides, bacterial biofilms can also cause biodeterioration of processing equipment and mechanical blockade leading to economic losses to the poultry industry (Aaron, 2009).

Several genes contributed to the virulence and survival of *C. jejuni* in the host and environment. These genes have diverse functions in *C. jejuni* such as motility, chemotaxis, attachment, stress response and quorum sensing (Bolton, 2015). The motility of *C. jejuni* is imparted by flagella and it has two major flagellin proteins (FlaA, FlaB) and a hook. The hook is composed of several proteins encoded by motility complex genes (*fliF*, *fliY*, *fliM*, *fliA*, *fliK*, *flgI*, *flgE*, *flgH*, *motA*, *motB*) (Bolton, 2015). In addition, various chemotaxis proteins (CheA, CheB, CheR, CheV, CheW, CheZ) and the energy taxis system (CetA, CetB) are responsible for motility in response to stimuli, attachment and biofilm formation on various surfaces (Kalmokoff et al., 2006). Similarly, the adhesion of *C. jejuni* to the biotic and abiotic surfaces is contributed by outer membrane proteins such as CadF, CapA, Peb1 and JlpA (Hermans et al., 2011b). The *C. jejuni* genome also consists of a response regulator (CosR), which regulates the transcription of stress response genes (*kata*, *sodB*, *ahpC*) for environmental survival (Hermans et al., 2011b; Bolton,



2015). The quorum sensing in *C. jejuni* is encoded by *luxS* gene which play an important role in the attachment for biofilm formation (Reeser et al., 2007) as well as for the survival at low temperature (4°C) in the poultry processing environment (Ligowska et al., 2011). Thus, effective interventions that reduce the transcription of aforementioned genes could directly impair the survival and virulence of *C. jejuni* in poultry, poultry products and environment.

### **MULTI-HURDLE APPROACHES FOR CONTROLLING *C. JEJUNI***

A hurdle approach is a method ensuring that the foodborne pathogens are either eliminated or inactivated in the food products (Geornaras et al., 2005). Despite good management practices and results from various studies that specific interventions can reduce *C. jejuni* counts in poultry and poultry products, *C. jejuni* related illnesses continue to occur (Marder et al., 2017). Therefore, multi-hurdle approaches combining several interventions from farm to fork are necessary for controlling *C. jejuni* in poultry products. Each step in a multi-hurdle approach provides an opportunity to control this pathogen, prevent cross-contamination and spread of *C. jejuni* to the environment. The main three pillars of the multi-hurdle approaches are discussed below.

#### ***A. Pre-harvest control strategies***

Controlling *Campylobacter* colonization in birds is the first step in reducing *Campylobacter* contamination of poultry products. Several strategies including supplementation of feed or water with plant-derived antimicrobials (Solis de Los Santos et al., 2010; Molatová et al., 2011; Arsi et al., 2014; Arambel et al., 2015; Wagle et al., 2017), probiotics (Arsi et al., 2015; Guyard-Nicodeme et al., 2015; Shrestha et al., 2017), bacteriophages (Carrillo, et al., 2005; Wagenaar, et al., 2005), bacteriocins (Stern et al., 2005; Svetoch and Stern, 2010), and vaccination of birds (Widders, et al., 1996; Buckley, et al., 2010) to reduce or prevent *Campylobacter* colonization in birds have been tested. The results from such studies are variable and have had

limited success in reducing *C. jejuni* colonization in broiler chickens. Previous studies from our laboratory demonstrated that supplementation of medium-chain fatty acids (caprylic, caproic, capric acids) consistently reduced colonization by 3-4 Log CFU/g in market age chickens (Solís de Los Santos, et al., 2010). However, inconsistent reductions were reported with several plant-derived antimicrobials including sodium caprate, thymol, carvacrol, eugenol, trans-cinnamaldehyde, benzoic acid, garlic and cranberry extracts as evaluated in various studies (Hermans et al., 2010, 2011a; Metcalf et al., 2011; Arsi et al., 2014; Garcia et al., 2015; Woo-Ming et al., 2016). The variability observed may be due to interactions with gut contents, differences in the bird's microbiome, age, immune status, and route of application. Therefore, any treatment developed for commercial poultry production has to be rugged enough to reduce *Campylobacter* counts in birds raised in diverse environments. In addition, intestinal mucus could also protect *C. jejuni* in the cecal crypts of birds thereby limiting the effect of antimicrobials (Hermans et al., 2010). Similar results were reported with probiotics, where the in-feed supplementation either delayed the colonization (Guyard-Nicodeme et al., 2015) or produced inconsistent reductions (Arsi et al., 2015) of *C. jejuni* in broilers. The use of bacteriophages is a promising approach in reducing *C. jejuni* colonization in poultry (Carrillo et al., 2005; Wagenaar et al., 2005); however, public concerns regarding the development of resistance (El-Shibiny et al., 2009; Carvalho et al., 2010) and consumer acceptability of poultry treated with viruses may limit their use in poultry (Janež and Loc-Carrillo, 2013). Similarly, in-feed or in-water supplementation of bacteriocins were reported to reduce cecal *C. jejuni* colonization in broilers and turkey poults (Stern et al., 2005; Svetoch and Stern, 2010). However, developing purified bacteriocins or employing strategies for targeted release of bacteriocins by protecting them from digestion before reaching to ceca (for e.g. microencapsulation) are expensive and thereby leading to significant increase in the cost of meat

production (Svetoch and Stern, 2010). Likewise, vaccination of birds against *C. jejuni* were effective in increasing immunoglobulins (IgG, IgA) but failed to prevent pathogen colonization in the ceca (Widders et al., 1996; Buckley et al., 2010). Due to aforementioned hurdles, there is a need to develop a multi-hurdle approach which combines several interventions strategies targeting the various steps of poultry production-supply chain to have significant impact on reducing the incidence of human *Campylobacter* infections.

### ***B. Post-harvest prevention strategies***

*C. jejuni* is highly prevalent in poultry and processing of birds from infected flocks can cause contamination of the processing plant leading to transmission of *C. jejuni* to carcasses from uninfected flocks (Klein et al., 2007). Therefore, post-harvest strategies are necessary to reduce cross-contamination of carcasses during processing and thereby, limit the prevalence of *Campylobacter* in retail poultry products. Post-harvest interventions are divided broadly into physical and chemical decontamination methods.

Physical methods comprise of application of different water treatments (for e.g. steam, electrolyzed, pressurized or ozonated water), irradiation, ultrasound, air chilling and freezing (Loretz et al., 2010). It has been reported that using physical methods such as steam, hot water or electrolyzed water significantly reduce *C. jejuni* (1-3 Log reductions) in the post-harvest poultry (Loretz et al., 2010). However, such treatments could adversely affect the organoleptic properties of meat (Dawson et al., 1963; Cox et al., 1974; Notermans and Kampelmacher, 1974; McMeekin and Thomas, 1978; Thomas and McMeekin, 1982; Whyte et al., 2003). Strategies such as freezing, air chilling, crust-freezing and steam-ultrasound may not affect the organoleptic properties and can reduce *Campylobacter* counts in poultry products (Boysen and Rosenquist, 2009). However, several reports have demonstrated the recovery of *Campylobacter* from refrigerated, frozen (-

20°C) or superchilled carcasses and thus, still pose a risk of human infections (Zhao et al., 2003; Bhaduri and Cottrell, 2004; Castro et al., 2018).

Chemical decontamination used during poultry processing include peracetic acid (PAA), hydrogen peroxide, chlorine, organic acids, and trisodium phosphate (TSP). In the commercial poultry processing, PAA is commonly used for washing poultry carcasses (McKee, 2011). Bauermeister et al. (2008) had reported that PAA at 0.01% has limited efficacy in reducing *C. jejuni* counts in post-harvest poultry. Studies have demonstrated that PAA in combination with hydrogen peroxide (85 ppm) can reduce *Campylobacter* in carcasses by 43% (Bauermeister et al., 2008). However, PAA alone or in combinations with other compounds can cause significant change in the color and sensory qualities of meat (Bauermeister et al., 2008). Reidel and coworkers (2009) have demonstrated the efficacy of various chlorine-based products to reduce *Campylobacter* counts on chicken skin. They found the most effective treatment was cetylpyridinium chloride (0.5%) or benzalkonium chloride (1%) in reducing *C. jejuni* counts (> 4.2 Log CFU/mL reduction). However, the efficacy of chlorine was significantly decreased in the presence of organic materials and a pH above 7.0 (Northcutt et al., 2005; Oyarzabal et al., 2005). The use of organic acids such as formic acid (2%), lactic acid (2.5%) and capric acid (5%) also reduced the counts significantly in the range from 1.75 to 3.8 Log CFU/mL (Riedel et al., 2009; Thormar et al., 2011). Similar reductions were observed with 10% TSP on chicken skin and meat (Riedel et al., 2009). However, studies have reported discoloration of skin after dipping in various organic acids including acetic acid, citric acid, lactic acid, malic acid, mandelic acid and tartaric acid (Bilgili et al., 1998). Moreover, other problems associated with chemical treatments include residues in the meat, problem in disposing of chemicals and high costs limit the use of conventional chemicals (SCVPH, 1998; EFSA BIOHAZ Panel, 2014). For a chemical compound to be

applicable in commercial processing, it should be taken into account that chemicals should be approved for use by the Food and Drug Administration, have well documented efficacy, concentrations and contact time should be suitable to use in processing plants, be cost-effective and should not produce any harmful effects on the personnel, processing equipment or product quality (Bauermeister et al., 2008).

With an increasing consumer preference for natural and minimally processed products, research has intensified to explore the potential of natural compounds as safe and effective antimicrobial treatments for reducing foodborne pathogens including *Campylobacter* in foods (Burt, 2004). Plants represent a vast resource of natural, safe and effective antimicrobials. Several plants based phytophenols and essential oils with significant antimicrobial properties have been identified (Burt, 2004). Moreover, the majority of the phytochemicals are Generally Recognized as Safe (GRAS) to use in foods by the United States Food and Drug Administration (US FDA 21 CFR Section, 184.1257). A number of studies demonstrated the potential use of phytochemicals for controlling *C. jejuni* on poultry meat. Riedel et al. (2009) had reported significant reductions (3 Log CFU/mL) in *C. jejuni* counts after washing of chicken skin with 1.6% grape fruit extract for 1 min. Recently, we observed a 2 Log reduction of *C. jejuni* on chicken meat after washing with  $\beta$ -resorcylic acid for 30 s (Wagle et al., 2017). In addition,  $\beta$ -resorcylic acid significantly downregulated genes responsible for virulence and survival of *C. jejuni* in the chicken meat juice. Similar reductions were obtained with an essential oil, caprylic acid, on chicken skin (Woo-Ming et al., unpublished data). However, essential oils are volatile and have low solubility which could hinder its antimicrobial efficacy for complete elimination of this pathogen on chicken skin. To address this issue, several studies have formulated food-grade emulsion and nanoemulsion of essential oils against *Salmonella* Enteritidis, *Escherichia coli* and *Listeria monocytogenes* on foods

and found effective in reducing the pathogens (Landry et al., 2014; Bhargava et al., 2015; Maté et al., 2016). To our knowledge, there are no studies on efficacy of essential oils nanoemulsions against *C. jejuni*. The essential oils can also use as an antimicrobial agent to enhance the efficacy of coating materials in reducing foodborne pathogens on foods and prevention of cross-contamination during storage and handling. In a study, Olaimat et al. (2014) used chitosan/ $\kappa$ -carrageenan combination coating on chicken breast. They found significant reductions (up to 2.78 Log CFU/g) of *C. jejuni* with the coating containing mustard extract. Our laboratory had also determined the potential of antimicrobial coatings in reducing *C. jejuni*. We found significant reductions of *C. jejuni* on chicken wingettes after coating with chitosan-based caprylic acid (Woo-Ming et al., unpublished data). Moreover, such coatings modulated the virulence and survival genes of *C. jejuni* in the chicken meat juice. The results of using phytochemicals for controlling *C. jejuni* in poultry meat are promising, however, an in-depth understanding of the potential mechanism(s) of antibacterial action, as well as the possible effect on the sensory qualities of poultry meat are warranted in the future.

### ***C. Strategies for controlling C. jejuni biofilm***

Prevention of biofilm formation or eliminating pre-formed biofilms on food processing surfaces is critical for controlling foodborne pathogens and thereby ensuring a safer food product to consumers. Disinfection procedures in the poultry processing plant employ various chemical methods for pathogen control. This involves pre-rinsing with water followed by washing or scrubbing with chemicals such as chlorine, PAA and quaternary ammonium-based compounds (Arnold, 2009). The common antimicrobials used for cleaning in poultry processing are discussed below.

**Conventional chemicals.** Effective cleaning is the first step to improve the sanitation of a processing plant (Hayes and Forsythe, 2013). The common chemicals used for cleaning and disinfection in the food processing industry are alkali and quaternary ammonium compounds. Most of these chemicals act by decreasing surface tension, emulsifying fats and denaturing proteins (Simoes et al., 2010). The effectiveness of chemicals relies on the ability to break extracellular polymeric substances of biofilms in order to access bacteria residing inside the biofilm. In addition, the use of pressure and high temperature reduces the necessity of scrubbing (Simoes et al., 2010). Regarding *C. jejuni* biofilms, Trachoo et al. (2002) examined the anti-biofilm potential of chlorine, quaternary ammonia or PAA. They developed a mixed biofilm containing *C. jejuni* and *Pseudomonas aeruginosa* on the polyvinyl chloride surfaces and exposed them to different concentrations (50 to 200 ppm) of chemical sanitizers (sodium hypochlorite, quaternary ammonia or PAA) for 45 or 180 s. Although they found decreasing numbers of *C. jejuni*, the biofilm was not completely inactivated by these chemicals (PAA, quaternary ammonia). Similarly, Somers et al. (1994) reported that TSP can reduce *C. jejuni* biofilms on a stainless steel surface. However, viable cells were still detected even after treating the surfaces with 8% TSP for 2 min. Further, treating with compounds such as hypochlorite, chlorhexidine and PAA were effective in reducing viable cells on a *C. jejuni* biofilm. However, such treatments may cause emergence of biocide tolerant strains (Melo et al., 2017). Studies have also reported the limited effectiveness of chemicals due to several factors, including presence of organic matter (fat, carbohydrates, and protein based materials), water hardness, temperature and contact time (Trachoo et al., 2002; Northcutt et al., 2005; Oyarzabal, 2005).

**Biofilm-degrading enzyme (DNase I).** The extracellular polymeric substance (EPS) matrix is composed of numerous polysaccharides, proteins and extracellular DNA (eDNA). The presence

of this matrix in the biofilm renders protection to bacteria from chemical disinfectants. Several studies have investigated the potential of biofilm-degrading enzyme (DNase I) to break EPS in several biofilms forming bacteria including *Listeria monocytogenes* (Nguyen and Burrows, 2014), *P. aeruginosa* (Nemoto et al., 2003; Eckhart et al., 2007), *Escherichia coli* (Tetz and Tetz, 2010) and *Staphylococcus aureus* (Eckhart et al., 2007; Tetz and Tetz, 2010, Kaplan et al., 2012). Since eDNA also plays a critical role in the *C. jejuni* biofilm formation (Svensson et al., 2014; Feng et al., 2018), disruption of the eDNA could potentially inhibit these biofilms. In this regard, few studies have used DNase I in preventing the biofilm formation and removal of the pre-existing biofilms of *C. jejuni* (Brown et al., 2015; Kim et al., 2017). Brown et al. (2015) determined that the presence of eDNase in the genome of *C. jejuni* RM 1221 (non-biofilm forming strain) inhibited the biofilm of *C. jejuni* NCTC 11168 after overnight co-incubation. In addition, they reported that inactivation of eDNase gene (cje1441) restores the ability of biofilm formation in the *C. jejuni* RM 1221 strain. Similarly, Kim et al. (2017) evaluated the biofilm forming ability of 78 isolates of *Campylobacter* obtained from raw chicken. They found that DNase I inhibited the biofilm formation in *C. jejuni* and *C. coli* in a dose dependent pattern with 90% of the reductions observed at a concentration of 0.1 unit/100 µL. However, the efficacy of the biofilm-degrading enzyme (DNase I) can be limited with the production of high quantities of EPS and proteolytic activity of exoenzymes produced by the mature biofilms (Whitchurch et al., 2002).

***Bacteriophages.*** Bacteriophages are the viruses that can infect and kill specific bacteria (Hughes et al., 1998). They act by producing phage proteins (EPS depolymerase) which are capable of degrading EPS matrix and by phage infection resulting in lysis of bacteria within the biofilms (Hughes et al., 1998; Chan and Abedon, 2015). The potential application of anti-biofilm effect of bacteriophages has been extensively studied against several bacteria including *L. monocytogenes*



(Hibma et al., 1997), *P. fluorescens* (Sillankorva et al., 2004), *S. epidermidis* (Curtin and Donlan, 2006), *E. coli* O157: H7 (Sharma et al., 2005) and *S. Enteritidis* (Garcia et al., 2017). These studies reported significant reductions of bacterial biofilms after phage treatments. In the context of *C. jejuni*, Siringan et al. (2011) had observed 1 to 3 Log CFU/cm<sup>2</sup> reduction in viable cells of *C. jejuni* biofilm after 24 h post-infections with *Campylobacter* specific phages CP8 and CP30. However, they also found that 90% of the surviving *C. jejuni* developed resistance following treatment with bacteriophages and aided in the dispersal of *C. jejuni* biofilms. Therefore, further studies should explore the potential of purified enzymes extracted from *Campylobacter* specific phages in reducing the survival of *C. jejuni* in the biofilms.

***Plant-derived antimicrobials.*** The use of plant-derived antimicrobials for controlling biofilms has been investigated in several foodborne pathogens including *L. monocytogenes* (Upadhyay et al., 2013), *Salmonella* (Miladi et al, 2016; Oh et al, 2017), *E. coli* (Bazargani et al., 2016; Kim et al., 2016; Oh et al., 2017), *S. aureus* (Espina et al., 2015; Vázquez-Sánchez et al., 2015; Bazargani et al., 2016), and *P. aeruginosa* (Kavanaugh et al., 2012; Pratiwi et al., 2015). Recently, researchers have determined the potential of an anti-biofilm effect of various essential oils against *Campylobacter*. Essential oils act mainly by disrupting the membrane integrity leading to an imbalance of pH homeostasis and equilibrium of inorganic ions (Lambert et al., 2001). Consequently, this can cause bacterial lysis due to weakening of cell wall and rupture of the cytoplasmic membrane (Burt, 2004). Essential oils also damage nucleic acids and inhibit the mitochondrial ATPase activity thereby inhibiting mitochondrial membrane potential in the cells (Burt, 2004). Since essential oils have several targets on bacterial cells, the chance of resistance development is less likely (Borges et al., 2016). Studies have demonstrated that selected essential oils (cassia, Peru balsam or red thyme) can reduce biofilms with higher efficiency than common

antibiotics (Kavanaugh et al., 2012). In combination with antibiotics, phenolic compounds (gallic acid and toxifolin) works synergistically to inhibit *C. jejuni* by increasing permeability to ciprofloxacin and erythromycin and by reducing the multi-drug efflux pump (CmeABC) (Oh and Jeon, 2015). Similarly, Lu et al. (2012) reported a complete inactivation of *C. jejuni* biofilms after treatment with diallyl sulphide (an antimicrobial agent from *Allium* spp.) for 24 h. They also reported that diallyl sulphide caused greater alterations in EPS proteins and polysaccharides than treatment with ciprofloxacin and erythromycin indicating greater anti-biofilm efficiency of plant compounds than antibiotics. In another study, by-products of food processing (resveratrol, skins and seeds of Pinot noir grape) also inhibited efflux pump and reduced adhesion of *C. jejuni* to abiotic and biotic surfaces (Klančnik et al., 2017a). Similar results of reduced adhesion of *C. jejuni* to plastic surfaces have been demonstrated with the extracts of thyme (Šikić Pogačar et al., 2016), olive leaf (Šikić Pogačar et al., 2016), *Alpinia katsumadai* seeds (Klančnik et al., 2017b) and juniper fruits (Klančnik et al., 2017c).

In addition to their antimicrobial actions, plant compounds also contain inhibitors (enzymes) of quorum sensing molecules. Quorum sensing is an intercellular signaling and regulatory mechanisms that involves the synthesis, secretion and detection of extracellular signaling molecules thereby triggering a signal transduction cascade for alteration in gene expression during *C. jejuni* biofilm formation (Reeser et al., 2007). A number of studies had observed that extract of citrus (Castillo et al., 2014), *Euodia ruticarpa* (Bezek et al., 2016) or resveratrol (Duarte et al., 2015) significantly inhibited quorum sensing in *C. jejuni* biofilms. However, the effect of plant-derived compounds in inactivating the pre-formed *C. jejuni* biofilms at conditions mimicking the poultry processing environment has not been conducted yet.

Moreover, future studies should also focused on delineating underlying mechanism of actions of such compounds.

### **CONCLUDING REMARKS**

This review of the available literature on controlling *Campylobacter* in birds, poultry products and in the environment, discusses several interventions with potential in reducing contamination of poultry meat. Due to interaction of antimicrobials with several extrinsic factors encountered at each stage of poultry production and the complexities of each system, a multi-hurdle approach from farm to fork combining several interventions is necessary for reducing pathogen load and the risk of infections associated with the consumption of contaminated poultry products.

### **HYPOTHESIS OF THE DISSERTATION**

Based on the published literature and preliminary research, it was hypothesized that plant-derived compounds exert significant antimicrobial effects and anti-biofilm effects against *C. jejuni* in post-harvest poultry and abiotic surfaces. Moreover, plant-derived antimicrobials reduce expression of associated genes/proteins.

The specific objectives of this dissertation were:

1. To investigate the efficacy of eugenol suspension, emulsion and nanoemulsion as an antimicrobial wash treatment to reduce *C. jejuni* counts on chicken skin.
2. To investigate the efficacy of pectin or chitosan coating fortified with eugenol to reduce *C. jejuni* counts on chicken wingettes.
3. To investigate the efficacy of *trans*-cinnamaldehyde, eugenol and carvacrol in inhibiting *C. jejuni* biofilm formation and inactivating matured *C. jejuni* biofilm.

## REFERENCES

- Achen, M., T. Y. Morishita, and E. C. Ley. 1998. Shedding and colonization of *Campylobacter jejuni* in broilers from day-of-hatch to slaughter age. *Avian Dis.* 42: 732-737.
- Allos, B. M. 2001. *Campylobacter jejuni* Infections: update on emerging issues and trends. *Clin. Infect. Dis.* 32: 1201-1206.
- Allos, B. M., and M. J. Blaser. 1995. *Campylobacter jejuni* and the expanding spectrum of related infections. *Clin. Infect. Dis.* 20: 1092-1099.
- Arambel, H. R., A. M. Donoghue, K. Arsi, A. Upadhyay, A. Woo-Ming, P. Blore, K. Venkitanarayanan, and D. Donoghue. 2015. Chitosan supplementation reduces enteric colonization of *Campylobacter jejuni* in broiler chickens and down-regulates expression of colonization genes. *Adv. Food Tech. Nutr. Sci.* 1: 104-111.
- Arnold, J. W. 2009. Biofilms in poultry processing. Pages 455-473 in *Biofilms in the Food and Beverage Industries*. Woodhead Publishing Limited, Cambridge, UK.
- Arsi, K., A. M. Donoghue, A. Woo-Ming, P. J. Blore, and D. J. Donoghue. 2015. The efficacy of selected probiotic and prebiotic combinations in reducing *Campylobacter* colonization in broiler chickens. *J. Appl. Poult. Res.* 24: 327-334.
- Arsi, K., A. M. Donoghue, K. Venkitanarayanan, A. K. Johnny, A. C. Fanatico, P. J. Blore and D. J. Donoghue. 2014. The efficacy of the natural plant extracts, thymol and carvacrol against *Campylobacter* colonization in broiler chickens. *J. Food Saf.* 34: 321-325.
- Asakura, H., M. Yamasaki, S. Yamamoto, and S. Igimi. 2007. Deletion of *peb4* gene impairs cell adhesion and biofilm formation in *Campylobacter jejuni*. *FEMS Microbiol. Lett.* 275: 278-285.
- Bauermeister, L. J., J. W. Bowers, J. C. Townsend, and S. R. McKee. 2008. Validating the efficacy of peracetic acid mixture as an antimicrobial in poultry chillers. *J. Food Prot.* 71: 1119-1122.
- Bazargani, M. M., and J. Rohloff. 2016. Antibiofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms. *Food Control.* 61: 156-164.
- Beery, J. T., M. B. Hugdahl, and M. P. Doyle. 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 54: 2365-2370.
- Bezek, K., M. Kurinčič, E. Knauder, A. Klančnik, P. Raspor, F. Bucar, and S. S. Možina. 2016. Attenuation of adhesion, biofilm formation and quorum sensing of *Campylobacter jejuni* by *Euodia ruticarpa*. *Phytother. Res.* 30: 1527-1532.
- Bhaduri, S., and B. Cottrell. 2004. Survival of cold-stressed *Campylobacter jejuni* on ground chicken and chicken skin during frozen storage. *Appl. Environ. Microbiol.* 70: 7103-7109.
- Bhargava, K., D. S. Conti, S. R. P. da Rocha, and Y. Zhang. 2015. Application of an oregano oil nanoemulsion to the control of foodborne bacteria on fresh lettuce. *Food Microbiol.* 47: 69-73.

- Bilgili, S., D. Conner, J. Pinion, and K. Tamblyn. 1998. Broiler skin color as affected by organic acids: Influence of concentration and method of application. *Poult. Sci.* 77: 752-757.
- Bolton, D. J. 2015. *Campylobacter* virulence and survival factors. *Food Microbiol.* 48: 99-108.
- Borges, A., A. C. Abreu, C. Dias, M. J. Saavedra, F. Borges, and M. Simões. 2016. New perspectives on the use of phytochemicals as an emergent strategy to control bacterial infections including biofilms. *Molecules* 21:877.
- Boysen, L., and H. Rosenquist. 2009. Reduction of thermotolerant *Campylobacter* species on broiler carcasses following physical decontamination at slaughter. *J. Food Prot.* 72: 497-502.
- Bronnec, V., H. Turoňová, A. Bouju, S. Cruveiller, R. Rodrigues, K. Demnerova, O. Tresse, N. Haddad, and M. Zagorec. 2016. Adhesion, biofilm formation, and genomic features of *Campylobacter jejuni* Bf, an atypical strain able to grow under aerobic conditions. *Front. Microbiol.* 7: 1002.
- Bronowski, C., C. E. James, and C. Winstanley. 2014. Role of environmental survival in transmission of *Campylobacter jejuni*. *FEMS Microbiol. Lett.* 356: 8-19.
- Brown, H. L., M. Reuter, K. Hanman, R. P. Betts, and A. H. M. van Vliet. 2015. Prevention of biofilm formation and removal of existing biofilms by extracellular DNases of *Campylobacter jejuni*. *PLoS One* 10: e0121680.
- Brown, H. L., M. Reuter, L. J. Salt, K. L. Cross, R. P. Betts, and A. H. M. van Vliet. 2014. Chicken juice enhances surface attachment and biofilm formation of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 80: 7053-7060.
- Buckley, A. M., J. Wang, D. L. Hudson, A. J. Grant, M. A. Jones, D. J. Maskell, and M. P. Stevens. 2010. Evaluation of live-attenuated *Salmonella* vaccines expressing *Campylobacter* antigens for control of *C. jejuni* in poultry. *Vaccine* 28: 1094-1105.
- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* 94: 223-253.
- Carrillo, C. Loc., R. J. Atterbury, A. El-Shibiny, P. L. Connerton, E. Dillon, A. Scott, and I. F. Connerton. 2005. Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Appl. Environ. Microbiol.* 71: 6554-6563.
- Carvalho, C. M., B. W. Gannon, D. E. Halfhide, S. B. Santos, C. M. Hayes, J. M. Roe, and J. Azeredo. 2010. The *in vivo* efficacy of two administration routes of a phage cocktail to reduce numbers of *Campylobacter coli* and *Campylobacter jejuni* in chickens. *BMC Microbiol.* 10: 232.
- Castillo, S., N. Heredia, E. Arechiga-Carvajal, and S. García. 2014. Citrus extracts as inhibitors of quorum sensing, biofilm formation and motility of *Campylobacter jejuni*. *Food Biotechnol.* 28: 106-122.
- Castro, A. G. S. A., E. Dorneles, E. L. S. Santos, T. M. Alves, G. R. Silva, T. C. Figueiredo, D. Assis, A. P. Lage, and S. V. Cançado. 2018. Viability of *Campylobacter* spp. in frozen and chilled broiler carcasses according to real-time PCR with propidium monoazide pretreatment. *Poult. Sci.* 0: 1-6.

- Chan, B. K., and S. T. Abedon. 2015. Bacteriophages and their enzymes in biofilm control. *Curr. Pharma. Des.* 21: 85-99
- Cox, N., A. Mercuri, J. Thomson, and D. Gregory. 1974. Quality of broiler carcasses as affected by hot water treatments. *Poult. Sci.* 53: 1566-1571.
- Curtin, J. J., and R. M. Donlan. 2006. Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* 50: 1268-1275.
- Dawson, L., R. Walker, M. Zabik, D. Bigbee, and W. Mallmann. 1963. Influence of surface pasteurization and chlortetracycline on bacterial incidence on fyers. *Food Tech.* 17: 218.
- Donlan, R. M., and J. W. Costerton. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15: 167-193.
- Duarte, A., A. C. Alves, S. Ferreira, F. Silva, and F. C. Domingues. 2015. Resveratrol inclusion complexes: antibacterial and anti-biofilm activity against *Campylobacter* spp. and *Arcobacter butzleri*. *Food Res. Int.* 77: 244-250.
- Eckhart, L., H. Fischer, K. B. Barken, T. Tolker-Nielsen, and E. Tschachler. 2007. DNase1L2 suppresses biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Br. J. Dermatol.* 156: 1342-1345.
- EFSA BIOHAZ Panel. 2014. Scientific opinion on the evaluation of the safety and efficacy of peroxyacetic acid solutions for reduction of pathogens on poultrycarcasses and meat. *EFSA Journal* 12. Accessed Feb. 2018.
- EFSA. 2010. Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008 - Part A: *Campylobacter* and *Salmonella* prevalence estimates. *EFSA Journal*. Accessed Feb. 2018.
- El-Shibiny, A., A. Scott, A. Timms, Y. Metawea, P. Connerton, and I. Connerton. 2009. Application of a group II *Campylobacter* bacteriophage to reduce strains of *Campylobacter jejuni* and *Campylobacter coli* colonizing broiler chickens. *J. Food Prot.* 72: 733-740.
- Espina, L., R. Pagán, D. López, and D. García-Gonzalo. 2015. Individual constituents from essential oils inhibit biofilm mass production by multi-drug resistant *Staphylococcus aureus*. *Molecules* 20: 11357-11372.
- Feng, J., L. Ma, J. Nie, M. E. Konkel, and X. Lu. 2018. Environmental stress-induced bacterial lysis and extracellular DNA release contribute to *Campylobacter jejuni* biofilm formation." *Appl. Environ. Microbiol.* 84: e02068-17.
- Friedman, C. R., R. M. Hoekstra, M. Samuel, R. Marcus, J. Bender, B. Shiferaw, S. Reddy, S. D. Ahuja, D. L. Helfrick, F. Hardnett, M. Carter, B. Anderson, R. V. Tauxe, and G. Emerging Infections Program FoodNet Working. 2004. Risk factors for sporadic *Campylobacter* infection in the United States: A case-control study in FoodNet sites. *Clin. Infect. Dis.* 3: S285-296.

- Garcia, K. C. O. D., I. M. O. Corrêa, L. Q. Pereira, T. M. Silva, M. S. R. Mioni, A. C. M. Izidoro, I. H. V. Bastos, G. A. M. Gonçalves, A. S. Okamoto, and R. L. A. Filho. 2017. Bacteriophage use to control *Salmonella* biofilm on surfaces present in chicken slaughterhouses. *Poult. Sci.* 96: 3392-3398.
- García-Sánchez, L., B. Melero, I. Jaime, M. Hänninen, M. Rossi, and J. Rovira. 2017. *Campylobacter jejuni* survival in a poultry processing plant environment. *Food Microbiol.* 65: 185-192
- Geornaras, I., and J. N. Sofos. 2005. Combining physical and chemical decontamination interventions for meat. Pages 433-460 in *Improving the Safety of Fresh Meat*.
- Gracia, M. I., C. Millán, J. Sánchez, M. Guyard-Nicodeme, J. Mayot, Y. Carre, A. Csorbai, M. Chemaly, and P. Medel. 2015. Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period: part B. *Poult. Sci.* 95: 886-892.
- Gradel, K. O., H. L. Nielsen, H. C. Schønheyder, T. Ejlersen, B. Kristensen, and H. Nielsen. 2009. Increased short- and long-term risk of inflammatory bowel disease after *Salmonella* or *Campylobacter* gastroenteritis. *Gastroenterol.* 137: 495-501.
- Guyard-Nicodeme, M., A. Keita, S. Quesne, M. Amelot, T. Poezevara, B. Le Berre, J. Sánchez, P. Vasseur, A. Martin, P. Medel, and M. Chemaly. 2015. Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period. *Poult. Sci.* 95: 298-305.
- Hayes, P. R., and S. J. Forsythe. 2013. *Food hygiene, microbiology and HACCP*. Springer Science & Business Media.
- Hermans, D., A. Martel, K. V. Deun, F. V. Immerseel, M. Heyndrickx, F. Haesebrouck, and F. Pasmans. 2011a. The cinnamon-oil ingredient trans-cinnamaldehyde fails to target *Campylobacter jejuni* strain KC 40 in the broiler chicken cecum despite marked *in vitro* activity. *J. Food Prot.* 74: 1729-1734.
- Hermans, D., A. Martel, K. V. Deun, M. Verlinden, F. V. Immerseel, A. Garmyn, W. Messens, M. Heyndrickx, F. Haesebrouck, and F. Pasmans. 2010. Intestinal mucus protects *Campylobacter jejuni* in the ceca of colonized broiler chickens against the bactericidal effects of medium-chain fatty acids. *Poult. Sci.* 89: 1144-1155.
- Hermans, D., K. Van Deun, A. Martel, F. Van Immerseel, W. Messens, M. Heyndrickx, F. Haesebrouck, and F. Pasmans. 2011b. Colonization factors of *Campylobacter jejuni* in the chicken gut. *Vet. Res.* 42: 82.
- Hibma, A. M., S. A. A. Jassim, and M. W. Griffiths. 1997. Infection and removal of L-forms of *Listeria monocytogenes* with bred bacteriophage. *Int. J. Food Microbiol.* 34: 197-207.
- Hoffmann, S., M. B. Batz, and J. G. Morris Jr. 2012. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. *J. Food Prot.* 75: 1292-1302.

- Hughes, K. A., I. W. Sutherland, J. Clark, and M. V. Jones. 1998. Bacteriophage and associated polysaccharide depolymerases—novel tools for study of bacterial biofilms. *J. Appl. Microbiol.* 85: 583-590.
- Jackson, D. N., B. Davis, S. M. Tirado, M. Duggal, J. K. Frankenhuyzen, D. Deaville, M. A. K. Wijesinghe, M. Tessaro, and J. T. Trevors. 2009. Survival mechanisms and culturability of *Campylobacter jejuni* under stress conditions. *Antonie Van Leeuwenhoek* 96: 377-394.
- Janež, N., and C. Loc-Carrillo. 2013. Use of phages to control *Campylobacter* spp. *J. Microbiol. Methods* 95:68-75.
- Joshua, G. W. P., C. Guthrie-Irons, A. V. Karlyshev, and B. W. Wren. 2006. Biofilm formation in *Campylobacter jejuni*. *Microbiol.* 152: 387-396.
- Kaplan, J. B., K. LoVetri, S. T. Cardona, S. Madhyastha, I. Sadovskaya, S. Jabbouri, and E. A. Izano. 2012. Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in *Staphylococci*. *J. Antibiot.* 65: 73.
- Kavanaugh, N. L., and K. Ribbeck. 2012. Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms. *Appl. Environ. Microbiol.* 78: 4057-4061.
- Kim, S., C. Park, E. Lee, W. Bang, Y. Kim, and J. Kim. 2017. Biofilm formation of *Campylobacter* strains isolated from raw chickens and its reduction with DNase I treatment. *Food Control* 71: 94-100.
- Kim, Y., J. Lee, G. Gwon, S. Kim, J. G. Park, and Jintae Lee. 2016. Essential oils and eugenols inhibit biofilm formation and the virulence of *Escherichia coli* O157: H7. *Sci. Rep.* 6: 36377.
- Klančnik, A., I. Gobin, D. Vučković, S. S. Možina, M. Abram, and B. Jeršek. 2017. Reduced contamination and infection via inhibition of adhesion of foodborne bacteria to abiotic polystyrene and biotic amoeba surfaces. *Int. J. Food Sci. Technol.* 53: 1013-1020.
- Klančnik, A., M. Šikić Pogačar, K. Trošt, M. Tušek Žnidarič, B. Mozetič Vodopivec, and S. Smole Možina. 2017. Anti-*Campylobacter* activity of resveratrol and an extract from waste Pinot noir grape skins and seeds, and resistance of *C. jejuni* planktonic and biofilm cells, mediated via the CmeABC efflux pump. *J. Appl. Microbiol.* 122: 65-77.
- Klančnik, A., Š. Zorko, N. Toplak, M. Kovač, F. Bucar, B. Jeršek, and S. S. Možina. 2017. Antiadhesion activity of juniper (*Juniperus communis* L.) preparations against *Campylobacter jejuni* evaluated with PCR-based methods. *Phytother. Res.* 32: 542-550.
- Klein, G., F. Reich, L. Beckmann, and V. Atanassova. 2007. Quantification of thermophilic *Campylobacter* spp. in broilers during meat processing. *Antonie van Leeuwenhoek* 92: 267-273.
- Lambert, R. J. W., P. N. Skandamis, P. J. Coote, and G. Nychas. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* 91: 453-462.



- Landry, K. S., Y. Chang, D. J. McClements, and L. McLandsborough. 2014. Effectiveness of a novel spontaneous carvacrol nanoemulsion against *Salmonella enterica* Enteritidis and *Escherichia coli* O157: H7 on contaminated mung bean and alfalfa seeds. *Int. J. Food Microbiol.* 187: 15-21.
- Lázaro, B., J. Cárcamo, A. Audicana, I. Perales, and A. Fernández-Astorga. 1999. Viability and DNA maintenance in nonculturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. *Appl. Environ. Microbiol.* 65: 4677-4681.
- Ligowska, M., M. T. Cohn, R. A. Stabler, B. W. Wren, and L. Brøndsted. 2011. Effect of chicken meat environment on gene expression of *Campylobacter jejuni* and its relevance to survival in food. *Int. J. Food Microbiol.* 145: 111-115.
- Lehtola, M. J., T. Pitkänen, L. Miebach, and I. T. Miettinen. 2006. Survival of *Campylobacter jejuni* in potable water biofilms: a comparative study with different detection methods. *Wat. Sci. Tech.* 54: 57-61.
- Lindsay, D., and I. Geornaras. 1996. Biofilms associated with poultry processing equipment. *Microbios* 86: 105-116.
- Loretz, M., R. Stephan, and C. Zweifel. 2010. Antimicrobial activity of decontamination treatments for poultry carcasses: a literature survey. *Food Control* 21: 791-804.
- Lu, X., D. R. Samuelson, B. A. Rasco, and M. E. Konkel. 2012. Antimicrobial effect of diallyl sulphide on *Campylobacter jejuni* biofilms. *J. Antimicrobial. Chemother.* : 1915-1926.
- Malik, H., S. Rajagunalan, M. S. Kumar, J. L. Kataria, P. Anjay, S. Sachan, A. K. Upadhyay, and A. Kumar. 2017. Assessment of Antibiotics Effect on Planktonic and Biofilm Forms of *Campylobacter* Isolates. *Israel J. Vet. Med.* 72: 4.
- Mangen, M. J., A. H. Havelaar, J. A. Haagsma, and M. E. E. Kretzschmar. 2016. The burden of *Campylobacter*-associated disease in six European countries. *Microb. Risk Anal.* 2: 48-52.
- Marder, E. P., P. R. Cieslak, A. B. Cronquist, J. Dunn, S. Lathrop, T. Rabatsky-Ehr, P. Ryan, K. Smith, M. Tobin-D'Angelo, D. J. Vugia, and S. Zansky. 2017. Incidence and trends of infections with pathogens transmitted commonly through food and the effect of increasing use of culture-independent diagnostic tests on surveillance-foodborne diseases active surveillance network, 10 US Sites, 2013-2016. *MMWR. Morb. Mortal. Wkly. Rep.* 66: 397-403. doi: 10.15585/mmwr.mm6615a1.
- Maté, J., M. P. Paula, and A. Palop. 2016. When nanoemulsified, d-limonene reduces *Listeria monocytogenes* heat resistance about one hundred times. *Food Control* 59: 824-828.
- McMeekin, T., and C. Thomas. 1978. Retention of bacteria on chicken skin after immersion in bacterial suspensions. *J. Appl. Bacteriol.* 45: 383-387.
- Melo, R. T., E. P. Mendonça, G. P. Monteiro, M. C. Siqueira, C. B. Pereira, P. A. B. M. Peres, H. Fernandez, and D. A. Rossi. 2017. Intrinsic and extrinsic aspects on *Campylobacter jejuni* biofilms. *Front. Microbiol.* 8: 1332.

- Metcalf, J. H., A. M. Donoghue, K. Venkitanarayanan, I. Reyes-Herrera, V. F. Aguiar, P. J. Blore, and D. J. Donoghue. 2011. Water administration of the medium-chain fatty acid caprylic acid produced variable efficacy against enteric *Campylobacter* colonization in broilers. *Poult. Sci.* 90: 494-497.
- Miladi, H., D. Mili, R. B. Slama, S. Zouari, E. Ammar, and A. Bakhrouf. 2016. Antibiofilm formation and anti-adhesive property of three mediterranean essential oils against a foodborne pathogen *Salmonella* strain. *Microb. Pathog.* 93: 22-31.
- Molatova, Z., E. Skřivanová, J. Baré, K. Houf, G. Bruggeman, and M. Marounek. 2011. Effect of coated and non-coated fatty acid supplementation on broiler chickens experimentally infected with *Campylobacter jejuni*. *J. Anim. Physiol. Anim. Nutr.* 95: 701-706.
- Murphy, C., C. Carroll, and K. N. Jordan. 2006. Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol.* 100: 623-632.
- Nemoto, K., K. Hirota, K. Murakami, K. Taniguti, H. Murata, D. Viducic, and Y. Miyake. 2003. Effect of Varidase (*streptodornase*) on biofilm formed by *Pseudomonas aeruginosa*. *Chemother.* 49: 121-125.
- Nguyen, U. T., and L. L. Burrows. 2014. DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms. *Int. j. Food Microbiol.* 187: 26-32.
- Northcutt, J. K., D. P. Smith, M. T. Musgrove, K. D. Ingram, and Arthur Hinton Jr. 2005. Microbiological impact of spray washing broiler carcasses using different chlorine concentrations and water temperatures. *Poult. Sci.* 84: 1648-1652.
- Notermans, S., and E. Kampelmacher. 1974. Attachment of some bacterial strains to the skin of broiler chickens. *Br. Poult. Sci.* 15: 573-585.
- Oh, E., and B. Jeon. 2015. Synergistic anti-*Campylobacter jejuni* activity of fluoroquinolone and macrolide antibiotics with phenolic compounds. *Front. Microbiol.* 6: 1129.
- Oh, S. Y., W. Yun, J. H. Lee, C. H. Lee, W. K. Kwak, and J. H. Cho. 2017. Effects of essential oil (blended and single essential oils) on anti-biofilm formation of *Salmonella* and *Escherichia coli*. *J. Anim. Sci. Technol.* 59: 4.
- Olaimat, A. N., Y. Fang, and R. A. Holley. 2014. Inhibition of *Campylobacter jejuni* on fresh chicken breasts by  $\kappa$ -carrageenan/chitosan-based coatings containing allyl isothiocyanate or deodorized oriental mustard extract. *Int. J. Food Microbiol.* 187: 77-82.
- Oyarzabal, O. A. 2005. Reduction of *Campylobacter* spp. by commercial antimicrobials applied during the processing of broiler chickens: a review from the United States perspective. *J. Food Prot.* 68: 1752-1760.
- Penesyan, A., M. Gillings, and I. T. Paulsen. 2015. Antibiotic discovery: combatting bacterial resistance in cells and in biofilm communities. *Molecules* 20: 5286-5298.

- Peyrat, M. B., C. Soumet, P. Maris, and P. Sanders. 2008. Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: analysis of a potential source of carcass contamination. *Int. J. Food Microbiol.* 124: 188-194.
- Pratiwi, S. U. T., E. L. Lagendijk, S. Weert, T. Hertiani, R. Idroes, and C. V. D. Hondel. 2015. Effect of *Cinnamomum burmannii* Nees ex Bl. and *Massoia aromatica* Becc. Essential oils on planktonic growth and biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* *In vitro*. *Int. J. Appl. Res. Nat. Prod.* 8: 1-13.
- Reeser, R. J., R. T. Medler, S. J. Billington, B. H. Jost, and L. A. Joens. 2007. Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. *Appl. Environ. Microbiol.* 73: 1908-1913.
- Reuter, M., A. Mallett, B. M. Pearson, and A. H. M. Vliet. 2010. Biofilm formation by *Campylobacter jejuni* is increased under aerobic conditions. *Appl. Environ. Microbiol.* 76: 2122-2128.
- Riedel, C. T., L. Brøndsted, H. Rosenquist, S. N. Haxgart, and B. B. Christensen. 2009. Chemical decontamination of *Campylobacter jejuni* on chicken skin and meat. *J. Food Prot.* 72: 1173-1180.
- Scharff, R. L. 2012. Economic burden from health losses due to foodborne illness in the United States. *J. Food Prot.* 75: 123-131.
- SCVPH. 1998. Report of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) on benefits and limitations of antimicrobial treatments for poultry carcasses. Accessed Feb 2018.
- Sharma, M., J. H. Ryu, and L. R. Beuchat. 2005. Inactivation of *Escherichia coli* O157: H7 in biofilm on stainless steel by treatment with an alkaline cleaner and a bacteriophage. *J. Appl. Microbiol.* 99: 449-459.
- Shrestha, S., K. Arsi, B. Wagle, A. Donoghue, and D. Donoghue. 2017. Ability of select probiotics to reduce enteric *Campylobacter* colonization in broiler chickens. *Int. J. Poult. Sci.* 16: 37-42.
- Šikić Pogačar, M., A. Klančnik, F. Bucar, T. Langerholc, and S. Smole Možina. 2016. Anti-adhesion activity of thyme (*Thymus vulgaris* L.) extract, thyme post-distillation waste, and olive (*Olea europea* L.) leaf extract against *Campylobacter jejuni* on polystyrene and intestine epithelial cells. *J. Sci. Food Agric.* 96: 2723-2730.
- Sillankorva, S., R. Oliveira, M. J. Vieira, I. Sutherland, and J. Azeredo. 2004. Bacteriophage  $\Phi$  S1 infection of *Pseudomonas fluorescens* planktonic cells versus biofilms. *Biofouling.* 20: 133-138.
- Simões, M., L. C. Simoes, and M. J. Vieira. 2010. A review of current and emergent biofilm control strategies. *LWT-Food Sci. Technol.* 43: 573-583.
- Siringan, P., P. L. Connerton, R. J. H. Payne, and I. F. Connerton. 2011. Bacteriophage-mediated dispersal of *Campylobacter jejuni* biofilms. *Appl. Environ. Microbiol.* 77: 3320-3326.

- Sofos, J. N., and I. Geornaras. 2010. Overview of current meat hygiene and safety risks and summary of recent studies on biofilms, and control of *Escherichia coli* O157: H7 in nonintact, and *Listeria monocytogenes* in ready-to-eat, meat products. *Meat Sci.* 86: 2-14.
- Solis de Los Santos, F. S., M. Hume, K. Venkitanarayanan, A. M. Donoghue, I. Hanning, M. F. Slavik, V. F. Aguiar, J. H. Metcalf, I. Reyes-Herrera, and P. J. Blore. 2010. Caprylic acid reduces enteric *Campylobacter* colonization in market-aged broiler chickens but does not appear to alter cecal microbial populations. *J. Food Prot.* 73: 251-257.
- Somers, E. B., J. L. Schoeni, and A. C. L. Wong. 1994. Effect of trisodium phosphate on biofilm and planktonic cells of *Campylobacter jejuni*, *Escherichia coli* O157: H7, *Listeria monocytogenes* and *Salmonella typhimurium*. *Int. J. Food Microbiol.* 22: 269-276.
- Spiller, R. C. 2007. Role of infection in irritable bowel syndrome. *J. Gastroenterol.* 42: 41-47.
- Stern, N. J., E. A. Svetoch, B. V. Eruslanov, Y. N. Kovalev, L. I. Volodina, V. V. Perelygin, E. V. Mitsevich, I. P. Mitsevich, and V. P. Levchuk. 2005. *Paenibacillus polymyxa* purified bacteriocin to control *Campylobacter jejuni* in chickens. *J. Food Prot.* 68: 1450-1453.
- Stern, N. J., M. R. S. Clavero, J. S. Bailey, N. A. Cox, and M. C. Robach. 1995. *Campylobacter* spp. in broilers on the farm and after transport. *Poult. Sci.* 74: 937-941.
- Svensson, S. L., M. Pryjma, and E. C. Gaynor. 2014. Flagella-mediated adhesion and extracellular DNA release contribute to biofilm formation and stress tolerance of *Campylobacter jejuni*. *PloS one* 9: e106063.
- Svetoch, E. A., and N. J. Stern. 2010. Bacteriocins to control *Campylobacter* spp. in poultry—a review. *Poult. Sci.* 89: 1763-1768.
- Teh, A. H. T., S. M. Lee, and G. A. Dykes. 2014. Does *Campylobacter jejuni* form biofilms in food-related environments?. *Appl. Environ. Microbiology* 80: 5154-5160.
- Tetz, V. V., and G. V. Tetz. 2010. Effect of extracellular DNA destruction by DNase I on characteristics of forming biofilms. *DNA Cell Biol.* 29: 399-405.
- Thomas, C. J., and T. A. McMeekin. 1982. Effect of water immersion on the microtopography of the skin of chicken carcasses. *J. Sci. Food Agric.* 33: 549-554.
- Thormar, H., H. Hilmarsson, J. Thrainsson, F. Georgsson, E. Gunnarsson, and S. Dadadottir. 2011. Treatment of fresh poultry carcasses with emulsions of glycerol monocaprinate (monocaprin) to reduce contamination with *Campylobacter* and psychrotrophic bacteria. *Br. Poult. Sci.* 52: 11-19.
- Trachoo, N., and J. F. Frank. 2002. Effectiveness of chemical sanitizers against *Campylobacter jejuni*-containing biofilms. *J. Food Prot.* 65: 1117-1121.
- Trachoo, N., J. F. Frank, and N. J. Stern. 2002. Survival of *Campylobacter jejuni* in biofilms isolated from chicken houses. *J. Food Prot.* 65: 1110-1116.
- Upadhyay, A., I. Upadhyaya, A. Kollanoor-Johny, and K. Venkitanarayanan. 2013. Antibiofilm effect of plant derived antimicrobials on *Listeria monocytogenes*. *Food Microbiol.* 36: 79-89.

- Vázquez-Sánchez, D., M. L. Cabo, and J. J. Rodríguez-Herrera. 2015. Antimicrobial activity of essential oils against *Staphylococcus aureus* biofilms. *Food Sci. Technol. Int.* 21: 559-570
- Wagenaar, J. A., M. A. P. Van Bergen, M. A. Mueller, T. M. Wassenaar, and R. M. Carlton. 2005. Phage therapy reduces *Campylobacter jejuni* colonization in broilers. *Vet. Microbiol.* 109: 275-283.
- Wagenaar, J.A., D. G. Newell, R. S. Kalupahana, and L. Mughini-Gras. 2015. *Campylobacter*: animal reservoirs, human infections, and options for control. Pages 159-177 In *Zoonoses-infections affecting humans and animals*. Springer: Dordrecht, Netherlands.
- Wagle, B. R., A. Upadhyay, K. Arsi, S. Shrestha, K. Venkitanarayanan, A. M. Donoghue, and D. J. Donoghue. 2017. Application of  $\beta$ -Resorcylic acid as potential antimicrobial feed additive to reduce *Campylobacter* colonization in broiler chickens. *Front. Microbiol.* 8: 599.
- Wagle, B. R., K. Arsi, A. Upadhyay, S. Shrestha, K. Venkitanarayanan, A. M. Donoghue, and D. J. Donoghue. 2017.  $\beta$ -Resorcylic acid, a phytophenolic compound, reduces *Campylobacter jejuni* in postharvest poultry. *J. Food Prot.* 80: 1243-1251.
- Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick. 2002. Extracellular DNA required for bacterial biofilm formation. *Science* 295: 1487-1487.
- Whyte, P., K. McGill, and J. Collins. 2003. An assessment of steam pasteurization and hot water immersion treatments for the microbiological decontamination of broiler carcasses. *Food Microbiol.* 20: 111-117.
- Widders, P. R., R. Perry, W. I. Muir, A. J. Husband, and K. A. Long. 1996. Immunisation of chickens to reduce intestinal colonization with *Campylobacter jejuni*. *Br. Poult. Sci.* 37: 765-778.
- Woo-Ming, A. N. 2015. Reduction of *Campylobacter jejuni* on chicken wingettes by treatment with caprylic acid, chitosan or protective cultures of *Lactobacillus* spp. PhD diss., University of Arkansas.
- Woo-Ming, A., K. Arsi, A. M. Donoghue, J. R. Moyle, and D. J. Donoghue. 2016. Efficacy of natural cranberry extracts against *Campylobacter* colonization in poultry. *Int. J. Food Eng.* 2: 66-70.
- Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. G. White, D. Wagner, and J. Meng. 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. *Appl. Environ. Microbiol.* 67: 5431-5436.
- Zhao, S., S. R. Young, E. Tong, J. W. Abbott, N. Womack, S. L. Friedman, and P. F. McDermott. 2010. Antimicrobial Resistance of *Campylobacter* isolates from retail meat in the United States between 2002 and 2007. *Appl. Environ. Microbiol.* 76: 7949-7956.
- Zhao, T., G. O. Ezeike, M. P. Doyle, Y. C. Hung, and R. S. Howell. 2003. Reduction of *Campylobacter jejuni* on poultry by low-temperature treatment. *J. Food Prot.* 66: 652-655.
- Zimmer, M., H. Barnhart, U. Idris, and M. D. Lee. 2003. Detection of *Campylobacter jejuni* strains in the water lines of a commercial broiler house and their relationship to the strains that colonized the chickens. *Avian Dis.* 47: 101-107.

**Table 1: Summary of key studies investigating strategies for controlling *C. jejuni* biofilms on different surfaces**

Treatments	Exposure time	Type of biofilm	Surfaces/Incubation time and temperature	<i>Campylobacter</i> spp.	Results	References
Sodium hypochlorite (4-6%)	50 and 200 ppm for 45 and 180 s	Mixed	Polyvinyl chloride; 24 h at 12°C	<i>C. jejuni</i> with <i>Pseudomonas</i>	Inactivated completely	Trachoo et al., 2002
Sodium hypochlorite and chlorhexidine	1% for 24 h	Monospecies	Nitrocellulose membrane; 72 h for 37°C microaerophilic	<i>C. jejuni</i>	5 Log CFU/mL reduction; emergence of resistant strain	Melo et al., 2017
Quaternary ammonium compounds	50 and 200 ppm for 45 and 180 s	Mixed	Polyvinyl chloride; 24 h at 12°C	<i>C. jejuni</i> with <i>Pseudomonas</i>	Reduce <i>C. jejuni</i> in biofilms	Trachoo et al., 2002
Peracetic acid	50 and 200 ppm for 45 and 180 s	Mixed	Polyvinyl chloride; 24 h at 12°C	<i>C. jejuni</i> with <i>Pseudomonas</i>	Reduce <i>C. jejuni</i> in biofilms	Trachoo et al., 2002
Peracetic acid	0.8% for 24 h	Monospecies	Nitrocellulose membrane; 72 h at 37°C microaerophilic	<i>C. jejuni</i>	4-5 Log CFU/mL reductions; emergence of resistant strains	Melo et al., 2017
Trisodium phosphate	1% for 30 s and 2 min	Monospecies	Stainless steel coupons and buna-N rubber surfaces; 48 h	<i>C. jejuni</i>	Reduce <i>C. jejuni</i> in biofilms	Somers et al., 1994
DNase	24 h co-incubation with biofilm	Monospecies	Borosilicate glass; 48 h at 37°C; microaerophilic and aerobic	<i>C. jejuni</i> NCTC 11168 and 81116	Degrade biofilm	Brown et al., 2015
DNase	1 h co-incubation	Monospecies	Polystyrene plates; 72 h at 37°C; microaerophilic	<i>C. jejuni</i> , <i>C. coli</i>	Reduced biofilm formation	Kim et al., 2017
Bacteriophages (CP8 and CP30)	24 h co-incubation	Monospecies	Glass coverslips; 120 h at 37°C; microaerophilic	<i>C. jejuni</i> NCTC 11168 and PT14	1 to 3 log CFU/cm <sup>2</sup> reduction	Siringan et al., 2011
Diallyl sulphide from plants, <i>Allium</i> spp	0.1mg/L for 24 h co-incubation	Monospecies	Nitrocellulose membrane; 72 h at 37°C microaerophilic	<i>C. jejuni</i>	Inactivated completely	Lu et al., 2012

## **Chapter 2**

### **Eugenol as an antimicrobial dip treatment reduces *Campylobacter jejuni* in postharvest poultry**

Running title: Eugenol reduces *C. jejuni* in poultry

**Title: Eugenol as an antimicrobial dip treatment reduces *Campylobacter jejuni* in postharvest poultry**

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## ABSTRACT

*Campylobacter jejuni*, a leading cause of foodborne illness in humans, is strongly associated with the consumption of contaminated poultry products. Interventions reducing *C. jejuni* contamination in poultry would reduce the risk of subsequent human infections. With increasing consumer demand for natural and minimally processed product, novel postharvest interventions, which are safe and environmentally friendly, are needed for controlling *C. jejuni* on poultry products. This study investigated the efficacy of a Generally Recognized as Safe compound, eugenol (EG), as an antimicrobial dip treatment to reduce *C. jejuni* in postharvest poultry. The antimicrobial efficacy of EG was studied in suspension, emulsion or nanoemulsion delivery systems (2 trials per delivery system). In each trial, chicken skin samples were inoculated with a mixture of four wild-type strains of *C. jejuni* (~7.2 Log CFU/sample). Inoculated skin samples were dipped in EG treatments (0, 0.125, 0.25, 0.5, 1 or 2%) for 1 min, drip dried for 2 min and then processed at 0, 8 and 24 h (n=5 samples/treatment/time point) of refrigerated storage for enumeration of surviving *C. jejuni* and total aerobic bacterial counts. In addition, the effect of EG on the color of chicken skin was evaluated. Bacterial counts were logarithmic transformed and data were analyzed by the PROC MIXED procedure of SAS. In both skin trials, all doses of the EG suspension reduced *C. jejuni* counts with the greatest reduction of >2.0 Log CFU/sample for the 2% dose of EG (P<0.05). The 0.5, 1 and 2% EG also consistently reduced the total aerobic counts by at least 0.57 Log CFU/sample. Eugenol emulsions or nanoemulsions did not provide any additional *Campylobacter* reduction when compared with suspension alone. Moreover, EG did not affect lightness, redness and yellowness of chicken skin (P>0.05). These findings suggest that EG could be an effective postharvest intervention for reducing *C. jejuni* contamination on poultry products.

## 1. INTRODUCTION

*Campylobacter* is a major cause of foodborne illness in humans resulting in significant economic losses worldwide (Mangen *et al.*, 2016; Marder *et al.*, 2017). Out of 17 species of *Campylobacter*, *Campylobacter jejuni* alone accounts for 90% of human campylobacteriosis (Wagenaar *et al.*, 2015). *C. jejuni* mainly causes gastroenteritis characterized by bloody diarrhea, cramping, abdominal pain and fever in humans. In certain cases, the pathogen leads to serious sequelae such as, reactive arthritis and Guillain-Barré syndrome (Spiller, 2007; Gradel *et al.*, 2009) that could lead to death. Foodborne illness surveillance has linked consumption and handling of poultry products to the majority of human campylobacteriosis cases (Friedman *et al.*, 2004; Danis *et al.*, 2009). *C. jejuni* colonizes the ceca in high numbers ( $10^8$  cells/g) without causing clinical illness in poultry and serve as source of infection in humans (Beery *et al.*, 1988; Achen *et al.*, 1998). Since low numbers (~500 cells) of *C. jejuni* can cause infection in humans (Black *et al.*, 1988), elimination or reduction of *C. jejuni* contamination in poultry can significantly reduce the incidence of human campylobacteriosis (Nauta *et al.*, 2016). However, limited success has been reported in interventions targeting the colonization of *C. jejuni* in birds (Hermans *et al.*, 2011; Arsi *et al.*, 2014; Gracia *et al.*, 2015; Guyard-Nicodème *et al.*, 2015; Wagenaar *et al.*, 2015). Such results highlight the need for novel postharvest interventions to reduce contamination of poultry meat and meat products.

Decontamination of the carcass during processing is beneficial to keep the products microbiologically safe at the various steps of poultry processing–supply continuum. In this regard, a number of studies have been conducted to reduce pathogen load using chemicals such as chlorine, trisodium phosphate, hydrogen peroxide, and organic acids (Zhao and Doyle, 2006; Bauermeister *et al.*, 2008; Riedel *et al.*, 2009; Birk *et al.*, 2010). However, consumer and poultry industry

acceptance has been limited owing to issues such as reduced effectiveness, high cost of chemicals, disposal of waste materials, and concerns over residues and discoloration (Bilgili *et al.*, 1998; SCVPH, 1998; EFSA BIOHAZ Panel, 2014). Additionally, the potential health risks associated with the use of synthetic chemicals as well as consumer preference towards natural antimicrobials has led to a rise in studies investigating the potential of natural, plant derived compounds to combat different foodborne pathogens (Calo *et al.*, 2015; Dore, 2015; Upadhyay *et al.*, 2015; Upadhyaya *et al.*, 2016).

Plant-derived antimicrobials have been used since ancient times as food preservatives and flavor enhancers in foods. They are secondary metabolites produced as a result of interaction between plants and environment (Burt, 2004; Holley and Patel, 2005). Due to their diverse mechanism of antibacterial action, the chances of resistance development in bacteria are relatively low (Borges *et al.*, 2016). Previously, antimicrobial activity of several active components of plant-derived antimicrobials has been reported (Burt, 2004; Calo *et al.*, 2015). Among them, eugenol (EG), an active component of clove oil (*Eugenia caryophyllus*), demonstrated significant antimicrobial action against foodborne pathogens such as *Listeria monocytogenes* (Upadhyay *et al.*, 2015), *Salmonella* (Mattson *et al.*, 2011; Upadhyaya *et al.*, 2016), and *Escherichia coli* CGMCC 1.487 (Pei *et al.*, 2009). The antibacterial activity of EG has also been reported against *C. jejuni in vitro* (Friedman *et al.*, 2002; Kollanoor Johny *et al.*, 2010) but the efficacy of EG in postharvest poultry has not been investigated. Eugenol is Generally Recognized as Safe (GRAS) to use in foods by the United States Food and Drug Administration (Code of Federal Regulations 21 Part 172).

The objective of this study was to determine the potential of EG as an antimicrobial dip treatment to reduce *C. jejuni* on chicken skin. The antimicrobial efficacy of EG was studied in

three different delivery systems; suspension, emulsion and nanoemulsion. The suspension, emulsion and nanoemulsion differ in the stability of solutions and droplet size of EG. We hypothesized that the antimicrobial efficacy of EG increases with the decreased in droplet size, which might reach into the crevices and empty feather follicle on chicken skin to inactivate the pathogen. In addition, the effect of EG dip treatment on the color of chicken skin was determined.

## **2. MATERIALS AND METHODS**

**2.1 *Campylobacter* strains and culture conditions.** Four wild-type strains (S1, S3, S4, S8) of *C. jejuni* were cultured separately as described previously (Wagle *et al.*, 2017) in *Campylobacter* enrichment broth (CEB; Neogen, Lansing, MI, USA) for 48 h under microaerophilic conditions at 42°C. All the strains were centrifuged at 3000 rpm for 10 min and washed twice in Butterfield's phosphate diluent (BPD; 0.625 mM potassium dihydrogen phosphate, pH 7.2). Each strain was appropriately diluted and equal portions of the four strains were combined to use as the inoculum for the study.

### **2.2 Study 1: Evaluation of efficacy of EG suspension as an antimicrobial dip treatment on chicken skin**

**2.2.1. Suspension preparation.** For the suspension, appropriate volume of eugenol (Sigma-Aldrich Co., St. Louis, MO, USA.) was added into the BPD solution, followed by vigorous mixing to obtain 0, 0.125, 0.25, 0.5, 1 or 2% EG concentration.

**2.2.2. Preparation, inoculation and treatment of chicken skin samples.** Chickens were obtained from the University of Arkansas pilot processing plant (Fayetteville, AR). Skin samples (4×4 cm<sup>2</sup>) were prepared aseptically and randomly assigned to 7 treatment groups (baseline, 0, 0.125, 0.25, 0.5, 1, and 2% EG; n=15 samples per treatment per trial). The effect of EG suspension in reducing *C. jejuni* on chicken skin was determined as described

previously (Wagle *et al.*, 2017). Two trials were conducted. In each trial, individual skin samples were inoculated with 50  $\mu$ L of a cocktail of four wild type strains of *C. jejuni* ( $\sim 7.2$  Log CFU/sample). After inoculation, the samples were incubated for 30 min to facilitate adherence followed by dipping the inoculated skin in 25 mL of treatment solution for 1 min and drip dried for 2 min. The samples were either processed immediately (0 h) or after 8 and 24 h of vacuum sealed storage at 4°C (n=5 samples per treatment per time point per trial).

**2.2.3. Sample processing and enumeration of *C. jejuni* and total aerobic bacteria.** The treated skin samples were transferred into 10 mL of Dey-Engley neutralizing broth (Difco Laboratories, Sparks, MD, USA) and vigorously vortexed for 30 s. Following serial dilution (1:10), each sample was plated on *Campylobacter* line agar plates (Line, 2001) for *C. jejuni* counts and incubated at 42°C for 48 h under microaerophilic conditions. For the total aerobic bacterial counts, each sample was plated on tryptic soy agar (Difco) plates and incubated at 37°C under aerobic condition for 24 h.

## **2.3 Study 2: Evaluation of efficacy of EG emulsion as an antimicrobial dip treatment on chicken skin**

**2.3.1 Emulsion preparation.** All the components used for the preparation of EG emulsion were designated as GRAS. A previously published method was used for the preparation of food grade EG emulsion by low-energy phase inversion method, which involves titrating the aqueous phase into an organic phase (Ostertag *et al.*, 2012). Briefly, an organic phase was prepared by adding EG and Tween-80 (Sigma-Aldrich) followed by mixing using a magnetic stirrer (750 rpm) for 30 min. Sodium phosphate buffer solution (5mM; pH 7.0; Sigma-Aldrich) was used as aqueous phase and added into the organic

phase at a flow rate of 2 mL/min with constant stirring at 750 rpm for 60 min. Emulsions with different surfactant-to-oil ratios (SOR) were prepared and evaluated for droplet size, polydispersity index (PDI), and stability. Based on the preliminary results, the most stable stock solution (SOR =0.75) was selected for further studies (Data not shown). The stock emulsion with SOR=0.75 was made by adding 10% oil, 7.5 % surfactant and 82.5% sodium phosphate buffer in the system at constant temperature (25°C). The stock solution (10% emulsion) was appropriately diluted to obtain 0.125, 0.25, 0.5, 1 and 2% solution.

**2.3.2 Characterization of emulsion.** The droplet size, zeta potential value and PDI of stock emulsion was determined as described previously (Zainol *et al.*, 2012; Abd-Elsalam and Khokhlov, 2015) using a dynamic light scattering method in Zeta-sizer Nano ZS (Malvern Instruments Ltd, Malvern, WR, UK) at room temperature. The measurements were taken from three replicates of stock solutions. The stability of EG emulsion was determined using a standard published method (Shafiq and Shakeel, 2010; Abd-Elsalam and Khokhlov, 2015). Briefly, formulations of EG were centrifuged at 3500 rpm for 20 min and evaluated for phase separation. The stability was further checked for heating and cooling cycles between 4°C and 40°C. Four cycles were performed with storage at each temperature for 48 h.

**2.3.3 Preparation, inoculation and treatment of chicken skin samples.** Chicken skin samples were prepared, inoculated and treated with EG emulsion as described above in Study 1. In this study, two trials were conducted with 12 different treatments (n=15 samples/ treatment/ trial) and 360 skin samples in totals were used. The treatments included BPD, 1.5% Tween-80, five doses (0.125, 0.25, 0.5, 1, and 2%) of EG emulsion and five doses (0.125, 0.25, 0.5, 1, and 2%) of EG suspension. The sample processing and

enumeration of *C. jejuni* and total aerobic bacteria were done according to the procedure described above in 2.2.3.

## **2.4 Study 3: Evaluation of efficacy of EG nanoemulsion as an antimicrobial dip treatment on chicken skin**

**2.4.1 Preparation and characterization of nanoemulsion.** Eugenol nanoemulsion was prepared by method described previously (Ostertag *et al.*, 2012). As in emulsion preparation, nanoemulsion with different SORs were prepared and were evaluated for their stability. Moreover, the droplet size, zeta potential and PDI of nanoemulsion was determined as described in Study 2. The most stable nanoemulsion, SOR=0.5, was selected for further studies. A coarse emulsion (SOR=0.5) was made by mixing the oil, surfactant (Tween-80) and water at 750 rpm for 30 min. The stock nanoemulsion was prepared by sonicating the coarse emulsion using an ultrasonicator (Qsonica Q700, Newtown, CT, USA) for 10 min. The stock solution was diluted to prepare 0.125, 0.25, 0.5, 1 and 2% EG nanoemulsion.

**2.4.2 Preparation, inoculation and treatment of chicken skin samples.** Chicken skin samples were prepared, inoculated and treated with EG nanoemulsion as described above. In addition, the experiment design was similar to that of Study 2. In total, two trials were conducted with 12 different groups (control, 1% Tween-80, five doses of nanoemulsion and five doses of suspension; n=15 samples per treatment per trial). The treated chicken skin samples were processed by method described previously in 2.2.3.

**2.5 Color Analysis.** The color of samples treated with EG was analyzed using Chroma meter (CR 400/410, Konica Minolta Sensing Americas, Inc., Ramsey, NJ, USA), which measures relative lightness ( $L^*$ ), redness ( $a^*$ ) and yellowness ( $b^*$ ). Two trials were conducted for each delivery

system, each trial with five treatments (0.125, 0.25, 0.5, 1, and 2%) and two time points (0 and 24 h). The instrument was calibrated against a white tile followed by recording color readings from three different locations on each sample (n=10 skin samples/treatments/time points for two trials).

**2.6 Statistical analysis.** The experiments were 6×3, 12×3 and 12×3 completely randomized design for EG suspension, emulsion and nanoemulsion trials respectively with six or twelve treatments and three time points (0, 8, and 24 h). For the bacterial data analysis, bacterial counts were logarithmic transformed to maintain the homogeneity of variance (Byrd *et al.*, 2001). For color analysis, data from both the trials were pooled for each treatment and control to minimize the variability in samples from different batches of processing used for this study. The data were analyzed using PROC MIXED procedure on SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA). The means were partitioned by least-squares means analysis and a P value of <0.05 was considered statistically significant.

### **3. RESULTS**

**3.1 Droplet properties and stability.** The distribution of particle size was unimodal and average size (Z-average) of emulsion and nanoemulsion was ~ 1000±200 and 175±15 nm respectively. The PDI was 0.531±0.12 and 0.296±0.08 for the emulsion and nanoemulsion respectively. The formulations were stable after centrifuging for 20 min at 3500 rpm and nanoemulsion resisted four cycles of heating and cooling.

**3.2 Evaluating the efficacy of EG suspension as dip treatment against *C. jejuni* and total aerobic bacteria on chicken skin**

**3.2.1 Effect of EG suspension on *C. jejuni*.** Figure 1 shows the effect of EG suspension in reducing *C. jejuni* on chicken skin. The number of *C. jejuni* recovered from baseline



(skin samples not subjected dip treatment) was  $6.8 \pm 0.1$  and  $6.5 \pm 0.08$  Log CFU/sample in trial 1 and 2 respectively. Dipping of chicken skin in BPD (control) significantly reduced *C. jejuni* population by 1.0-1.5 Log CFU/sample in both trials. All EG suspension doses reduced *C. jejuni* counts whereas the 2.0% dose consistently reduced *C. jejuni* by greater than 2.0 Log CFU/sample compared to BPD control ( $P < 0.05$ ). Two percent EG was the most effective treatment in reducing *C. jejuni* counts on chicken skin at 0 and 8 h in trial 1 and across all time points in trial 2. After washing of chicken skin, there was  $\sim 5.5$  Log CFU/mL *C. jejuni* survived in the control solution, however, the counts were below detection limit ( $< 1$  Log CFU/mL) in all EG suspension solution.

**3.2.2 Effect of EG suspension on aerobic bacteria.** Figure 2 shows the effect of EG suspension on total aerobic bacterial counts. The number of aerobic bacteria present on chicken skin samples was  $\sim 4.5$  Log CFU/sample. Dipping of skin samples in BPD did not reduce aerobic counts in either trial ( $P > 0.05$ ). Doses of EG reduced aerobic counts ranging from 0.5 to 2.0 Log CFU/sample compared to control in the both trials with the exception of 0.125 and 0.25% EG at 8 h in trial 1.

### **3.3 Evaluating the efficacy of EG emulsion as dip treatment against *C. jejuni* and total aerobic bacteria on chicken skin**

**3.3.1 Effect of EG emulsion on *C. jejuni*.** The effect of EG emulsion on *C. jejuni* counts on chicken skin is presented in Table 1. *C. jejuni* recovered from skin samples dipped in BPD was  $6.29 \pm 0.04$  and  $5.38 \pm 0.1$  Log CFU/sample at 0 h in trial 1 and 2 respectively. Treatment of skin samples with 1.5% Tween-80 (control for EG emulsion) did not significantly reduce *C. jejuni* counts compared to BPD in either trial ( $P > 0.05$ ). All the EG emulsion treatments consistently reduced *C. jejuni* counts on

chicken skin as compared to Tween-80 and BPD controls across all time points ( $P < 0.05$ ). When the suspension versus emulsion treatments were compared within time points and doses, only the 2% dose at 0 h in trial 2 provided an additional reduction in *Campylobacter* counts.

**3.3.2 Effect of EG emulsion on aerobic bacteria.** The effect of EG emulsion against aerobic bacterial counts on chicken skin is shown in Table 2. The total aerobic bacterial counts on chicken skin dipped in BPD was approximately  $6.8 \pm 0.02$  and  $5.3 \pm 0.24$  Log CFU/sample in trial 1 and 2 respectively. Dipping of skin samples in Tween-80 alone did not reduce the counts when compared with BPD controls. All EG emulsion doses reduced total aerobic counts as compared to Tween-80. When the suspension versus emulsion treatments were compared within time points and doses, only the 0.5% dose at 0 h in trial 1 provided an additional reduction in aerobic counts.

### **3.4 Evaluating the efficacy of EG nanoemulsion as dip treatment against *C. jejuni* and total aerobic bacteria on chicken skin**

**3.4.1 Effect of EG nanoemulsion on *C. jejuni*.** The effect of EG nanoemulsion against *C. jejuni* on chicken skin is presented in Table 3. The skin samples dipped in BPD had  $6.81 \pm 0.08$  Log CFU/sample of *C. jejuni* surviving on the surface. The treatment with 1% Tween-80 did not significantly reduce the counts (reductions ranged from 0.02 to 0.3) versus BPD controls in either trial. Dipping of skin samples in 0.125% EG suspension or nanoemulsion treatments reduced *C. jejuni* counts by 0.5 Log CFU/sample and higher reduction (up to 3 Log CFU/sample) was obtained with 2% treatment as compared to Tween-80 or BPD controls across all time points ( $P < 0.05$ ). When the suspension versus nanoemulsion treatments were compared within time

points and doses, only the 1% dose at 0, 8 or 24 h or 0.5% at 8 h in only trial 1 provided an additional reduction in *Campylobacter* counts.

**3.4.2 Effect of EG nanoemulsion on aerobic bacteria.** Table 4 shows the effect of EG nanoemulsion in reducing aerobic bacteria on chicken skin. The total number of aerobic bacterial population on skin after dipping with BPD was ~5 and 7 Log CFU/sample in trial 1 and 2 respectively. Only treatment of skin samples with 2% EG suspension or nanoemulsion reduced aerobic bacteria consistently in trial 1 (> 1.5 Log CFU/sample) whereas all EG doses reduced counts in trial 2. When the suspension versus nanoemulsion treatments were compared within time points and doses, the 0.5 or 1 % dose at 0 h in trial 1 and only 1% dose at 0 h in trial 2 provided an additional reduction in aerobic counts.

**3.5 Effect of EG treatments on the color of chicken skin.** The lightness ( $L^*$ ), redness ( $a^*$ ), and yellowness ( $b^*$ ) values of the chicken samples dipped in BPD (Control) at 0 h were  $74.98 \pm 1.31$ ,  $1.50 \pm 0.4$ , and  $12.34 \pm 1.53$  respectively (Data not shown). These color values did not change significantly with storage for 24 h. Similar results were observed with 1.5% Tween-80. Dipping of chicken skin in EG suspension, emulsion and nanoemulsion did not affect the lightness, redness and yellowness of samples at 0 h or at 24 h ( $P > 0.05$ ).

#### 4. DISCUSSION

In spite of continuous efforts in developing effective intervention strategies, *Campylobacter* infections remain one of the most common cause of bacterial gastroenteritis for humans (Mangen *et al.*, 2016; Marder, 2017). The Food Safety Inspection Service, United States Department of Agriculture, has recently enacted a rule requiring additional performance standards for testing of *Campylobacter* on the raw chickens to achieve 33% reduction in human illness by

2020 (USDA FSIS, 2015). It is estimated that a 2-Log reduction of *C. jejuni* counts in poultry carcass can bring about a 90% decrease in the risk of human *Campylobacter* infections (Nauta *et al.*, 2016). Therefore, reducing or eliminating *Campylobacter* on poultry during processing is critical to produce microbiologically safe products and ultimately controlling *Campylobacter* related illness in humans. In this study, we used chicken skin as a model to represent carcass surface. Previously, a similar skin model has been used to test the efficacy of antimicrobials for use in poultry processing (Goode *et al.*, 2003; Mehvar *et al.*, 2005; Riedel *et al.*, 2009).

Antimicrobial wash treatment studies have been extensively investigated in poultry (Zhao and Doyle, 2006; Bauermeister *et al.*, 2008; Riedel *et al.*, 2009; Birk *et al.*, 2010; Thormar *et al.*, 2011; Kim *et al.*, 2017), however, limited research has been conducted using essential oils against *C. jejuni*. In the present study, utilization of 1% and 2% EG suspension as an antimicrobial dip treatment demonstrated 2 and 3 Log reductions respectively on chicken skin samples (Fig. 1). Eugenol has been found to be effective in killing other foodborne pathogens. For example, ~ 5 Log reductions in *Salmonella* counts were reported by Upadhyaya *et al.* (2016) when 0.25% EG was used as coating on egg shells. Similarly, antimicrobial wash treatment with 0.75% EG reduced *Salmonella* by ~6 Log CFU on tomatoes (Mattson *et al.*, 2011). In addition to investigating the antimicrobial potential of EG against *C. jejuni*, we have studied its efficacy against total aerobic bacteria on chicken skin samples as these bacteria could reduce the shelf life of products and impair the quality of the product during storage and handling (Kim and Marshall, 2000). In the present study, washing of chicken skin with EG also reduced aerobic counts indicating lower bacterial load and potentially safer food products (Fig. 2). We also observed that aerobic bacteria significantly increased after 24 h in controls. Similar results were reported on the chicken breast

(Jiménez *et al.*, 1997) and drumstick (Ílhak *et al.*, 2017) due to the growth of psychrophilic bacteria.

In an effort to improve the efficacy of EG, we formulated emulsions of this essential oil for evaluation on skin samples. Several studies have shown that formulation of emulsion and nanoemulsion significantly improved the antimicrobial activity of essential oils against various pathogens (Donsi *et al.*, 2011, 2012; Ghosh *et al.*, 2013; Bhargava *et al.*, 2015; Speranza *et al.*, 2015). The emulsion is characterized by particle size of 2000 nm whereas in nanoemulsion the droplet size is less than 200 nm (Ostertag *et al.*, 2012). The decrease in droplet size leads to an increase in the surface area for interaction between chemicals and bacteria and ultimately enhancing the antimicrobial efficacy of compounds (Speranza *et al.*, 2015). However, the effect of EG emulsion and its nanoemulsion against *C. jejuni* on poultry products has not been previously reported. Stable formulation of emulsion (SOR=0.75) and nanoemulsion (SOR=0.5) were selected from a range of SOR based on size, PDI, zeta potential and thermodynamic stability. In addition, nanoemulsion was relatively more stable than emulsion based on PDI ( $0.296\pm 0.08$  vs  $0.531\pm 0.12$ ). Since solubility is enhanced and droplets size is significantly reduced with the formulation of emulsion and nanoemulsion respectively, we hypothesized that the antibacterial activity of EG as a dip treatment on chicken skin would also increase. Eugenol suspension was included in the study with emulsion and nanoemulsion for the purpose of comparison. We observed that the efficacy of EG emulsion or nanoemulsion against *C. jejuni* on chicken skin samples did not improve the beneficial effects against *Campylobacter* loads versus the suspension solution by itself (Tables 1, 3). In this study, complete elimination of *C. jejuni* on chicken skin was not achieved even after exposure to the highest dose of EG. One possible explanation is that bacteria present in the crevices and empty feather follicles of the chicken skin are not killed since treatments could not reach these

sites in sufficient concentrations. Previous studies have demonstrated that *Campylobacter* resides in the pores of the chicken skin (Chantarapanont *et al.*, 2003; Jang *et al.*, 2007). Moreover, the viability of *Campylobacter* transformed with P<sub>c</sub>gfp plasmid located in sites up to 30 µm beneath the chicken skin surfaces did not change after rinsing (Chantarapanont *et al.*, 2003). Therefore, future studies should focus on novel methods to inactivate *C. jejuni* present in the crevices and feather follicles on skin.

Visual appearance of meat is one of the primary factor that drives acceptability/purchasing decisions by consumers. Decrease in lightness (L\*) indicates an increase in the paleness of meat and the redness of meat is influence by the myoglobin and heme content. We observed that EG dip treatments had no significant effect on the color of samples as compared to controls at 0 h and 24 h post storage (Data not shown). This indicates that washing of poultry products with EG would not adversely affect the meat color. Khan *et al* (2015) had observed similar results with 0.05% EG on raw chicken when stored for 10 days. In addition, they also reported that EG was effective in preventing lipid oxidation in chicken patties due to its hydrophobic nature resulting in accumulation of EG in lipid phase.

In conclusion, EG as an antimicrobial dip on poultry products was effective in reducing *C. jejuni*. In addition, preparation of emulsion and nanoemulsion is not necessary to observe the positive effect of EG in reducing *C. jejuni*. The applications of EG dip treatments represent a safe, effective and natural approach that would have significant impact on the food safety. However, follow-up studies are warranted for testing the effect of EG on the organoleptic properties of meat before recommending to use in the commercial setting.

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## 6. REFERENCES

- Abd-Elsalam KA, Khokhlov AR. Eugenol oil nanoemulsion: antifungal activity against *Fusarium oxysporum f. sp. vasinfectum* and phytotoxicity on cottonseeds. *Appl Nanosci* 2015; 5:255-265.
- Achen M, Morishita TY, Ley EC. Shedding and colonization of *Campylobacter jejuni* in broilers from day-of-hatch to slaughter age. *Avian Dis* 1998; 42:732-737.
- Arsi K, Donoghue AM, Venkitanarayanan K, Kollanoor-Johny A, Fanatico AC, Blore PJ, Donoghue DJ. The efficacy of the natural plant extracts, thymol and carvacrol against *Campylobacter* colonization in broiler chickens. *J Food Saf* 2014; 34:321-325.
- Bauermeister LJ, Bowers JW, Townsend JC, McKEE SR. Validating the efficacy of peracetic acid mixture as an antimicrobial in poultry chillers. *J Food Prot* 2008; 71:1119-1122.
- Beery JT, Hugdahl MB, Doyle MP. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl Environ Microbiol* 1988; 54:2365-2370.
- Bhargava K, Conti DS, da Rocha SR, Zhang Y. Application of an oregano oil nanoemulsion to the control of foodborne bacteria on fresh lettuce. *Food Microbiol* 2015; 47:69-73.
- Bilgili SF, Conner DE, Pinion JL, Tamblyn KC. Broiler skin color as affected by organic acids: Influence of concentration and method of application. *Poult Sci* 1998; 77:752-757.
- Birk T, Grønlund AC, Christensen BB, Knøchel S, Lohse K, Rosenquist H. Effect of organic acids and marination ingredients on the survival of *Campylobacter jejuni* on meat. *J Food Prot* 2010; 73:258-265.
- Black RE, Levine MM, Clements ML, Hughes TP, Blaser MJ. Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis* 1988; 157:472-479.
- Borges A, Abreu AC, Dias C, Saavedra MJ, Borges F, Simões M. New perspectives on the use of phytochemicals as an emergent strategy to control bacterial infections including biofilms. *Molecules* 2016; 21:877.
- Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int J Food Microbiol* 2004; 94:223-253.
- Byrd JA, Hargis BM, Caldwell DJ, Bailey RH, Herron KL, McReynolds JL, Brewer RL, Anderson RC, Bischoff KM, Callaway TR, Kubena LF. Effect of Lactic Acid Administration in the Drinking Water During Preslaughter Feed Withdrawal on *Salmonella* and *Campylobacter* Contamination of Broilers 2. *Poult Sci* 2001; 80:278-283.
- Calo JR, Crandall PG, O'Bryan CA, Ricke SC. Essential oils as antimicrobials in food systems—A review. *Food Control* 2015; 54:111-119.

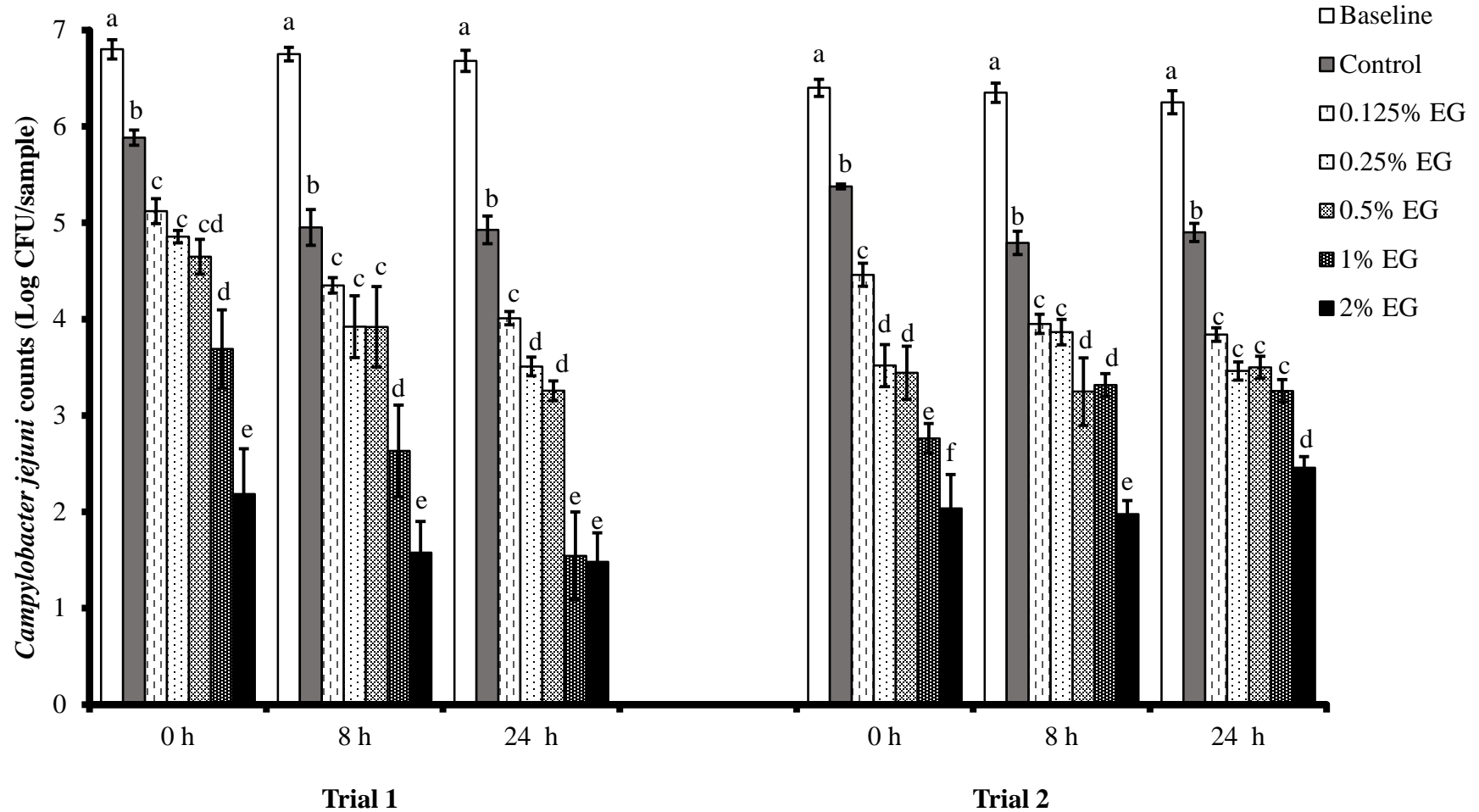


- Chantarapanont W, Berrang M, Frank JF. Direct microscopic observation and viability determination of *Campylobacter jejuni* on chicken skin. *J Food Prot* 2003; 66:2222-2230.
- Danis K, Di Renzi M, O'Neill W, Smyth B, McKeown P, Foley B, Tohani V, Devine M. Risk factors for sporadic *Campylobacter* infection: an all-Ireland case-control study. *Euro Surveill* 2009; 14:19123.
- Donsì F, Annunziata M, Sessa M, Ferrari G. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *Food Sci Technol* 2011; 44:1908-1914.
- Donsì F, Annunziata M, Vincensi M, Ferrari G. Design of nanoemulsion-based delivery systems of natural antimicrobials: effect of the emulsifier. *J Biotechnol* 2012; 159:342-50.
- Dore MH. Threats to human health: Use of chlorine, an obsolete treatment technology. In: *Global drinking water management and conservation*. Springer International Publishing, 2015, pp. 197-212.
- EFSA BIOHAZ Panel. Scientific opinion on the evaluation of the safety and efficacy of peroxyacetic acid solutions for reduction of pathogens on poultry carcasses and meat. *EFSA Journal* 2014; 12:3599.
- Friedman M, Henika PR, Mandrell RE. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J Food Prot*. 2002; 65:1545-1560.
- Friedman CR, Hoekstra RM, Samuel M, Marcus R, Bender J, Shiferaw B, Reddy S, Ahuja SD, Helfrick DL, Hardnett F, Carter M. Risk factors for sporadic *Campylobacter* infection in the United States: a case-control study in FoodNet sites. *Clin Infect Dis* 2004; 38:285-296.
- Ghosh V, Mukherjee A, Chandrasekaran N. Ultrasonic emulsification of food-grade nanoemulsion formulation and evaluation of its bactericidal activity. *Ultrason Sonochem* 2013; 20:338-344.
- Goode D, Allen VM, Barrow PA. Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. *Appl Environ Microbiol* 2003; 69:5032-5036.
- Gracia MI, Millán C, Sánchez J, Guyard-Nicodème M, Mayot J, Carre Y, Csorbai A, Chemaly M, Medel P. Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period: Part B. *Poult Sci* 2015; 95:886-892.

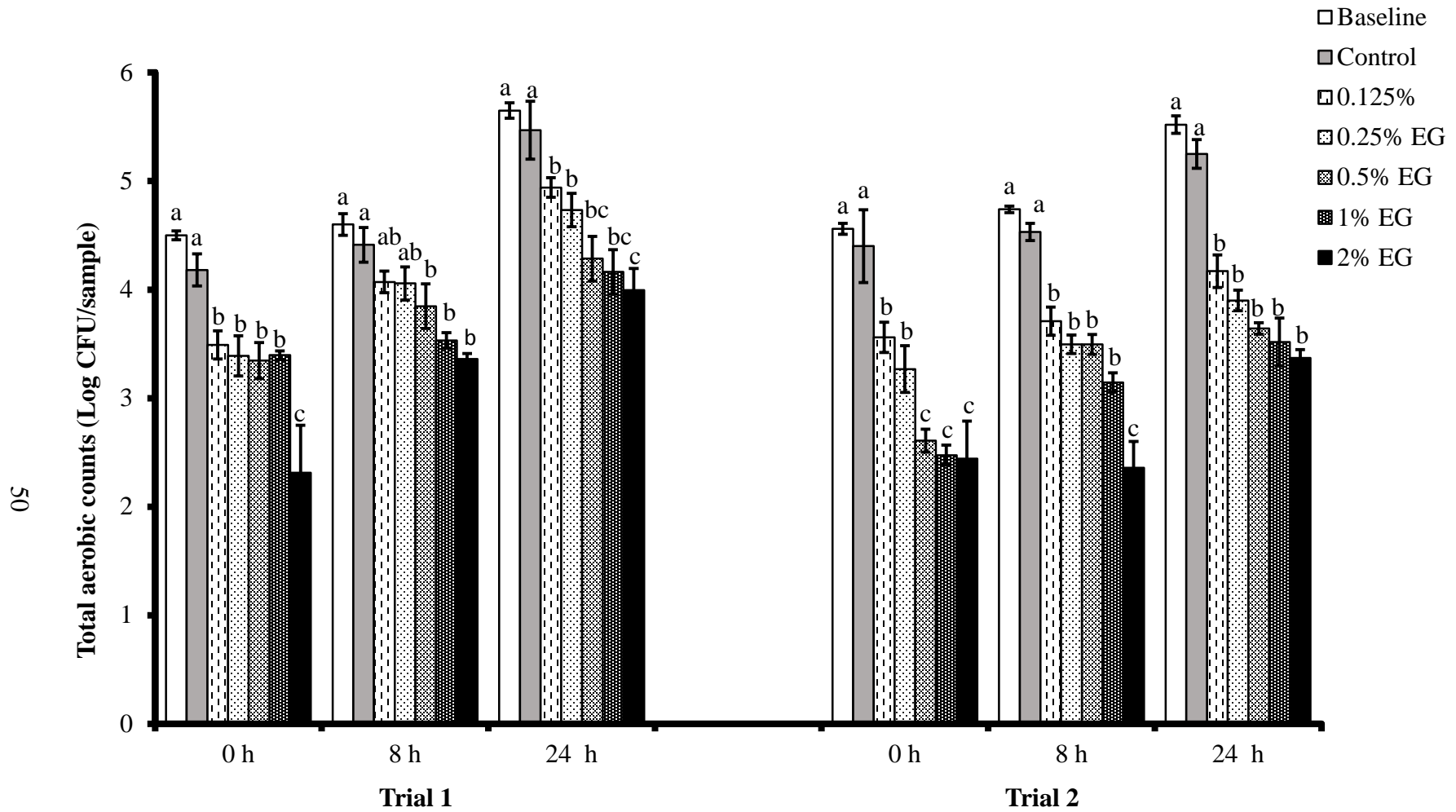
- Gradel KO, Nielsen HL, Schønheyder HC, Ejlersen T, Kristensen B, Nielsen H. Increased short- and long-term risk of inflammatory bowel disease after *Salmonella* or *Campylobacter* gastroenteritis. *Gastroenterology* 2009; 137:495-501.
- Guyard-Nicodème M, Keita A, Quesne S, Amelot M, Poezevara T, Le Berre B, Sánchez J, Vesseur P, Martín Á, Medel P, Chemaly M. Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period. *Poult Sci* 2015; 95:298-305.
- Hermans D, Van Deun K, Messens W, Martel A, Van Immerseel F, Haesebrouck F, Rasschaert G, Heyndrickx M, Pasmans F. *Campylobacter* control in poultry by current intervention measures ineffective: urgent need for intensified fundamental research. *Vet Microbiol* 2011; 152:219-228.
- Holley RA, Patel D. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiol* 2005; 22:273-292.
- İlhak Oİ, İncili GK, Durmuşoğlu H. Evaluation of effect of thymol combined with lactic acid or sodium lactate on psychrophilic bacteria and *Salmonella* spp. on chicken drumstick. *Ann Anim Sci* 2017; 17:271-80.
- Jang K, Kim M, Ha S, Kim K, Lee K, Chung D, Kim C, Kim K. Morphology and adhesion of *Campylobacter jejuni* to chicken skin under varying conditions. *J Microbiol Biotechnol* 2007; 17:202.
- Jiménez SM, Salsi MS, Tiburzi MC, Rafaghelli RC, Tessi MA, Coutaz VR. Spoilage microflora in fresh chicken breast stored at 4° C: influence of packaging methods. *J Appl Microbiol* 1997; 83:613-618.
- Khan A, Allen K, Wang X. Effect of Type I and Type II Antioxidants on oxidative stability, microbial growth, pH, and color in raw poultry meat. *Food Nutr Sci* 2015; 6:1541-1551.
- Kim SA, Park SH, Lee SI, Owens CM, Ricke SC. Assessment of chicken carcass microbiome responses during processing in the presence of commercial antimicrobials using a next generation sequencing approach. *Sci Rep* 2017; 7:43354.
- Kim CR, Marshall DL. Quality evaluation of refrigerated chicken wings treated with organic acids. *J Food Qual* 2000; 23:327-335.
- Kollanoor Johny A, Darre MJ, Donoghue AM, Donoghue DJ, Venkitanarayanan K. Antibacterial effect of trans-cinnamaldehyde, eugenol, carvacrol, and thymol on *Salmonella Enteritidis* and *Campylobacter jejuni* in chicken cecal contents *in vitro*. *J Appl Poult Res* 2010; 19:237-244.

- Line JE. Development of a selective differential agar for isolation and enumeration of *Campylobacter* spp. J Food Prot 2001; 64:1711-1715.
- Mangen MJ, Havelaar AH, Haagsma JA, Kretzschmar ME. The burden of *Campylobacter*-associated disease in six European countries. Microb Risk Anal 2016; 2:48-52.
- Marder EP, Cieslak PR, Cronquist AB, Dunn J, Lathrop S, Rabatsky-Ehr T, Ryan P, Smith K, Tobin-D'Angelo M, Vugia DJ, Zansky S. Incidence and trends of infections with pathogens transmitted commonly through food and the effect of increasing use of culture-independent diagnostic tests on surveillance-foodborne diseases active surveillance network, 10 US Sites, 2013-2016. MMWR Morb Mortal Wkly Rep 2017; 66:397.
- Mattson TE, Johny AK, Amalaradjou MA, More K, Schreiber DT, Patel J, Venkitanarayanan K. Inactivation of *Salmonella* spp. on tomatoes by plant molecules. Int J Food Microbiol 2011; 144:464-468.
- Mehyar G, Blank G, Han JH, Hydamaka A, Holley RA. Effectiveness of trisodium phosphate, lactic acid and commercial anti-microbials against pathogenic bacteria on chicken skin. Food Prot Trends 2005; 25:351-362.
- Nauta MJ, Johannessen G, Adame LL, Williams N, Rosenquist H. The effect of reducing numbers of *Campylobacter* in broiler intestines on human health risk. Microb Risk Anal 2016; 2:68-77.
- Ostertag F, Weiss J, McClements DJ. Low-energy formation of edible nanoemulsions: factors influencing droplet size produced by emulsion phase inversion. J Colloid Interface Sci 2012; 388:95-102.
- Pei RS, Zhou F, Ji BP, Xu J. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method. J Food Sci 2009; 74:379-383.
- Riedel CT, Brøndsted L, Rosenquist H, Haxgart SN, Christensen BB. Chemical decontamination of *Campylobacter jejuni* on chicken skin and meat. J Food Prot 2009; 72:1173-1180.
- SCVPH. Report of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) on benefits and limitations of antimicrobial treatments for poultry carcasses. 1998. Available at: [https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com\\_scv\\_out14\\_en.pdf](https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scv_out14_en.pdf) . Accessed 22 September 2017.
- Shafiq S, Shakeel F. Stability and self-nanoemulsification efficiency of ramipril nanoemulsion containing labrasol and pluroleique. Clin Res Regul Aff 2010; 27:7-12.

- Speranza P, Ribeiro AP, Cunha RL, Macedo JA, Macedo GA. Influence of emulsion droplet size on antimicrobial activity of interesterified Amazonian oils. *Food Sci Technol* 2015; 60:207-212.
- Spiller RC. Role of infection in irritable bowel syndrome. *J Gastroenterol* 2007; 42:41-47.
- Thormar H, Hilmarsson H, Thráinsson JH, Georgsson F, Gunnarsson E, Dadadóttir S. Treatment of fresh poultry carcasses with emulsions of glycerol monocaprinate (monocaprin) to reduce contamination with *Campylobacter* and psychrotrophic bacteria. *Br Poult Sci* 2011; 52:11-19.
- Upadhyay A, Upadhyaya I, Karumathil DP, Yin HB, Nair MS, Bhattaram V, Chen CH, Flock G, Mooyottu S, Venkitanarayanan K. Control of *Listeria monocytogenes* on skinless frankfurters by coating with phytochemicals. *Food Sci Technol* 2015; 63:37-42.
- Upadhyaya I, Yin HB, Surendran Nair M, Chen CH, Lang R, Darre MJ, Venkitanarayanan K. Inactivation of *Salmonella enteritidis* on shell eggs by coating with phytochemicals. *Poult Sci* 2016; 95:2106-2111.
- USDA FSIS. Changes to the *Salmonella* and *Campylobacter* verification testing program: proposed performance standards for *Salmonella* and *Campylobacter* in not-ready-to-eat comminuted chicken and turkey products and raw chicken parts and related agency verification procedures and other changes to agency sampling. *Fed Regist* 2015; 80:3940-3950.
- Wagenaar JA, Newell DG, Kalupahana RS, Mughini-Gras L. *Campylobacter*: animal reservoirs, human infections, and options for control. In: *Zoonoses-infections affecting humans and animals*. Springer: Dordrecht, Netherlands, 2015, pp. 159-177.
- Wagle BR, Arsi K, Upadhyay A, Shrestha S, Venkitanarayanan K, Donoghue AM, Donoghue DJ.  $\beta$ -Resorcylic Acid, a phytophenolic compound, reduces *Campylobacter jejuni* in postharvest poultry. *J Food Prot* 2017; 80:1243-1251.
- Zainol S, Basri M, Basri HB, Shamsuddin AF, Abdul-Gani SS, Karjiban RA, Abdul-Malek E. Formulation optimization of a palm-based nanoemulsion system containing levodopa. *Int J Mol Sci* 2012; 13:13049-13064.
- Zhao T, Doyle MP. Reduction of *Campylobacter jejuni* on chicken wings by chemical treatments. *J Food Prot* 2006; 69:762-767.



**Figure 1:** Evaluating the efficacy of eugenol suspension (0, 0.125, 0.25, 0.5, 1, and 2% EG) as a dip treatment against *C. jejuni* on chicken skin. Inoculated chicken skin samples (~7.2 Log CFU/sample) were dipped in treatment solution for 1 min followed by drip dried for 2 min and processed at 0, 8 or 24 h of refrigerated storage. In each trial, “a-f” designate the statistical difference among the treatments within the same time points ( $P < 0.05$ ).



**Figure 2:** Evaluating the efficacy of eugenol suspension (0, 0.125, 0.25, 0.5, 1, and 2% EG) as a dip treatment against total aerobic bacteria on chicken skin at 0, 8 or 24 h of refrigerated storage. In each trial, “a-c” designate the statistical difference among the treatments within the same time points ( $P < 0.05$ ).

**Table 1:** Antimicrobial efficacy of eugenol suspension versus emulsion as a dip treatment against *C. jejuni* on chicken skin<sup>1</sup>.

Time points	Treatments	Trial 1		Trial 2	
		Suspension	Emulsion	Suspension	Emulsion
0 h	Control*	6.29±0.04 <sup>a</sup> (BPD)	6.31±0.05 <sup>a</sup> (1.5% Tween-80)	5.38±0.1 <sup>a</sup> (BPD)	5.29±0.08 <sup>a</sup> (1.5% Tween-80)
	0.125%	5.34±0.11 <sup>b</sup>	5.28±0.13 <sup>b</sup>	4.46±0.16 <sup>b</sup>	4.44±0.09 <sup>b</sup>
	0.25%	5.12±0.09 <sup>b</sup>	5.12±0.08 <sup>b</sup>	3.83±0.24 <sup>bc</sup>	3.60±0.51 <sup>c</sup>
	0.5%	4.39±0.13 <sup>c</sup>	4.29±0.12 <sup>cd</sup>	3.49±0.31 <sup>c</sup>	3.84±0.20 <sup>bc</sup>
	1%	4.11±0.10 <sup>de</sup>	3.94±0.09 <sup>e</sup>	3.88±0.13 <sup>bc</sup>	3.31±0.20 <sup>c</sup>
	2%	2.89±0.23 <sup>f</sup>	3.02±0.05 <sup>f</sup>	2.62±0.26 <sup>d</sup>	1.84±0.41 <sup>e</sup>
8 h	Control	6.47±0.05 <sup>a</sup> (BPD)	6.31±0.07 <sup>a</sup> (1.5% Tween-80)	5.25±0.07 <sup>a</sup> (BPD)	5.07±0.13 <sup>a</sup> (1.5% Tween-80)
	0.125%	5.24±0.05 <sup>b</sup>	5.10±0.03 <sup>b</sup>	3.94±0.07 <sup>bc</sup>	4.18±0.06 <sup>b</sup>
	0.25%	5.11±0.04 <sup>b</sup>	5.13±0.07 <sup>b</sup>	3.37±0.20 <sup>cde</sup>	3.68±0.19 <sup>bcd</sup>
	0.5%	4.41±0.05 <sup>c</sup>	4.30±0.06 <sup>cd</sup>	3.75±0.23 <sup>bcd</sup>	3.26±0.14 <sup>de</sup>
	1%	4.09±0.06 <sup>de</sup>	3.81±0.17 <sup>e</sup>	2.81±0.20 <sup>ef</sup>	2.82±0.33 <sup>ef</sup>
	2%	2.99±0.07 <sup>f</sup>	2.98±0.05 <sup>f</sup>	1.98±0.29 <sup>g</sup>	2.43±0.39 <sup>fg</sup>
24 h	Control	6.30±0.07 <sup>a</sup> (BPD)	6.32±0.04 <sup>a</sup> (1.5% Tween-80)	5.41±0.11 <sup>a</sup> (BPD)	5.35±0.07 <sup>a</sup> (1.5% Tween-80)
	0.125%	4.73±0.07 <sup>b</sup>	4.78±0.10 <sup>b</sup>	4.20±0.07 <sup>b</sup>	4.24±0.14 <sup>b</sup>
	0.25%	3.83±0.15 <sup>cd</sup>	3.90±0.12 <sup>c</sup>	3.85±0.24 <sup>bc</sup>	3.90±0.25 <sup>b</sup>
	0.5%	3.64±0.08 <sup>cd</sup>	3.53±0.09 <sup>de</sup>	2.99±0.11 <sup>de</sup>	3.67±0.37 <sup>bc</sup>
	1%	3.21±0.07 <sup>ef</sup>	3.16±0.09 <sup>f</sup>	2.41±0.18 <sup>ef</sup>	3.22±0.41 <sup>cd</sup>
	2%	2.96±0.15 <sup>f</sup>	2.97±0.14 <sup>f</sup>	1.79±0.36 <sup>fg</sup>	1.64±0.31 <sup>g</sup>

<sup>1</sup>Chicken skin samples were inoculated with 7.15 and 6.5 Log CFU/sample of *C. jejuni* in trial 1 and trial 2 respectively. Data presented as mean ± standard error of the mean. Data within storage time (0, 8 or 24 h) for both suspension and emulsion within trial with different superscript are statistically different (P<0.05). \*BPD is the control of eugenol suspension and 1.5% Tween-80 is the control of eugenol emulsion.

**Table 2:** Antimicrobial efficacy of eugenol suspension versus emulsion as a dip treatment against total aerobic bacteria on chicken skin<sup>1</sup>.

Time points	Treatments	Trial 1		Trial 2	
		Suspension	Emulsion	Suspension	Emulsion
0 h	Control*	6.85±0.02 <sup>a</sup> (BPD)	6.84±0.02 <sup>a</sup> (1.5% Tween-80)	5.34±0.24 <sup>a</sup> (BPD)	5.43±0.28 <sup>a</sup> (1.5% Tween-80)
	0.125%	6.26±0.05 <sup>b</sup>	6.35±0.05 <sup>b</sup>	3.83±0.04 <sup>bc</sup>	3.92±0.13 <sup>b</sup>
	0.25%	6.17±0.10 <sup>b</sup>	6.14±0.06 <sup>b</sup>	3.79±0.15 <sup>bcd</sup>	3.65±0.11 <sup>bcd</sup>
	0.5%	6.16±0.07 <sup>b</sup>	5.83±0.10 <sup>c</sup>	3.46±0.17 <sup>cde</sup>	3.63±0.09 <sup>bcd</sup>
	1%	5.73±0.12 <sup>cd</sup>	5.63±0.05 <sup>cd</sup>	3.42±0.12 <sup>de</sup>	3.71±0.12 <sup>bcd</sup>
	2%	5.50±0.04 <sup>d</sup>	5.49±0.09 <sup>d</sup>	2.82±0.13 <sup>f</sup>	3.17±0.16 <sup>ef</sup>
8 h	Control	7.13±0.07 <sup>a</sup> (BPD)	7.09±0.08 <sup>a</sup> (1.5% Tween-80)	5.34±0.23 <sup>a</sup> (BPD)	5.59±0.14 <sup>a</sup> (1.5% Tween-80)
	0.125%	6.19±0.04 <sup>bc</sup>	6.38±0.06 <sup>b</sup>	4.63±0.08 <sup>b</sup>	4.58±0.08 <sup>bc</sup>
	0.25%	6.05±0.04 <sup>cd</sup>	6.12±0.06 <sup>cd</sup>	4.08±0.07 <sup>def</sup>	4.45±0.13 <sup>bcd</sup>
	0.5%	5.97±0.03 <sup>cd</sup>	5.89±0.04 <sup>de</sup>	4.20±0.08 <sup>cde</sup>	4.29±0.08 <sup>bcd</sup>
	1%	5.63±0.05 <sup>ef</sup>	5.54±0.06 <sup>f</sup>	3.95±0.06 <sup>efg</sup>	3.78±0.08 <sup>fg</sup>
	2%	5.49±0.06 <sup>f</sup>	5.56±0.08 <sup>f</sup>	3.67±0.05 <sup>g</sup>	3.62±0.09 <sup>g</sup>
24 h	Control	7.28±0.19 <sup>a</sup> (BPD)	7.32±0.13 <sup>a</sup> (1.5% Tween-80)	5.98±0.03 <sup>a</sup> (BPD)	5.97±0.01 <sup>a</sup> (1.5% Tween-80)
	0.125%	6.25±0.13 <sup>b</sup>	6.40±0.11 <sup>b</sup>	4.70±0.11 <sup>b</sup>	4.45±0.14 <sup>bc</sup>
	0.25%	5.75±0.16 <sup>c</sup>	5.66±0.19 <sup>c</sup>	4.47±0.13 <sup>bc</sup>	4.52±0.11 <sup>bc</sup>
	0.5%	5.50±0.15 <sup>c</sup>	5.67±0.08 <sup>c</sup>	4.21±0.21 <sup>cd</sup>	4.17±0.24 <sup>cd</sup>
	1%	5.61±0.07 <sup>c</sup>	5.58±0.06 <sup>c</sup>	3.99±0.18 <sup>de</sup>	4.52±0.07 <sup>bc</sup>
	2%	5.65±0.14 <sup>c</sup>	5.71±0.11 <sup>c</sup>	3.73±0.25 <sup>e</sup>	3.92±0.14 <sup>de</sup>

<sup>1</sup>Data presented as mean ± standard error of the mean. Data within storage time (0, 8 or 24 h) for both suspension and emulsion within trial with different superscript are statistically different (P<0.05). \*BPD is the control of eugenol suspension and 1.5% Tween-80 is the control of eugenol emulsion.



**Table 3:** Antimicrobial efficacy of eugenol suspension versus nanoemulsion as a dip treatment against *C. jejuni* on chicken skin<sup>1</sup>.

Time points	Treatments	Trial 1		Trial 2	
		Suspension	Nanoemulsion	Suspension	Nanoemulsion
0 h	Control*	6.81±0.08 <sup>a</sup> (BPD)	6.52±0.10 <sup>a</sup> (1% Tween-80)	6.25±0.06 <sup>a</sup> (BPD)	6.21±0.06 <sup>a</sup> (1% Tween-80)
	0.125%	5.79±0.06 <sup>b</sup>	5.78±0.06 <sup>b</sup>	5.60±0.10 <sup>b</sup>	5.09±0.18 <sup>bc</sup>
	0.25%	5.34±0.03 <sup>c</sup>	5.33±0.04 <sup>c</sup>	4.62±0.26 <sup>c</sup>	4.64±0.19 <sup>c</sup>
	0.5%	5.33±0.03 <sup>c</sup>	5.17±0.04 <sup>cd</sup>	4.61±0.10 <sup>c</sup>	4.61±0.08 <sup>c</sup>
	1%	4.94±0.31 <sup>d</sup>	4.40±0.15 <sup>e</sup>	4.43±0.09 <sup>c</sup>	4.45±0.06 <sup>c</sup>
	2%	3.59±0.04 <sup>f</sup>	3.54±0.06 <sup>f</sup>	3.81±0.23 <sup>d</sup>	3.80±0.04 <sup>d</sup>
8 h	Control	6.47±0.05 <sup>a</sup> (BPD)	6.31±0.07 <sup>a</sup> (1% Tween-80)	6.28±0.08 <sup>a</sup> (BPD)	6.26±0.10 <sup>a</sup> (1% Tween-80)
	0.125%	5.79±0.07 <sup>b</sup>	5.83±0.05 <sup>b</sup>	5.46±0.14 <sup>b</sup>	5.05±0.04 <sup>bc</sup>
	0.25%	5.71±0.13 <sup>bc</sup>	5.50±0.08 <sup>cd</sup>	4.64±0.06 <sup>cd</sup>	4.60±0.06 <sup>d</sup>
	0.5%	5.31±0.13 <sup>d</sup>	4.89±0.15 <sup>e</sup>	4.50±0.05 <sup>d</sup>	4.55±0.06 <sup>d</sup>
	1%	4.99±0.09 <sup>e</sup>	4.59±0.16 <sup>f</sup>	4.51±0.12 <sup>d</sup>	4.31±0.08 <sup>d</sup>
	2%	3.48±0.04 <sup>g</sup>	3.40±0.07 <sup>g</sup>	3.59±0.16 <sup>e</sup>	3.36±0.09 <sup>e</sup>
24 h	Control	6.36±0.18 <sup>a</sup> (BPD)	6.06±0.22 <sup>ab</sup> (1% Tween-80)	6.23±0.12 <sup>a</sup> (BPD)	6.01±0.10 <sup>a</sup> (1% Tween-80)
	0.125%	5.50±0.13 <sup>bc</sup>	5.26±0.06 <sup>cd</sup>	5.02±0.05 <sup>b</sup>	4.84±0.05 <sup>b</sup>
	0.25%	5.21±0.11 <sup>cd</sup>	5.30±0.14 <sup>cd</sup>	4.73±0.08 <sup>b</sup>	4.68±0.21 <sup>bc</sup>
	0.5%	4.83±0.30 <sup>de</sup>	4.54±0.46 <sup>ef</sup>	4.47±0.15 <sup>bc</sup>	4.46±0.12 <sup>bc</sup>
	1%	4.85±0.07 <sup>de</sup>	4.22±0.17 <sup>f</sup>	4.58±0.07 <sup>bc</sup>	4.00±0.29 <sup>c</sup>
	2%	3.29±0.06 <sup>g</sup>	3.27±0.05 <sup>g</sup>	3.01±0.51 <sup>d</sup>	2.98±0.25 <sup>d</sup>

<sup>1</sup>Chicken skin samples were inoculated with 7.45 and 6.9 Log CFU/sample of *C. jejuni* in trial 1 and trial 2 respectively. Data presented as mean ± standard error of the mean. Data within storage time (0, 8 or 24 h) for both suspension and nanoemulsion within trial with different superscript are statistically different (P<0.05). \*BPD is the control of eugenol suspension and 1% Tween-80 is the control of nanoemulsion.

**Table 4:** Antimicrobial efficacy of eugenol suspension versus nanoemulsion as a dip treatment against total aerobic bacteria on chicken skin<sup>1</sup>.

Time points	Treatments	Trial 1		Trial 2	
		Suspension	Nanoemulsion	Suspension	Nanoemulsion
0 h	Control*	4.91±0.05 <sup>ab</sup> (BPD)	5.03±0.24 <sup>a</sup> (1% Tween-80)	7.04±0.04 <sup>a</sup> (BPD)	6.94±0.14 <sup>a</sup> (1% Tween-80)
	0.125%	4.85±0.16 <sup>abc</sup>	4.91±0.05 <sup>ab</sup>	6.52±0.10 <sup>b</sup>	6.52±0.09 <sup>b</sup>
	0.25%	4.58±0.03 <sup>bcd</sup>	4.55±0.13 <sup>bcd</sup>	6.48±0.11 <sup>bc</sup>	6.42±0.12 <sup>bc</sup>
	0.5%	4.52±0.07 <sup>cd</sup>	4.16±0.17 <sup>e</sup>	6.50±0.28 <sup>bc</sup>	6.18±0.23 <sup>cd</sup>
	1%	4.34±0.15 <sup>de</sup>	3.58±0.16 <sup>f</sup>	6.59±0.34 <sup>b</sup>	6.01±0.18 <sup>d</sup>
	2%	3.50±0.05 <sup>f</sup>	3.41±0.07 <sup>f</sup>	6.34±0.23 <sup>bcd</sup>	6.44±0.08 <sup>bc</sup>
8 h	Control	5.32±0.13 <sup>ab</sup> (BPD)	5.46±0.21 <sup>a</sup> (1% Tween-80)	7.52±0.07 <sup>a</sup> (BPD)	7.60±0.13 <sup>a</sup> (1% Tween-80)
	0.125%	5.21±0.13 <sup>abc</sup>	5.35±0.08 <sup>ab</sup>	6.51±0.14 <sup>b</sup>	6.54±0.06 <sup>b</sup>
	0.25%	5.18±0.06 <sup>abc</sup>	5.26±0.09 <sup>abc</sup>	6.48±0.07 <sup>b</sup>	6.49±0.06 <sup>b</sup>
	0.5%	5.00±0.03 <sup>bc</sup>	4.94±0.04 <sup>c</sup>	6.53±0.07 <sup>b</sup>	6.33±0.07 <sup>b</sup>
	1%	5.09±0.09 <sup>bc</sup>	4.91±0.06 <sup>c</sup>	6.49±0.06 <sup>b</sup>	6.33±0.11 <sup>b</sup>
	2%	3.70±0.04 <sup>d</sup>	3.70±0.06 <sup>d</sup>	6.36±0.09 <sup>b</sup>	6.39±0.02 <sup>b</sup>
24 h	Control	5.79±0.03 <sup>a</sup> (BPD)	5.67±0.11 <sup>a</sup> (1% Tween-80)	7.46±0.06 <sup>a</sup> (BPD)	7.57±0.03 <sup>a</sup> (1% Tween-80)
	0.125%	5.32±0.04 <sup>b</sup>	5.27±0.04 <sup>bc</sup>	6.95±0.03 <sup>b</sup>	6.72±0.07 <sup>bc</sup>
	0.25%	5.20±0.07 <sup>bc</sup>	5.20±0.05 <sup>bc</sup>	6.81±0.04 <sup>bc</sup>	6.75±0.05 <sup>bc</sup>
	0.5%	5.17±0.11 <sup>bc</sup>	5.16±0.06 <sup>bc</sup>	6.76±0.03 <sup>bc</sup>	6.45±0.05 <sup>c</sup>
	1%	5.11±0.04 <sup>cd</sup>	4.97±0.03 <sup>d</sup>	6.58±0.04 <sup>bc</sup>	6.41±0.04 <sup>c</sup>
	2%	3.97±0.03 <sup>e</sup>	3.95±0.05 <sup>e</sup>	6.40±0.04 <sup>c</sup>	6.31±0.09 <sup>c</sup>

<sup>1</sup>Data presented as mean ± standard error of the mean. Data within storage time (0, 8 or 24 h) for both suspension and nanoemulsion within trial with different superscript are statistically different (P<0.05). \*BPD is the control of eugenol suspension and 1% Tween-80 is the control of nanoemulsion.



March 26, 2018

**MEMORANDUM**

**TO:** Dr. Dan Donoghue

**FROM:** Bob Beitle, Acting Biosafety Committee Chair

**RE:** Protocol Renewal

**PROTOCOL #:** 06021

**PROTOCOL TITLE:** Reducing Food Borne Pathogens in Poultry

**APPROVED PROJECT PERIOD:** Start Date February 14, 2006 Expiration Date February 13, 2021

The Institutional Biosafety Committee (IBC) has approved your request, dated January 26, 2018, to renew IBC # 06021, "Reducing Food Borne Pathogens in Poultry".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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*The University of Arkansas is an equal opportunity/affirmative action institution.*

**PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:**

List all personnel (including PI and Co-PI) to be involved in this project:

**Name:** (first and last) - **POSITION** (Title, academic degrees, certifications, and material field of expertise)

**QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE**  
Describe previous work or training with biohazardous and/or recombinant DNA and include Biosafety Levels)

**Example:**

Bob Biohazard - Associate Professor, PhD Microbiology

14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.

Dan J. Donoghue, Ph.D. Professor, Food Safety	over 20 years experience as PI running research laboratory working with BL-2 pathogens.
Ann M. Donoghue, Ph.D. Research Leader, Food safety	over 20 years experience as PI running research laboratory working with BL-2 pathogens.
Komala Arsi, Ph. D, Post-doctoral fellow	Post-doctoral fellow trained under Dr. Donoghue's supervision
Abhinav Upadhyay, Ph.D. Post-doctoral fellow	Post-doctoral fellow trained under Dr. Donoghue's supervision
Indu Upadhyaya, Ph. D. Post-doctoral fellow	Post-doctoral fellow trained under Dr. Donoghue's supervision
Basanta Raj Wagle, Graduate Student	Graduate assistant trained under Dr. Donoghue's supervision
Sandip Shreshta, Graduate Student	Graduate assistant trained under Dr. Donoghue's supervision

Additional Personnel Information (if needed):

### **Chapter 3**

**Pectin or chitosan coating fortified with eugenol reduces *Campylobacter jejuni* on chicken wingettes and modulates expression of critical survival genes**

RUNNING TITLE: EUGENOL COATING REDUCES *C. JEJUNI*

**Title: Pectin or chitosan coating fortified with eugenol reduces *Campylobacter jejuni* on chicken wingettes and modulates expression of critical survival genes**

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**This manuscript is under review in Poultry Science journal**

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## ABSTRACT

*Campylobacter jejuni* infection in humans is strongly associated with the consumption of contaminated poultry products. With increasing consumer demand for minimally processed and natural product, there is a need for novel intervention strategies for controlling *C. jejuni*. Antimicrobial coatings are increasingly being used for preventing food contamination due to their efficacy and continuous protection of product. This study investigated the efficacy of pectin and chitosan coating fortified with eugenol to reduce *C. jejuni* on chicken wingettes. Pectin, chitosan and eugenol are GRAS status compounds derived from berries, crustaceans and cloves respectively. Each wingette was inoculated with a mixture of four wild-type strains of *C. jejuni* ( $\sim 10^7$  CFU/sample) and randomly assigned to controls, pectin (3%), chitosan (2%), eugenol (0.5, 1 or 2%), or their combinations. Following 1 min of coating, wingettes were air-dried, vacuum sealed and sampled on d 0, 1, 3, 5, and 7 of refrigerated storage for *C. jejuni* and aerobic counts (n=5 wingettes/treatment/day). In addition, the effect of treatments on wingette color and expression of *C. jejuni* survival/virulence genes was evaluated. All three doses of eugenol or chitosan significantly reduced *C. jejuni* and aerobic bacteria from d 0 through d 7. Incorporation of 2% eugenol in chitosan improved coating efficiency and reduced *C. jejuni* counts by  $\sim 3$  Log CFU/sample at the end of 7 days of storage ( $P < 0.05$ ). Similarly, the antimicrobial efficacy of pectin was improved by 2% eugenol and the coating reduced *C. jejuni* by  $\sim 2$  Log CFU/sample at day 7 of storage. Chitosan coating with 2% eugenol also showed greater reductions of total aerobic counts as compared to individual treatments of eugenol and chitosan. No significant difference in the color of chicken wingettes was observed between treatments. Exposure of *C. jejuni* to eugenol, chitosan or combination significantly modulated select genes encoding for motility, quorum sensing and stress response. Results demonstrate the potential of pectin or chitosan coating

fortified with eugenol as a postharvest intervention against *C. jejuni* contamination on poultry products.

**Keywords:** Antimicrobial coating, eugenol, *Campylobacter*, gene expression, postharvest poultry



## INTRODUCTION

*Campylobacter* is a major foodborne pathogen causing bacterial illness in humans worldwide (Mangen et al., 2016; Marder et al., 2017). The incidence of this pathogen recently surpassed the incidence of *Salmonella* (17.43 vs 16.66 per 100,000) in the United States with the incorporation of culture independent diagnostic tests (Marder et al., 2017). Out of 17 species of *Campylobacter*, *Campylobacter jejuni* is responsible for 90% of the campylobacteriosis in humans (Hermans et al., 2011). *C. jejuni* is frequently associated with gastroenteritis, reactive arthritis and Guillain-Barré syndrome (Spiller, 2007; Gradel et al., 2009). The primary source of human *Campylobacter* infection reported through risk assessment studies is the consumption and handling of poultry products (Friedman et al., 2004; Danis et al., 2009). The high level of *Campylobacter* in the ceca of birds ( $\sim 10^8$  CFU/g) and low infective dose ( $\sim 500$  CFU) poses a serious public health concern if carcasses are not properly decontaminated (Beery et al., 1988; Black et al., 1988; Achen et al., 1998).

Studies have shown that *C. jejuni* survives during poultry processing and can cross-contaminate poultry carcasses (Stern et al., 2001; Allen et al., 2007). The poultry producers rely on the use of chlorinated water to decrease the microbial load of poultry carcass; however, it results in minimal reduction and the efficacy further reduces in the presence of organic matter (Northcutt et al., 2005; Oyarzabal, 2005). The generation of potential mutagens from the reaction of chlorine and organic materials further raises concerns owing to associated health hazards, including cancer (Donato and Zani, 2010; Dore, 2015). As an alternative to chlorine, various other chemicals including trisodium phosphate, hydrogen peroxide, and organic acids have been studied (Zhao and Doyle, 2006; Bauermeister et al., 2008; Riedel et al., 2009; Birk et al., 2010). However, these chemical treatments have not been completely accepted due to limited effectiveness, high cost,

discoloration of carcass and residues in meat (Bilgili et al., 1998; SCVPH, 1998; EFSA BIOHAZ Panel, 2014).

Numerous studies have focused on plant-derived antimicrobials as an alternative of conventional chemical-based treatments to decontaminate food products (Pei et al., 2009; Mattson et al., 2011; Olaimat et al., 2014; Calo et al., 2015; Olaimat and Holley, 2015; Upadhyay et al., 2015; Woo-Ming, 2015; Upadhyaya et al., 2016; Wagle et al., 2017b). The antimicrobial coating on poultry products represents a viable intervention to reduce or eliminate foodborne pathogens (Cagri et al., 2004; Ricke and Hanning, 2013). However, few studies have utilized antimicrobial coating on poultry cuts to reduce *Campylobacter* (Olaimat et al., 2014; Woo-Ming, 2015), and there are no reports on the efficacy of pectin and chitosan coating fortified with eugenol in reducing *C. jejuni* load on chicken wingettes. The incorporation of antimicrobial agents in the coatings offers several advantages such as increased contact time, possible synergism between two compounds thereby requiring low concentrations to inhibit or reduce foodborne pathogens (Cagri et al., 2004; Sangsuwan et al., 2009). Additionally, the coatings remain on the food product thereby protecting foods from contamination during storage and handling. Pectin is a plant-derived heteropolysaccharide and commonly used as a gelling and thickening agent in jelly, marmalades and confectionaries and as edible coating in foods (Moalemiyan et al., 2012). Chitosan is a linear polysaccharide obtained from crustaceans with significant antimicrobial activity against *Salmonella*, *Listeria* and *C. jejuni* (Ganan et al., 2009; Olaimat et al., 2014; Olaimat and Holley, 2015; Upadhyay et al., 2015; Woo-Ming, 2015).

Various active components with significant antimicrobial efficacy from plant sources have been reported (Burt, 2004; Calo et al., 2015). Eugenol (EG) is the active component of clove oil (*Eugenia caryophyllus*) and has shown significant antimicrobial efficacy as an antimicrobial wash

or as chitosan-based coating on food products against various foodborne pathogens including *Listeria monocytogenes* (Upadhyay et al., 2015), *Salmonella* (Mattson et al., 2011; Upadhyaya et al., 2016), and *Escherichia coli* (Pei et al., 2009). All of the aforementioned compounds are classified as Generally Recognized as Safe by the US FDA for use in foods (Code of Federal Regulations 21 part 184, 170 and 172 respectively for pectin, chitosan and eugenol).

The objective of this study was to investigate the efficacy of pectin and chitosan coating fortified with eugenol to reduce *C. jejuni* on chicken wingettes. In addition, the effect of treatments on the color of chicken wingettes was evaluated. Moreover, the effect of treatments on the expression of *C. jejuni* genes essential for survival and virulence was also determined.

## **MATERIALS AND METHODS**

### ***Campylobacter Strains and Culture Conditions***

Four wild type strains (S-1, S-3, S-4, S-8) of *C. jejuni* isolated from commercial poultry were cultured according to a standard published method (Wagle et al., 2017a, b). Each *C. jejuni* strain was cultured in *Campylobacter* enrichment broth (CEB; catalogue no. 7526A, Neogen Corp., Lansing, MI) for 48 h followed by subculture for 24 h under microaerophilic conditions at 42°C. The strains were centrifuged at 3000 rpm for 10 min and washed twice in Butterfield's phosphate diluent (BPD; 0.625 mM potassium dihydrogen phosphate, pH 7.2). Each strain was appropriately diluted for plating and equal portions of the strains were combined to use as the inoculum for the study.

### ***Preparation of Coating Treatments***

Two coating materials namely pectin and chitosan were used as carrier of eugenol on chicken wingettes. For the study with pectin, 3% pectin solution was prepared as described previously (Upadhyaya et al., 2013). Briefly, 3 g of pectin powder obtained from citrus peel

(catalogue no. P9135, Sigma-Aldrich Co., St. Louis, MO) were added to BPD and heated to 60°C for 15 min. For the study with chitosan, medium molecular weight (MMW; 190-310 kDa) chitosan (catalogue no. 448877, Sigma-Aldrich) was chosen as carrier for eugenol and its 2% solution was prepared in 50 mM acetic acid (catalogue no. UN2789, ThermoFisher Scientific, Fair Lawn, NJ) according to a previously published method with slight modifications (Upadhyay et al., 2015). Three concentrations of eugenol (0.5, 1 and 2%) were prepared by adding required volume of eugenol (catalogue no. E51791, Sigma-Aldrich) into BPD solution followed by mixing with a magnetic stirrer for 30 min. The eugenol coating treatments (0.5, 1 and 2% EG) were prepared by adding appropriate quantity of eugenol in 3% pectin or 2% chitosan solution. The concentrations of coating treatments were selected based on preliminary experiments on adherence and antimicrobial strength of the coating on chicken wingettes. The BPD and 50 mM acetic acid in BPD were included as controls. The pH of all the solutions were adjusted to  $6.5 \pm 0.2$ .

### ***Evaluation of Antimicrobial Efficacy of Coating Treatments on Chicken Wingettes***

A published method was used to evaluate the antimicrobial activity of coating treatments (Olaimat et al., 2014). Briefly, chicken wingettes were made from chicken wings procured from University of Arkansas pilot processing plant and inoculated with 50  $\mu$ L mixture of *C. jejuni* ( $\sim 10^7$  CFU/sample) followed by air drying for 30 min to facilitate bacterial adherence. Wingettes were coated with controls (BPD, acetic acid), eugenol (0.5, 1, and 2%), coating materials (3% pectin or 2% chitosan) or coating materials fortified with eugenol for 1 min followed by air drying for 30 min on each side. All the samples were vacuum-sealed and stored at 4°C until sampling on d 0, 1, 3, 5, and 7.

### ***Enumeration of C. jejuni and Aerobic Bacteria on Chicken Wingettes***

For the processing of chicken wingettes, each wingettes was dipped in 30 mL of Dey-Engley neutralizing broth (catalogue no. C7371, Hardy Diagnostics, Santa Maria, CA) and blended using stomacher (Stomacher<sup>®</sup> 400 Circulator, Steward Ltd., Worthing, West Sussex, UK) at 250 rpm for 30 s. For all samples, a serial dilution (1:10) of each sample was made and plated on *Campylobacter* line agar plates (Line, 2001) and tryptic soy agar (catalogue no. DF0369-17-6, Becton, Dickinson and Company, Sparks, MD) plates. *C. jejuni* counts were enumerated after incubation at 42°C for 48 h and aerobic bacteria were enumerated after incubation at 37°C for 24 h.

### ***Color Analysis***

Following air drying of wingettes samples, color of the samples was measured using Chroma meter (CR 400/410, Konica Minolta Sensing Americas, Inc., Ramsey, NJ) as described previously (Wagle et al., 2017b). The Chroma meter provides information about three different colors (L, a, and b indicating relative lightness, redness and yellowness respectively). The instrument was calibrated against a tile and average color values were recorded from three different locations on each sample. The samples were then vacuum-sealed to store at 4°C.

### ***Gene Expression Analysis Using Real-Time Quantitative PCR***

The effect of eugenol, chitosan and their combination on the expression of survival and virulence genes of *C. jejuni* was studied as described previously (Wagle et al, 2017a, b) using real-time quantitative PCR (RT-qPCR) in the presence of chicken meat exudate. Chicken meat exudate was prepared according to a standard published method (Birk et al., 2004). Following the mid-log growth of a wild strain of *C. jejuni* (S-8) in CEB at 42°C under microaerophilic condition, bacteria were exposed to chicken meat exudate treated with subinhibitory concentrations (SICs) of eugenol

or chitosan or their combination for 1 h at 25°C. The total RNA was extracted using RNA mini kit (catalogue no. 12183018A, Invitrogen, Carlsbad, CA) and treated with DNase I (catalogue no. 18068015, Thermo Fisher Scientific, Foster city, CA). The complementary DNAs (cDNA) were prepared using iScript cDNA synthesis kit (catalogue no. 1708890, Bio-Rad Laboratories, Inc., Hercules, CA). Primer 3 software (National Center for Biotechnology Information, Bethesda, MD) was used for designing all the primers from Gene Bank and obtained from Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). The amplified products were detected by using SYBR Green reagent (iQ SYBR Green Supermix, catalogue no. 1708880, Bio-Rad Laboratories, Inc.). Data were normalized to endogenous control (16S rRNA) and relative quantification of amplified genes were calculated using comparative critical threshold ( $\Delta\Delta C_t$ ) method on Quant Studio 3 real-time PCR system (Applied Biosystems, Thermo Fisher). Duplicate samples were used and the study was repeated three times.

### *Statistical Analysis*

The study was a completely randomized design. In total, four trials were conducted on the chicken wingettes with 5 wingettes per treatment per storage day for 225 and 250 wingettes in total per pectin and chitosan trial respectively. Each trial was replicated twice (n=950 chicken wingettes per total trials). For the analysis, bacterial counts were logarithmic transferred to maintain the homogeneity of variance (Byrd et al., 2001) and the data of color analysis and gene expression were pooled before analysis. The data were analyzed by using PROC MIXED procedure in SAS version 9.3 software (SAS Institute Inc., Cary, NC). The treatment means were separated by least-square means analysis and the significance levels was  $P < 0.05$  for statistical difference.

## RESULTS

### *Antimicrobial Efficacy of Coating Treatment With or Without Eugenol Against C. jejuni on Chicken Wingettes*

The effect of eugenol in reducing *C. jejuni* on chicken wingettes was evaluated in both pectin and chitosan trials presented in Tables 2 and 3 respectively. *C. jejuni* counts recovered from the wingettes not subjected to coating treatment (baseline) ranged from 5.27 to 6.72 Log CFU/sample in all the trials. Washing with BPD produced a maximum reduction of ~1.44 Log CFU/sample compared to baseline in pectin trials (Table 2); however, in the chitosan trial 2 (Table 3), the reduction was not significant on d 0 and d 3. The 0.5, 1 and 2% eugenol consistently reduced *C. jejuni* counts on chicken wingettes by at least 0.8, 0.56, 0.66 and 0.72 Log CFU/sample compared to BPD control in trials 1 and 2 of pectin and chitosan studies respectively. Among the three doses of eugenol, 2% produced significantly greater reductions than 0.5% on d 0 and d 7 in both pectin trials, d 7 in chitosan trial 1 and on d 0 and d 1 in chitosan trial 2. Similarly, 2% EG was more effective than 1% EG on d 0 and d 7 in pectin trial 1, d 1 and d 3 in pectin trial 2, d 7 in chitosan trial 1, and d 0 and d 1 in chitosan trial 2. There was significant difference in anti-*Campylobacter* effect between 0.5 and 1% EG on d 7 in pectin trial 2, however the results were not consistent between trials.

The effect of pectin as a coating material and the eugenol-pectin coating combinations against *C. jejuni* is presented in Table 2. Pectin consistently reduced *C. jejuni* counts on majority of storage time points by at least 0.6 Log CFU/sample as compared to non-coated (baseline) chicken wingettes. However, there was no consistent differences between pectin and BPD controls in both trials. Incorporation of eugenol in the pectin coating consistently improved the anti-*Campylobacter* activity of pectin ( $P < 0.05$ ) but the combinations was similar in efficacy as

compared with eugenol in majority of storage days ( $P>0.05$ ). Among the three combination treatments, 2% eugenol-pectin combination produced at least 2.1 Log CFU/sample reduction and this was significantly greater when compared with 0.5% combinations in most of the storage days (d 0, 1, 5 and 7 in trial 1 and d 0, 1 and 5 in trial 2). Similarly, this reduction was significantly greater in two storage days (d 0 and 5 in trial 1 and on d 1 and 3 in trial 2) as compared to 1% combination treatment. There was no significant differences between 0.5 and 1% combination treatments of pectin and eugenol with the exception on d 1 in trial 1.

Table 3 shows the effect of chitosan coating either alone or in combination with eugenol against *C. jejuni* on chicken wingettes. Chitosan coating consistently reduced *C. jejuni* counts with a range from 0.74 to 2.06 Log CFU/sample in both trials. Acetic acid (control for chitosan coating with or without eugenol) reduced *C. jejuni* counts in trial 1 from d 1 to d 7; however in trial 2, an inconsistent reduction was observed as compared to baseline. When compared with BPD control, acetic acid did not significantly reduce *C. jejuni* counts on majority of storage days. The combination of eugenol and chitosan produced consistent reduction of at least 0.9 Log CFU/sample when compared with acetic acid in both trials. Among the three combinations of eugenol and chitosan when compared with acetic acid control, the maximum reduction was ~3 Log CFU/sample on d 0 of both trials observed with 1 and 2% eugenol-chitosan combinations. The 2% eugenol-chitosan coating produced greater reduction of *C. jejuni* counts than that by chitosan alone in both trials ( $P<0.05$ ). This reduction was also significantly different from 2% eugenol alone in majority of storage days (d 0, 1, 3, and 5) in trial 1 and only on d 5 in trial 2. Additionally, there was no consistent differences among 0.5, 1 and 2% eugenol-chitosan combinations.



### ***Antimicrobial Efficacy of Coating Treatment With or Without Eugenol Against Aerobic Bacteria on Chicken Wingettes***

Tables 4 and 5 show the effect of eugenol on the total aerobic bacterial counts on chicken wingettes (from pectin and chitosan study respectively). The aerobic counts on chicken wingettes not subjected to any treatment (baseline) was ~ 4.5 Log CFU/sample on d 0. The aerobic counts increased by at least 1.2 Log CFU/sample by the end of d 7 in all trials ( $P < 0.05$ ). The treatment with BPD failed to reduce aerobic bacteria in all the trials except on d 0 and d 1 in chitosan trial 1 (Table 5). All the tested doses of eugenol consistently reduced aerobic counts by at least 0.51 Log CFU/sample from d 0 to d 7 when compared with BPD controls. There was no significant difference among 0.5, 1 and 2% eugenol treatments on majority of days (d 3, 5, and 7) in both pectin trials and chitosan trial 2 and on d 0 and d 1 in chitosan trial 1.

Table 4 also shows the effect of pectin and eugenol-pectin coating on the total aerobic counts on chicken wingettes. Pectin coating significantly reduced aerobic counts starting from d 3 to d 7 in both trials. Eugenol-pectin combination coating also reduced the counts consistently and improved the antibacterial efficacy of pectin at the beginning (d 0 to 5 in trial 1 and d 0 to 3 in trial 2) but not on d 7 in both trials. However, no significant differences have been observed among 0.5, 1 and 2% eugenol-pectin combinations on majority of storage days (d 0, 1, 3, 7) in trial 1, and on d 3 and d 7 in trial 2.

The effect of chitosan and eugenol-chitosan combination on the total aerobic counts on chicken wingettes is shown in Table 5. Treatment of chicken wingettes with acetic acid did not significantly reduce aerobic counts with the exception on d 0 and d 3 in trial 1. In contrast, chitosan coating significantly reduced the counts across all storage days in both trials. This reduction ranged from 0.72 to 1.2 and from 0.86 to 1.37 Log CFU/sample in trial 1 and trial 2 respectively. Similar

result was observed with 0.5 and 1% eugenol-chitosan combination coating. The 2% eugenol-chitosan coating showed greater reductions as compared to 0.5% combination treatments beginning from d 3 in both trials whereas in comparison with 1% combination treatments, it was significantly different on d 1, 3, 5 and 7 in trial 1 and on d 1, 5, 7 in trial 2. Moreover, the reduction obtained with 2% eugenol-chitosan was significantly different from the individual's treatment of chitosan and eugenol across all storage days in both trials with the exception on d 0 in trial 2.

### ***Effect of Coating Treatments on the Color of Chicken Wingettes***

The coating of chicken wingettes with eugenol, pectin, chitosan and their combination did not affect the lightness, redness and yellowness of meat (Data not shown). However, the refrigerated storage time had a significant effect on the yellowness of chicken wingettes. Similarly, storage time significantly increased the lightness of chicken wingettes treated with chitosan alone or in combination with 0.5 or 1% EG.

### ***Effect of Eugenol and Chitosan on Expression of *C. jejuni* Virulence Genes***

The gene expression profile of *C. jejuni* in response to SICs of eugenol, chitosan and their combinations is shown in Table 6. The presence of SICs of eugenol, chitosan, and their combination significantly changed the expression of select genes coding for pathogen motility, stress response, quorum sensing and attachment to epithelial cells. The SIC of eugenol significantly downregulated the expression of genes coding for motility (*motA*, *motB*), stress response (*katA*) and quorum sensing (*luxS*). However, energy taxis genes (*cetA*, *cetB*) responsible for directional motility, attachment genes (*cadF*, *ciaB*, *jlpA*) and two-component regulatory proteins (RacR-RacS) were not affected ( $P > 0.05$ ). Chitosan at SIC level, downregulated *motA* gene, however, upregulated select genes for motility (*motB*), attachment (*ciaB*, *jlpA*), and stress response (*sodB*). Other genes essential for *C. jejuni* motility (*fliA*, *cetA*, *cetB*), stress response

(*katA*), quorum sensing (*luxS*) and two-component regulatory system (*racS-racR*) were not changed by chitosan ( $P>0.05$ ). Similar to chitosan, the eugenol-chitosan combination downregulated *motA* gene and upregulated genes *motB*, *ciaB*, *jlpA*, *sodB*. In addition, the combination also downregulated genes *luxS* and *katA*, an effect similar to eugenol treatment. The combination of eugenol and chitosan reduced the expression of *cetA* as compared to control ( $P<0.05$ ). The individual treatments did not modulate the expression of *cetA* ( $P>0.05$ ). The acetic acid treatment did not affect the expression of tested genes ( $P>0.05$ ).

## DISCUSSION

*Campylobacter* contamination of poultry product is one of the major risk factor for human campylobacteriosis (Friedman et al., 2004). Despite rigorous search for interventions to be utilized in the processing facility, the pathogen is widely present on raw poultry products (Stern et al., 2001). In addition, there is an increase in consumer preference towards product with minimal processing and chemical treatment. A potential strategy for controlling *Campylobacter* is by antimicrobial coating of raw poultry products. In this study, we investigated the efficacy of eugenol as a coating treatment of chicken wingettes and hypothesized that increasing contact time between compounds and bacteria could improve the antibacterial activity of eugenol.

In order to coat the chicken wingettes with eugenol, we selected two coating materials, pectin and chitosan, which are extensively studied as films in the food industry as an alternative of conventional packaging materials (Aider, 2010; Moalemiyan et al., 2012). Pectin dissolves at neutral pH while chitosan requires acidification. Therefore, we used acetic acid at 50mM to dissolve the compound. Pectin itself did not exhibit antimicrobial activity against *C. jejuni* (Table 2). Similar findings were reported previously where pectin coating did not significantly reduce coliforms on shrimp (Alvarez et al., 2014) and *Salmonella* on eggs (Upadhyaya et al., 2016). In

contrast to pectin, coating of chicken wingettes with MMW chitosan exerted significant antimicrobial activity against *C. jejuni* (Table 3). Olaimat et al. (2014) used chitosan/ $\kappa$ -carrageenan combination coating on chicken breast. They found significant reduction (up to 2.78 Log CFU/g) of *C. jejuni* with the coating containing mustard extract. In the present study, the incorporation of select concentrations of eugenol in coating materials significantly improved the efficacy of pectin and chitosan coating materials. This finding was also similar to previous reports from other studies where the incorporation of eugenol significantly improved the efficacy of pectin coating against *Salmonella* Enteritidis (Upadhyaya et al., 2016) and chitosan coating against *L. monocytogenes* (Upadhyay et al., 2015).

Reducing aerobic counts on chicken wingettes is important to increase the shelf life of product during refrigeration (Kim and Marshall, 2000). Coating of raw chicken wingettes with eugenol and its coating materials significantly reduced total aerobic counts (Tables 4 and 5). However, none of the treatments checked further growth of aerobic bacteria on chicken wingettes with storage days. Kim and Marshall (2000) had similar findings when 1% organic acids treated chicken wings were stored at 4°C for 12 days. Since chicken skin harbors diverse bacteria including psychrophiles (Cox et al., 1998; Kim et al., 2017), the increase in aerobic plate count could be due to growth of these bacteria. Even though there was an increase in aerobic plate counts by at least 1.2 Log CFU/sample on d 7 in controls, the counts in controls as well as in treatments were below the critical point (8 Log CFU/cm<sup>2</sup>) where fresh meat produce sliminess due to bacterial spoilage (Cox et al., 1998).

We investigated the effect of the treatments on color of chicken wingettes since it is one of the key factor to assess the quality of poultry products for purchaser. We observed that there were no significant differences in color (lightness, redness, yellowness) of chicken wingettes between

treatments and controls (Data not shown). Khan et al (2015) had also observed similar results with 0.05% eugenol on raw chicken. During storage, studies have shown that changes in color values are more pronounced within 6 h after post-mortem and become less variable later on (Petracci and Fletcher, 2002). It was also reported previously that color of poultry meat changed to lighter and more brownish with time due to growth of microbes, pH, lipid oxidation and other deteriorating factors (Khan et al., 2015). We did not observe any significant change in color except yellowness with storage days probably due to the effect of coating material.

Previous studies from our lab (Arambel et al., 2015; Upadhyay et al., 2017; Wagle et al., 2017a, b) as well as other researchers (Castillo et al., 2014; Oh and Jeon, 2015; Kovács et al., 2016) have determined that phytochemicals at subinhibitory concentrations modulates the expression of genes in various microbes including *C. jejuni*. We investigated the effect of SICs of eugenol and chitosan on the expression of *C. jejuni* genes associated with survival and virulence to delineate their potential mechanism of action. Since pectin failed to reduce *C. jejuni* counts compared to BPD controls (Table 2), its effect on *C. jejuni* gene expression was not determined. Gene expression analysis was studied in the presence of chicken meat juice to represent the meat environment, especially because chicken meat juice is known to modulate the physiology of *C. jejuni* thereby enhancing their survival in the poultry products (Birk et al., 2004; Brown et al., 2014). Several researchers have used 16S rRNA as an endogenous control in real-time qPCR (Klančnik et al., 2006; Tasara and Stephan, 2007; Hays, 2009; Koolman et al., 2016) and we used the same gene for calibrating the expression of other genes. A variety of genes responsible for bacterial virulence has been characterized for *C. jejuni* (Hermans et al., 2011). The movement of *C. jejuni* towards substrate at low temperature (4°C) is responsible for their survival in meat (Hazeleger et al., 1998). The motility of *C. jejuni* is imparted through flagella and encoded by the

genes *motA*, *motB*, and *fliA*, which also play a role in the pathogenesis of human *Campylobacter* infection (Young et al., 2007). In addition, the energy taxis genes (*cetA*, *cetB*) are essential for motility in response to stimuli, attachment and biofilm formation on various surfaces (Kalmokoff et al., 2006; Hermans et al., 2011). Moreover, *cadF* and *jlpA* are responsible for cell surface attachment (Jin et al., 2003; Hermans et al., 2011). The two-component regulatory proteins (RacR-RacS) are necessary for temperature-dependent growth of *C. jejuni* (Hermans et al., 2011). Previously, it was shown that *C. jejuni luxS* mutants were unable to survive in meat environment (Ligowska et al., 2011). Similarly, stress response (*katA*, *sodB*) genes are important for adaptation and survival of *C. jejuni* (Atack and Kelly, 2009). We observed that eugenol significantly downregulated the expression of select genes encoding motility (*motA*, *motB*) and quorum sensing (*luxS*) in *C. jejuni* thereby potentially limiting the survival in meat environment (Table 6). Similarly, eugenol also downregulated *katA* gene, which is in contrast to the previous reports (Kovács et al., 2016) with clove oils possibly due to difference in *C. jejuni* strains (Wild type vs NCTC 11168). The expression level of majority of genes in eugenol-chitosan combination was similar to that of either eugenol or chitosan except *cetA* and *jlpA*. We observed an upregulation of few virulence genes (*motB*, *ciaB*, *jlpA*) and stress gene (*sodB*) in response to chitosan. This could potentially be due to trigger of a compensatory or stress response pathway. A transcriptomic study would shed more light on the effect of chitosan on virulence and other critical genes. Overall, these findings suggest that the aforementioned treatments could affect the potential of *C. jejuni* to survive in meat and cause disease in humans.

## CONCLUSION

In conclusion, pectin and chitosan coating fortified with eugenol on the poultry cuts consistently reduce *C. jejuni*. In addition, eugenol, chitosan and their combination modulated transcription of several genes essential for survival and virulence of *C. jejuni*. Since a 2-Log reduction of *C. jejuni* from poultry carcass translates into more than 90% reduction in the risk of human *Campylobacter* infections (Nauta et al., 2016), the aforementioned treatments represent a safe, effective and natural approach that could improve poultry product safety. Follow-up studies testing the effect of the coating on the organoleptic properties of meat are warranted.

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## REFERENCES

- Achen, M., T. Y. Morishita, and E. C. Ley. 1998. Shedding and colonization of *Campylobacter jejuni* in broilers from day-of-hatch to slaughter age. *Avian Dis.* 42: 732-737.
- Aider, Mohammed. 2010. Chitosan application for active bio-based films production and potential in the food industry. *Food Sci. Technol.* 43: 837-842.
- Allen, V. M., S. A. Bull, J. E. L. Corry, G. Domingue, F. Jørgensen, J. A. Frost, R. Whyte, A. Gonzalez, N. Elviss, and T. J. Humphrey. 2007. *Campylobacter* spp. contamination of chicken carcasses during processing in relation to flock colonisation. *Int. J. Food Microbiol.* 113: 54-61.
- Alvarez, M. V., L. A. Ortega-Ramirez, M. M. Gutierrez-Pacheco, A. T. Bernal-Mercado, I. Rodriguez-Garcia, G. A. Gonzalez-Aguilar, A. Ponce, M. D. R. Moreira, S. I. Roura, and J. F. Ayala-Zavala. 2014. Oregano essential oil-pectin edible films as anti-quorum sensing and food antimicrobial agents. *Front. Microbiol.* 5:699.
- Arambel, H. R., A. N. Donoghue, K. Arsi, A. Upadhyay, A. Woo-Ming, P. Blore, K. Venkitanarayanan, and D. Donoghue. 2015. Chitosan supplementation reduces enteric colonization of *Campylobacter jejuni* in broiler chickens and down-regulates expression of colonization genes. *Adv. Food Technol. Nutr. Sci. Open J.* 1: 104-111.
- Atack, J. M., and D. J. Kelly. 2009. Oxidative stress in *Campylobacter jejuni*: responses, resistance and regulation. *Future Microbiol.* 4: 677-690.
- Bauermeister, L. J., J. W. Bowers, J. C. Townsend, and S. R. McKEE. 2008. Validating the efficacy of peracetic acid mixture as an antimicrobial in poultry chillers. *J. Food Prot.* 71: 1119-1122.
- Beery, J. T., M. B. Hugdahl, and M. P. Doyle. 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 54: 2365-2370.
- Bilgili, S., D. Conner, J. Pinion, and K. Tamblyn. 1998. Broiler skin color as affected by organic acids: Influence of concentration and method of application. *Poult. Sci.* 77:752-757.
- Birk, T., A. C. Grønlund, B. B. Christensen, S. Knøchel, K. Lohse, and H. Rosenquist. 2010. Effect of organic acids and marination ingredients on the survival of *Campylobacter jejuni* on meat. *J. Food Prot.* 73:258-265.
- Birk, T., H. Ingmer, M. T. Andersen, K. Jørgensen and L. Brøndsted. 2004. Chicken juice, a food-based model system suitable to study survival of *Campylobacter jejuni*. *Lett. Appl. Microbiol.* 38:66-71.
- Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser. 1988. Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Dis.* 157: 472-479.

- Brown, H. L., M. Reuter, L. J. Salt, K. L. Cross, R. P. Betts, and A. HM van Vliet. 2014. Chicken juice enhances surface attachment and biofilm formation of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 80: 7053-7060.
- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food. Microbiol.* 94: 223-253.
- Byrd, J. A., B. M. Hargis, D. J. Caldwell, R. H. Bailey, K. L. Herron, J. L. McReynolds, R. L. Brewer, R. C. Anderson, K. M. Bischoff, T. R. Callaway, and L. F. Kubena. 2001. Effect of lactic acid administration in the drinking water during preslaughter feed withdrawal on *Salmonella* and *Campylobacter* contamination of broilers. *Poult. Sci.* 80:278-283.
- Cagri, A., Z. Ustunol, and E. T. Ryser. 2004. Antimicrobial edible films and coatings. *J. Food Prot.* 67: 833-848.
- Calo, J. R., P. G. Crandall, C. A. O'Bryan, and S. C. Ricke. 2015. Essential oils as antimicrobials in food systems—A review. *Food Control* 54: 111-119.
- Castillo, S., N. Heredia, E. Arechiga-Carvajal, and S. García. 2014. Citrus extracts as inhibitors of quorum sensing, biofilm formation and motility of *Campylobacter jejuni*. *Food Biotechnol.* 28:106-122.
- Chantarapanont, W., M. Berrang, and J. F. Frank. 2003. Direct microscopic observation and viability determination of *Campylobacter jejuni* on chicken skin. *J Food Prot* 66: 2222-2230.
- Cox, N. A., S. M. Russell, and J. S. Bailey. 1998. The microbiology of stored poultry. Pages 266-287 in *The Microbiology of Meat and Poultry*. Blackie Academic & Professional. New York.
- Danis, K., M. Di Renzi, W. O'Neill, B. Smyth, P. McKeown, B. Foley, V. Tohani, and M. Devine. 2009. Risk factors for sporadic *Campylobacter* infection: an all-Ireland case-control study. *Euro. Surveill.* 14: 19123.
- Donato, F., and C. Zani. 2009. Chronic exposure to organochlorine compounds and health effects in adults: cancer, non-Hodgkin lymphoma. Review of literature. *Ann. Ig.* 22: 357-367.
- Dore, M. H. 2015. Threats to Human Health: Use of chlorine, an obsolete treatment technology. Pages 197-212 in *Global Drinking Water Management and Conservation*. Springer Water. Springer, Cham. doi.org/10.1007/978-3-319-11032-5\_9.
- EFSA BIOHAZ Panel. 2014. Scientific opinion on the evaluation of the safety and efficacy of peroxyacetic acid solutions for reduction of pathogens on poultry carcasses and meat. *EFSA J.* 12:3599.

- Friedman, C. R., R. M. Hoekstra, M. Samuel, R. Marcus, J. Bender, B. Shiferaw, S. Reddy, S. D. Ahuja, D. L. Helfrick, F. Hardnett, and M. Carter. 2004. Risk factors for sporadic *Campylobacter* infection in the United States: a case-control study in FoodNet sites. *Clin. Infect. Dis.* 38: 285-S296.
- Ganan, M., A. V. Carrascosa, and A. J. Martinez-Rodriguez. 2009. Antimicrobial activity of chitosan against *Campylobacter* spp. and other microorganisms and its mechanism of action. *J. Food Prot.* 72: 1735-1738.
- Gradel, K. O., H. L. Nielsen, H. C. Schønheyder, T. Ejlersen, B. Kristensen, and H. Nielsen. 2009. Increased short-and long-term risk of inflammatory bowel disease after salmonella or campylobacter gastroenteritis. *Gastroenterology* 137: 495-501.
- Hays, J. P. 2009. The evaluation of putative endogenous control housekeeping genes for real-time polymerase chain reaction expression studies in *Moraxella catarrhalis*. *Diag. Microbiol. Infect. Dis.* 65:323-326.
- Hazeleger, W. C., J. A. Wouters, F. M. Rombouts, and T. Abee. 1998. Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. *Appl. Environ. Microbiol.* 64: 3917-3922.
- Helander, I. M., E. L. Nurmiäho-Lassila, R. Ahvenainen, J. Rhoades, and S. Roller. 2001. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *Int. J. Food Microbiol.* 71: 235-244.
- Hermans, D., K. Van Deun, A. Martel, F. Van Immerseel, W. Messens, M. Heyndrickx, F. Haesebrouck, and F. Pasmans. 2011. Colonization factors of *Campylobacter jejuni* in the chicken gut. *Vet. Res.* 42: 82.
- Jiménez, S. M., M. S. Salsi, M. C. Tiburzi, R. C. Rafaghelli, M. A. Tessi, and V. R. Coutaz. 1997. Spoilage microflora in fresh chicken breast stored at 4 C: influence of packaging methods. *J. Appl. Microbiol.* 83: 613-618.
- Jin, S., Y. C. Song, A. Emili, P. M. Sherman, and V. L. Chan. 2003. JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90 $\alpha$  and triggers signalling pathways leading to the activation of NF- $\kappa$ B and p38 MAP kinase in epithelial cells. *Cell. Microbiol.* 5: 165-174.
- Kalmokoff, M., P. Lanthier, T. L. Tremblay, M. Foss, P. C. Lau, G. Sanders, J. Austin, J. Kelly, and C. M. Szymanski. 2006. Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. *J. Bacteriol.* 188: 4312-4320.
- Khan, A., K. Allen, and X. Wang. 2015. Effect of Type I and Type II antioxidants on oxidative stability, microbial growth, pH, and color in raw poultry meat. *Food Nutr. Sci.* 6: 1541-1551.

- Kim, C. R., and D. L. Marshall. 2000. Quality evaluation of refrigerated chicken wings treated with organic acids. *J. Food Qual.* 23: 327-335.
- Klančnik, A., N. Botteldoorn, L. Herman, and S. S. Možina. 2006. Survival and stress induced expression of groEL and rpoD of *Campylobacter jejuni* from different growth phases. *Int. J. Food Microbiol.* 112: 200-207.
- Koolman, L., P. Whyte, C. Burgess, and D. Bolton. 2016. Virulence gene expression, adhesion and invasion of *Campylobacter jejuni* exposed to oxidative stress (H<sub>2</sub>O<sub>2</sub>). *Int. J. Food Microbiol.* 220: 33-38.
- Kovács, J. K., P. Felső, L. Makszin, Z. Pápai, G. Horváth, H. Ábrahám, T. Palkovics, A. Böszörményi, L. Emödy, and G. Schneider. 2016. Antimicrobial and virulence-modulating effects of clove essential oil on the foodborne pathogen *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 82:6158-6166.
- Ligowska, M., M. T. Cohn, R. A. Stabler, B. W. Wren, and L. Brøndsted. 2011. Effect of chicken meat environment on gene expression of *Campylobacter jejuni* and its relevance to survival in food. *Int. J. Food Microbiol.* 145: 111-115.
- Line, J. E. 2001. Development of a selective differential agar for isolation and enumeration of *Campylobacter* spp. *J. Food Prot.* 64:1711-1715.
- Mangen, M. J., A. H. Havelaar, J. A. Haagisma, and M. E. E. Kretzschmar. 2016. The burden of *Campylobacter*-associated disease in six European countries. *Microb. Risk Anal.* 2: 48-52.
- Marder, E. P., P. R. Cieslak, A. B. Cronquist, J. Dunn, S. Lathrop, T. Rabatsky-Ehr, P. Ryan, K. Smith, M. Tobin-D'Angelo, D. J. Vugia, and S. Zansky. 2017. Incidence and trends of infections with pathogens transmitted commonly through food and the effect of increasing use of culture-independent diagnostic tests on surveillance-foodborne diseases active surveillance network, 10 US Sites, 2013-2016. *MMWR. Morb. Mortal. Wkly. Rep.* 66: 397-403. doi: 10.15585/mmwr.mm6615a1.
- Mattson, T. E., A. K. Johnny, M. A. R. Amalaradjou, K. More, D. T. Schreiber, J. Patel, and K. Venkitanarayanan. 2011. Inactivation of *Salmonella* spp. on tomatoes by plant molecules. *Int. J. Food Microbiol.* 144: 464-468.
- Moalemiyan, M., H. S. Ramaswamy, and N. Maftoonazad. 2012. Pectin-based edible coating for shelf-life extension of ataulfo mango. *J. Food Process Eng.* 35: 572-600.
- Nauta, M. J., G. Johannessen, L. L. Adame, N. Williams, and H. Rosenquist. 2016. The effect of reducing numbers of *Campylobacter* in broiler intestines on human health risk. *Microb. Risk Anal.* 2: 68-77.

- Northcutt, J. K., D. P. Smith, M. T. Musgrove, K. D. Ingram, and Arthur Hinton Jr. 2005. Microbiological impact of spray washing broiler carcasses using different chlorine concentrations and water temperatures. *Poult. Sci.* 84: 1648-1652.
- Oh, E. and B. Jeon. 2015. Synergistic anti-*Campylobacter jejuni* activity of fluoroquinolone and macrolide antibiotics with phenolic compounds. *Front. Microbiol.* 6: 1129.
- Olaimat, A. N., and R. A. Holley. 2015. Control of *Salmonella* on fresh chicken breasts by  $\kappa$ -carrageenan/chitosan-based coatings containing allyl isothiocyanate or deodorized Oriental mustard extract plus EDTA. *Food Microbiol.* 48: 83-88.
- Olaimat, A. N., Y. Fang, and R. A. Holley. 2014. Inhibition of *Campylobacter jejuni* on fresh chicken breasts by  $\kappa$ -carrageenan/chitosan-based coatings containing allyl isothiocyanate or deodorized oriental mustard extract. *Int. J. Food Microbiol.* 187:77–82.
- Oyarzabal, Omar A. 2005. Reduction of *Campylobacter* spp. by commercial antimicrobials applied during the processing of broiler chickens: a review from the United States perspective. *J. Food Prot.* 68: 1752-1760.
- Pei, R. S, F. Zhou, B. P. Ji, and J. Xu. 2009. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method. *J. Food Sci.* 74: 379-383.
- Petracci, M., and D. L. Fletcher. 2002. Broiler skin and meat color changes during storage. *Poult. Sci.* 81: 1589-1597.
- Ricke, S. C., and I. Hanning. 2013. Food safety applications of nanoparticles. Pages 115–125 in *Nanotechnology Safety*. Asmatulu, R., ed. Elsevier, Amsterdam.
- Riedel, C. T., L. Brøndsted, H. Rosenquist, S. N. Haxgart, and B. B. Christensen. 2009. Chemical decontamination of *Campylobacter jejuni* on chicken skin and meat. *J. Food Prot.* 72:1173-1180.
- Sangsuwan, J., N. Rattanapanone, R. A. Auras, B. R. Harte, and P. Rachtanapun. 2009. Factors affecting migration of vanillin from chitosan/methyl cellulose films. *J Food Sci.* 74: 549-555.
- SCVPH. 1998. Report of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) on benefits and limitations of antimicrobial treatments for poultry carcasses. Available at: [https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com\\_scv\\_out14\\_en.pdf](https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scv_out14_en.pdf) . Accessed 22 September 2017.
- Spiller, R. C. 2007. Role of infection in irritable bowel syndrome. *J. Gastroenterol.* 42:41-47.

- Stern, N. J., P. Fedorka-Cray, J. S. Bailey, N. A. Cox, S. E. Craven, K. L. Hiett, M. T. Musgrove, Scott Ladely, Douglas Cosby, and G. C. Mead. 2001. Distribution of *Campylobacter* spp. in selected US poultry production and processing operations. *J. Food Prot.* 64: 1705-1710.
- Tasara, T., and R. Stephan. 2007. Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. *FEMS Microbiol. Lett.* 269:265-272.
- Upadhyay, A., I. Upadhyaya, D. P. Karumathil, H. Yin, M. S. Nair, V. Bhattaram, C. Chen, G. Flock, S. Mooyottu, and K. Venkitanarayanan. 2015. Control of *Listeria monocytogenes* on skinless frankfurters by coating with phytochemicals. *LWT-Food Sci. Technol.* 63:37-42.
- Upadhyay, A., K. Arsi, B. R. Wagle, I. Upadhyaya, S. Shrestha, A. M. Donoghue, and D. J. Donoghue. 2017. Trans-Cinnamaldehyde, carvacrol, and eugenol reduce *Campylobacter jejuni* colonization factors and expression of virulence genes in Vitro. *Front. Microbiol.* 8: 713.
- Upadhyaya, I., H. B. Yin, M. S. Nair, C. H. Chen, R. Lang, M. J. Darre, and K. Venkitanarayanan. 2016. Inactivation of *Salmonella* Enteritidis on shell eggs by coating with phytochemicals. *Poult. Sci.* 95: 2106-2111.
- Wagle, B. R., A. Upadhyay, K. Arsi, S. Shrestha, K. Venkitanarayanan, A. M. Donoghue, and D. J. Donoghue. 2017a. Application of  $\beta$ -Resorcylic acid as potential antimicrobial feed additive to reduce *Campylobacter* colonization in broiler chickens. *Front. Microbiol.* 8: 599.
- Wagle, B. R., K. Arsi, A. Upadhyay, S. Shrestha, K. Venkitanarayanan, A. M. Donoghue, and D. J. Donoghue. 2017b.  $\beta$ -Resorcylic Acid, a phytophenolic compound, reduces *Campylobacter jejuni* in postharvest poultry. *J. Food Prot.* 80: 1243-1251.
- Woo-Ming, A. N. 2015. Reduction of *Campylobacter jejuni* on chicken wingettes by treatment with caprylic acid, chitosan or protective cultures of *Lactobacillus* spp. PhD diss., University of Arkansas.
- Young, K. T., L. M. Davis, and V. J. DiRita. 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev. Microbiol.* 5: 665-679.
- Zhao, T., and M. P. Doyle. 2006. Reduction of *Campylobacter jejuni* on chicken wings by chemical treatments. *J. Food Prot.* 69:762-767.

**Table 1:** Primers used for gene expression analysis using real-time quantitative PCR

<b>Gene with Accession no.</b>	<b>Primer</b>	<b>Sequence (5'-3')</b>
16S-rRNA (NC_002163.1) (product length 78 bp)	Forward	5'-ATAAGCACCGGCTAACTCCG-3'
	Reverse	5'-TTACGCCCAGTGATTCCGAG-3'
<i>motA</i> (NC_002163.1) (product length 75 bp)	Forward	5'-AGCGGGTATTTTCAGGTGCTT-3'
	Reverse	5'-CCCCAAGGAGCAAAAAGTGC-3'
<i>motB</i> (NC_002163.1) (product length 51 bp)	Forward	5'-AATGCCCAGAATGTCCAGCA-3'
	Reverse	5'-AGTCTGCATAAGGCACAGCC-3'
<i>fliA</i> (NC_002163.1) (product length 56 bp)	Forward	5'-AGCTTTCACGCCGTTACGAT-3'
	Reverse	5'-TCTTGCAAAACCCAGAAAGT-3'
<i>cetA</i> (NC_002163.1) (product length 78 bp)	Forward	5'-CCTACCATGCTCTCCTGCAC -3'
	Reverse	5'-CGCGATATAGCCGATCAAACC-3'
<i>cetB</i> (NC_002163.1) (product length 88 bp)	Forward	5'-GCCTTGTTGCTGTTCTGCTC -3'
	Reverse	5'-TTCCGTTTCGTGATGCCAA -3'
<i>cadF</i> (NC_002163.1) (product length 135 bp)	Forward	5'-CGCGGGTGTAATAATCCGTC-3'
	Reverse	5'-TCCTTTTTGCCACCAAACCA-3'
<i>ciaB</i> (NC_002163.1) (product length 50 bp)	Forward	5'-TCTCAGCTCAAGTCGTTCCA-3'
	Reverse	5'-GCCCCGCTTAGAACTTACAA-3'
<i>jlpA</i> (NC_002163.1) (product length 66 bp)	Forward	5'-AGCACACAGGGAATCGACAG-3'
	Reverse	5'-TAACGCTTCTGTGGCGTCTT-3'
<i>sodB</i> (NC_002163.1) (product length 98 bp)	Forward	5'-CAAACTTCAAATGGGGGCGT-3'
	Reverse	5'-CACAGCCACAGCCTGTACTT -3'
<i>katA</i> (NC_002163.1) (product length 99 bp)	Forward	5'-ATGCTGAACGCGATGTGAGA -3'
	Reverse	5'-CGCGGATGAAGAATGTCGGA-3'
<i>luxS</i> (NC_002163.1) (product length 106 bp)	Forward	5'-AGTGTTGCAAAAGCTTGGGA-3'
	Reverse	5'-GCATTGCACAAGTTCCGCAT-3'
<i>racS</i> (NC_002163.1) (product length 79 bp)	Forward	5'-AGACAAGTTGCCGAAGTTGC -3'
	Reverse	5'-AGGCGATCTTGCCCTACTTCA -3'
<i>racR</i> (NC_002163.1) (product length 83 bp)	Forward	5'-AGAGAACAGCTTGTAAGTCGCT-3'
	Reverse	5'-ACCCTTAAGCGACCGATGAT -3'

**Table 2:** The efficacy of eugenol (0, 0.5, 1 or 2%), pectin (0 or 3%) and their combinations as coating treatment to reduce *C. jejuni* on chicken wingettes<sup>1</sup>

Trial no.	Treatments	Day 0	Day 1	Day 3	Day 5	Day 7
1	Baseline	6.55±0.07 <sup>a,x</sup>	6.14±0.14 <sup>a,xy</sup>	5.77±0.11 <sup>a,y</sup>	6.06±0.05 <sup>a,y</sup>	5.81±0.05 <sup>a,y</sup>
	BPD control	5.41±0.05 <sup>b,x</sup>	5.21±0.15 <sup>b,xy</sup>	5.03±0.06 <sup>b,xy</sup>	4.98±0.07 <sup>b,xy</sup>	4.83±0.11 <sup>b,y</sup>
	0.5% Eugenol	4.57±0.21 <sup>c,x</sup>	4.04±0.11 <sup>cd,y</sup>	3.98±0.17 <sup>c,y</sup>	3.98±0.19 <sup>c,y</sup>	3.58±0.11 <sup>cd,y</sup>
	1% Eugenol	4.51±0.11 <sup>c,x</sup>	4.01±0.11 <sup>cd,y</sup>	3.94±0.12 <sup>cd,yz</sup>	3.98±0.13 <sup>c,y</sup>	3.29±0.39 <sup>d,z</sup>
	2% Eugenol	3.95±0.12 <sup>d,x</sup>	3.88±0.09 <sup>cde,x</sup>	3.60±0.14 <sup>cd,x</sup>	3.55±0.27 <sup>cd,x</sup>	2.58±0.37 <sup>e,y</sup>
	3% Pectin	5.57±0.12 <sup>b,x</sup>	5.35±0.18 <sup>b,xy</sup>	4.79±0.11 <sup>b,z</sup>	5.03±0.17 <sup>b,yz</sup>	4.82±0.23 <sup>b,z</sup>
	0.5% Eugenol +3% Pectin	4.66±0.16 <sup>c,x</sup>	4.31±0.08 <sup>c,xy</sup>	3.97±0.11 <sup>cd,y</sup>	3.81±0.12 <sup>c,z</sup>	3.83±0.07 <sup>c,z</sup>
	1% Eugenol + 3% Pectin	4.38±0.13 <sup>cd,x</sup>	3.75±0.14 <sup>de,y</sup>	3.73±0.10 <sup>cd,y</sup>	3.86±0.20 <sup>c,y</sup>	3.60±0.23 <sup>cd,y</sup>
2% Eugenol + 3% Pectin	3.40±0.27 <sup>e,x</sup>	3.50±0.27 <sup>e,x</sup>	3.50±0.11 <sup>d,x</sup>	3.16±0.30 <sup>d,x</sup>	3.17±0.18 <sup>d,x</sup>	
2	Baseline	6.72±0.05 <sup>a,w</sup>	6.11±0.06 <sup>a,x</sup>	6.17±0.16 <sup>a,x</sup>	5.63±0.06 <sup>a,y</sup>	5.27±0.05 <sup>a,z</sup>
	BPD control	5.69±0.07 <sup>b,w</sup>	4.92±0.05 <sup>c,x</sup>	4.73±0.05 <sup>b,xy</sup>	4.53±0.08 <sup>c,xy</sup>	4.51±0.08 <sup>b,y</sup>
	0.5% Eugenol	4.74±0.13 <sup>cd,x</sup>	4.19±0.17 <sup>de,y</sup>	3.93±0.08 <sup>cd,y</sup>	3.85±0.16 <sup>de,y</sup>	3.95±0.08 <sup>c,y</sup>
	1% Eugenol	4.42±0.23 <sup>def,x</sup>	4.37±0.07 <sup>d,x</sup>	4.11±0.05 <sup>c,x</sup>	3.48±0.17 <sup>e,y</sup>	3.48±0.26 <sup>d,y</sup>
	2% Eugenol	4.12±0.27 <sup>f,x</sup>	3.75±0.15 <sup>e,xy</sup>	3.62±0.22 <sup>d,y</sup>	3.46±0.18 <sup>e,y</sup>	3.31±0.24 <sup>d,y</sup>
	3% Pectin	5.55±0.14 <sup>b,w</sup>	5.49±0.17 <sup>b,xw</sup>	5.06±0.05 <sup>b,xy</sup>	5.03±0.11 <sup>b,y</sup>	4.55±0.14 <sup>b,z</sup>
	0.5% Eugenol +3% Pectin	4.90±0.13 <sup>c,w</sup>	4.31±0.37 <sup>d,x</sup>	4.07±0.12 <sup>cd,xy</sup>	4.05±0.14 <sup>d,xy</sup>	3.73±0.19 <sup>cd,y</sup>
	1% Eugenol + 3% Pectin	4.61±0.18 <sup>cde,x</sup>	4.41±0.16 <sup>d,x</sup>	4.20±0.06 <sup>c,xy</sup>	3.83±0.19 <sup>de,y</sup>	3.57±0.12 <sup>cd,y</sup>
2% Eugenol + 3% Pectin	4.22±0.33 <sup>ef,w</sup>	3.85±0.17 <sup>e,wx</sup>	3.65±0.13 <sup>d,xy</sup>	3.47±0.15 <sup>e,xy</sup>	3.37±0.23 <sup>d,y</sup>	

<sup>1</sup> n=5 replicates per treatment per day per trial. Values (Log CFU/sample) presented as mean ± standard error of the mean. Within the same trial, different superscript a-f in columns and w-z in rows differ significantly at P<0.05.



**Table 3:** The efficacy of eugenol (0, 0.5, 1 or 2%), chitosan (0 or 2%) and their combinations as coating treatment to reduce *C. jejuni* on chicken wingettes<sup>1</sup>

Trial no.	Treatments	Day 0	Day 1	Day 3	Day 5	Day 7
1	Baseline	6.09±0.08 <sup>a,x</sup>	5.78±0.09 <sup>a,x</sup>	5.85±0.11 <sup>a,x</sup>	5.89±0.11 <sup>a,x</sup>	5.93±0.12 <sup>a,x</sup>
	BPD control	5.08±0.03 <sup>b,x</sup>	4.98±0.12 <sup>b,x</sup>	4.88±0.15 <sup>b,x</sup>	4.90±0.10 <sup>b,x</sup>	4.82±0.08 <sup>b,x</sup>
	0.5% Eugenol	4.28±0.09 <sup>c,x</sup>	4.30±0.10 <sup>cd,x</sup>	4.07±0.06 <sup>c,x</sup>	4.20±0.06 <sup>c,x</sup>	4.16±0.09 <sup>c,x</sup>
	1% Eugenol	3.88±0.41 <sup>cd,x</sup>	4.24±0.08 <sup>cd,x</sup>	3.95±0.23 <sup>c,x</sup>	4.05±0.15 <sup>c,x</sup>	4.15±0.08 <sup>c,x</sup>
	2% Eugenol	3.81±0.07 <sup>cd,x</sup>	3.92±0.27 <sup>cde,x</sup>	3.67±0.21 <sup>c,x</sup>	3.56±0.32 <sup>c,x</sup>	2.61±0.51 <sup>d,y</sup>
	Acetic acid control	6.00±0.12 <sup>a,x</sup>	4.81±0.06 <sup>b,y</sup>	5.07±0.08 <sup>b,y</sup>	4.97±0.05 <sup>b,y</sup>	4.83±0.12 <sup>b,y</sup>
	2% Chitosan	3.94±0.10 <sup>cd,x</sup>	3.82±0.27 <sup>def,x</sup>	3.95±0.07 <sup>c,x</sup>	3.97±0.14 <sup>c,x</sup>	3.71±0.14 <sup>c,x</sup>
	0.5% Eugenol +2% Chitosan	3.50±0.16 <sup>de,x</sup>	3.24±0.29 <sup>efg,xy</sup>	3.66±0.20 <sup>c,x</sup>	3.83±0.31 <sup>c,x</sup>	2.75±0.61 <sup>d,y</sup>
	1% Eugenol + 2% Chitosan	2.93±0.17 <sup>e,y</sup>	3.16±0.24 <sup>fg,xy</sup>	3.85±0.39 <sup>c,x</sup>	3.53±0.08 <sup>c,xy</sup>	2.89±0.58 <sup>d,y</sup>
	2% Eugenol + 2% Chitosan	2.91±0.20 <sup>e,x</sup>	2.86±0.25 <sup>g,x</sup>	2.85±0.58 <sup>d,x</sup>	2.66±0.50 <sup>d,x</sup>	2.45±0.48 <sup>d,x</sup>
2	Baseline	6.40±0.03 <sup>a,x</sup>	6.11±0.06 <sup>a,xy</sup>	5.32±0.22 <sup>a,z</sup>	5.62±0.10 <sup>a,y</sup>	5.87±0.02 <sup>a,xyz</sup>
	BPD control	5.69±0.09 <sup>a,x</sup>	4.98±0.06 <sup>b,xy</sup>	4.78±0.10 <sup>a,y</sup>	4.70±0.09 <sup>b,y</sup>	5.02±0.07 <sup>b,xy</sup>
	0.5% Eugenol	4.32±0.31 <sup>b,x</sup>	4.11±0.05 <sup>c,x</sup>	4.01±0.15 <sup>b,x</sup>	3.98±0.10 <sup>c,x</sup>	3.83±0.26 <sup>cd,x</sup>
	1% Eugenol	4.36±0.16 <sup>b,x</sup>	3.80±0.27 <sup>c,x</sup>	4.01±0.08 <sup>b,x</sup>	3.95±0.08 <sup>c,x</sup>	3.81±0.31 <sup>cd,x</sup>
	2% Eugenol	3.34±0.25 <sup>cd,xy</sup>	2.99±0.23 <sup>de,y</sup>	3.30±0.28 <sup>bcd,xy</sup>	3.78±0.11 <sup>cd,x</sup>	3.22±0.67 <sup>de,xy</sup>
	Acetic acid control	5.79±0.07 <sup>a,x</sup>	5.00±0.07 <sup>b,y</sup>	4.79±0.09 <sup>a,y</sup>	5.00±0.09 <sup>ab,y</sup>	5.16±0.06 <sup>ab,xy</sup>
	2% Chitosan	4.30±0.09 <sup>b,x</sup>	3.93±0.16 <sup>c,x</sup>	4.01±0.25 <sup>b,x</sup>	4.07±0.21 <sup>c,x</sup>	4.20±0.08 <sup>c,x</sup>
	0.5% Eugenol +2% Chitosan	3.95±0.19 <sup>bc,x</sup>	3.55±0.45 <sup>cd,x</sup>	3.89±0.15 <sup>bc,x</sup>	3.76±0.37 <sup>cd,x</sup>	3.87±0.33 <sup>cd,x</sup>
	1% Eugenol + 2% Chitosan	3.11±0.58 <sup>d,x</sup>	3.44±0.30 <sup>cd,x</sup>	3.26±0.20 <sup>cd,x</sup>	3.14±0.35 <sup>de,x</sup>	3.05±0.62 <sup>e,x</sup>
	2% Eugenol + 2% Chitosan	2.62±0.54 <sup>d,x</sup>	2.70±0.20 <sup>e,x</sup>	3.05±0.18 <sup>d,x</sup>	2.73±0.50 <sup>e,x</sup>	3.03±0.28 <sup>e,x</sup>

<sup>1</sup> n=5 replicates per treatment per day per trial. Values (Log CFU/sample) presented as mean ± standard error of the mean. Within the same trial, different superscript a-g in columns and x-z in rows differ significantly at P<0.05.

**Table 4:** The efficacy of eugenol (0, 0.5, 1 or 2%), pectin (0 or 3%) and their combinations as coating treatment against aerobic bacteria on chicken wingettes<sup>1</sup>

Trial no.	Treatments	Day 0	Day 1	Day 3	Day 5	Day 7
1	Baseline	4.69±0.03 <sup>a,z</sup>	4.73±0.13 <sup>a,z</sup>	5.50±0.18 <sup>a,y</sup>	6.65±0.05 <sup>a,x</sup>	7.15±0.10 <sup>a,w</sup>
	BPD control	4.39±0.07 <sup>a,z</sup>	4.43±0.17 <sup>a,z</sup>	5.23±0.20 <sup>a,y</sup>	6.46±0.21 <sup>a,x</sup>	6.98±0.06 <sup>a,w</sup>
	0.5% Eugenol	3.86±0.16 <sup>b,y</sup>	3.32±0.24 <sup>cd,z</sup>	4.03±0.11 <sup>cd,y</sup>	5.26±0.08 <sup>c,x</sup>	6.29±0.07 <sup>bc,w</sup>
	1% Eugenol	3.73±0.09 <sup>bc,z</sup>	3.69±0.12 <sup>c,z</sup>	3.71±0.22 <sup>cd,z</sup>	5.03±0.16 <sup>cd,y</sup>	6.34±0.08 <sup>bc,x</sup>
	2% Eugenol	3.42±0.07 <sup>cd,z</sup>	3.66±0.16 <sup>cd,z</sup>	3.71±0.14 <sup>cd,z</sup>	5.22±0.10 <sup>c,y</sup>	6.16±0.13 <sup>bc,x</sup>
	3% Pectin	4.35±0.11 <sup>a,z</sup>	4.43±0.18 <sup>a,z</sup>	4.73±0.18 <sup>b,z</sup>	5.99±0.12 <sup>b,y</sup>	6.53±0.22 <sup>b,x</sup>
	0.5% Eugenol +3% Pectin	3.55±0.13 <sup>bcd,z</sup>	3.47±0.14 <sup>cd,z</sup>	3.86±0.12 <sup>cd,z</sup>	5.29±0.08 <sup>c,y</sup>	6.26±0.10 <sup>bc,x</sup>
	1% Eugenol + 3% Pectin	3.42±0.10 <sup>cd,z</sup>	3.62±0.15 <sup>cd,z</sup>	3.81±0.08 <sup>cd,z</sup>	4.71±0.13 <sup>d,y</sup>	6.36±0.05 <sup>bc,x</sup>
2% Eugenol + 3% Pectin	3.29±0.18 <sup>d,z</sup>	3.27±0.22 <sup>d,z</sup>	3.57±0.16 <sup>d,z</sup>	4.19±0.10 <sup>e,y</sup>	5.98±0.19 <sup>c,x</sup>	
2	Baseline	4.56±0.08 <sup>a,z</sup>	5.32±0.12 <sup>a,y</sup>	5.99±0.17 <sup>a,x</sup>	7.05±0.19 <sup>a,w</sup>	7.05±0.12 <sup>a,w</sup>
	BPD control	4.35±0.14 <sup>a,z</sup>	5.25±0.18 <sup>a,y</sup>	6.16±0.14 <sup>a,x</sup>	6.95±0.10 <sup>a,w</sup>	7.04±0.14 <sup>a,w</sup>
	0.5% Eugenol	3.81±0.06 <sup>b,z</sup>	4.41±0.20 <sup>b,y</sup>	5.00±0.13 <sup>c,x</sup>	5.98±0.09 <sup>cd,w</sup>	6.19±0.06 <sup>bc,w</sup>
	1% Eugenol	3.68±0.23 <sup>b,z</sup>	4.15±0.28 <sup>bc,y</sup>	5.02±0.13 <sup>bc,x</sup>	5.67±0.22 <sup>d,w</sup>	6.35±0.02 <sup>bc,v</sup>
	2% Eugenol	3.68±0.11 <sup>b,y</sup>	3.34±0.19 <sup>d,y</sup>	5.05±0.14 <sup>bc,x</sup>	5.77±0.12 <sup>cd,w</sup>	6.04±0.09 <sup>c,w</sup>
	3% Pectin	4.26±0.24 <sup>a,y</sup>	5.58±0.10 <sup>a,x</sup>	5.43±0.26 <sup>b,x</sup>	6.42±0.03 <sup>b,w</sup>	6.53±0.21 <sup>b,w</sup>
	0.5% Eugenol +3% Pectin	3.84±0.06 <sup>b,y</sup>	4.01±0.06 <sup>bc,y</sup>	4.99±0.18 <sup>c,x</sup>	6.18±0.06 <sup>bc,w</sup>	6.26±0.06 <sup>bc,w</sup>
	1% Eugenol + 3% Pectin	3.55±0.11 <sup>b,y</sup>	3.80±0.10 <sup>c,y</sup>	4.96±0.08 <sup>c,x</sup>	5.77±0.11 <sup>cd,w</sup>	6.18±0.05 <sup>bc,w</sup>
2% Eugenol + 3% Pectin	2.98±0.37 <sup>c,y</sup>	3.35±0.32 <sup>d,y</sup>	4.76±0.15 <sup>c,x</sup>	5.74±0.07 <sup>d,w</sup>	5.96±0.12 <sup>c,w</sup>	

<sup>1</sup> n=5 replicates per treatment per day per trial. Values (Log CFU/sample) presented as mean ± standard error of the mean. Within the same trial, different superscript a-e in columns and v-z in rows differ significantly at P<0.05.

**Table 5:** The effect of eugenol (0, 0.5, 1 or 2%), chitosan (0 or 2%) and their combinations as coating treatment against aerobic bacteria on chicken wingettes<sup>1</sup>

Trial no.	Treatments	Day 0	Day 1	Day 3	Day 5	Day 7
1	Baseline	4.30±0.10 <sup>a, z</sup>	4.84±0.08 <sup>a, y</sup>	5.59±0.15 <sup>a, wx</sup>	5.85±0.06 <sup>a, w</sup>	5.54±0.06 <sup>ab, x</sup>
	BPD control	3.93±0.07 <sup>b, z</sup>	4.63±0.11 <sup>b, y</sup>	5.46±0.10 <sup>ab, x</sup>	5.85±0.04 <sup>a, w</sup>	5.42±0.16 <sup>b, x</sup>
	0.5% Eugenol	3.62±0.10 <sup>c, z</sup>	4.09±0.08 <sup>c, y</sup>	4.59±0.07 <sup>c, xy</sup>	5.33±0.02 <sup>b, w</sup>	4.81±0.09 <sup>cd, x</sup>
	1% Eugenol	3.28±0.11 <sup>d, z</sup>	3.99±0.09 <sup>c, y</sup>	4.58±0.05 <sup>c, y</sup>	5.35±0.03 <sup>b, w</sup>	5.00±0.11 <sup>c, x</sup>
	2% Eugenol	3.32±0.18 <sup>cd, y</sup>	3.94±0.09 <sup>c, x</sup>	4.04±0.16 <sup>d, x</sup>	4.93±0.09 <sup>c, w</sup>	4.68±0.06 <sup>d, w</sup>
	Acetic acid control	3.96±0.07 <sup>b, z</sup>	4.46±0.06 <sup>ab, y</sup>	5.28±0.14 <sup>b, x</sup>	5.74±0.11 <sup>a, w</sup>	5.76±0.06 <sup>a, w</sup>
	2% Chitosan	3.10±0.09 <sup>d, y</sup>	3.95±0.10 <sup>c, x</sup>	4.85±0.13 <sup>c, w</sup>	4.87±0.12 <sup>c, w</sup>	4.71±0.04 <sup>cd, w</sup>
	0.5% Eugenol +2% Chitosan	2.73±0.06 <sup>e, z</sup>	3.51±0.06 <sup>d, y</sup>	4.20±0.10 <sup>d, x</sup>	4.81±0.05 <sup>c, w</sup>	4.59±0.02 <sup>d, w</sup>
	1% Eugenol + 2% Chitosan	2.53±0.19 <sup>e, y</sup>	3.83±0.05 <sup>c, x</sup>	4.08±0.25 <sup>d, x</sup>	4.63±0.09 <sup>c, w</sup>	4.53±0.05 <sup>d, w</sup>
	2% Eugenol + 2% Chitosan	2.61±0.18 <sup>e, z</sup>	3.23±0.15 <sup>d, y</sup>	3.54±0.15 <sup>e, x</sup>	3.96±0.09 <sup>d, w</sup>	3.94±0.13 <sup>e, w</sup>
2	Baseline	4.79±0.20 <sup>a, y</sup>	5.38±0.03 <sup>a, x</sup>	5.52±0.05 <sup>a, x</sup>	5.77±0.02 <sup>a, x</sup>	6.61±0.05 <sup>a, w</sup>
	BPD control	4.50±0.11 <sup>a, y</sup>	5.31±0.21 <sup>a, x</sup>	5.34±0.06 <sup>a, x</sup>	5.63±0.03 <sup>a, x</sup>	6.49±0.08 <sup>a, w</sup>
	0.5% Eugenol	3.74±0.10 <sup>bc, y</sup>	4.79±0.11 <sup>b, x</sup>	4.83±0.07 <sup>b, x</sup>	5.01±0.08 <sup>b, x</sup>	5.65±0.04 <sup>b, w</sup>
	1% Eugenol	3.99±0.17 <sup>b, y</sup>	4.22±0.24 <sup>cd, y</sup>	4.75±0.07 <sup>bc, x</sup>	4.97±0.12 <sup>b, w</sup>	5.58±0.06 <sup>b, w</sup>
	2% Eugenol	3.71±0.13 <sup>bc, y</sup>	3.73±0.16 <sup>e, y</sup>	4.53±0.08 <sup>bc, x</sup>	4.96±0.06 <sup>b, x</sup>	5.45±0.17 <sup>b, w</sup>
	Acetic acid control	4.58±0.16 <sup>a, y</sup>	5.34±0.12 <sup>a, x</sup>	5.31±0.17 <sup>a, x</sup>	5.60±0.05 <sup>a, x</sup>	6.26±0.17 <sup>a, w</sup>
	2% Chitosan	3.42±0.16 <sup>ed, z</sup>	4.52±0.14 <sup>bc, x</sup>	4.51±0.11 <sup>bc, x</sup>	4.75±0.05 <sup>b, wx</sup>	5.21±0.14 <sup>b, w</sup>
	0.5% Eugenol +2% Chitosan	3.14±0.14 <sup>de, y</sup>	4.75±0.23 <sup>b, x</sup>	4.70±0.15 <sup>bc, x</sup>	4.95±0.08 <sup>b, wx</sup>	5.34±0.16 <sup>b, w</sup>
	1% Eugenol + 2% Chitosan	2.92±0.22 <sup>e, z</sup>	3.98±0.24 <sup>de, y</sup>	4.31±0.13 <sup>cd, y</sup>	4.80±0.19 <sup>b, x</sup>	5.33±0.32 <sup>b, w</sup>
	2% Eugenol + 2% Chitosan	2.90±0.17 <sup>e, y</sup>	2.79±0.51 <sup>f, y</sup>	3.94±0.10 <sup>d, x</sup>	4.15±0.18 <sup>c, x</sup>	4.71±0.32 <sup>c, w</sup>

<sup>1</sup> n=5 replicates per treatment per day per trial. Values (Log CFU/sample) presented as mean ± standard error of the mean. Within the same trial, different superscript a-e in columns and w-z in rows differ significantly at P<0.05.

**Table 6:** The effect of 0.0125% MMW chitosan and 0.0125% eugenol on the expression of *C. jejuni* genes essential for survival and virulence

Gene	Gene product function	Relative gene expression (Log <sub>10</sub> RQ) <sup>1</sup>				
		Treatments				
		Control	Acetic Acid	Chitosan	Eugenol	Eugenol + Chitosan
<i>motA</i>	Motility	0 <sup>a</sup>	-0.09±0.03 <sup>a</sup>	-0.35±0.17 <sup>b</sup>	-0.51±0.15 <sup>b</sup>	-0.32±0.11 <sup>b</sup>
<i>motB</i>	Motility	0 <sup>b</sup>	0.13±0.09 <sup>b</sup>	0.21±0.03 <sup>a</sup>	-0.13±0.06 <sup>c</sup>	0.24±0.08 <sup>a</sup>
<i>fliA</i>	Motility	0 <sup>a</sup>	0.09±0.04 <sup>a</sup>	0.08±0.09 <sup>a</sup>	-0.04±0.02 <sup>a</sup>	0.13±0.10 <sup>a</sup>
<i>cetA</i>	Energy taxis protein/motility	0 <sup>b</sup>	0.08±0.14 <sup>b</sup>	0.13±0.07 <sup>b</sup>	0.05±0.06 <sup>b</sup>	0.20±0.08 <sup>a</sup>
<i>cetB</i>	Energy taxis protein/motility	0 <sup>a</sup>	-0.05±0.14 <sup>a</sup>	-0.05±0.29 <sup>a</sup>	-0.02±0.10 <sup>a</sup>	0.08±0.36 <sup>a</sup>
<i>cadF</i>	Attachment	0 <sup>a</sup>	0.11±0.06 <sup>a</sup>	0.08±0.09 <sup>a</sup>	-0.10±0.04 <sup>a</sup>	0.10±0.09 <sup>a</sup>
<i>ciaB</i>	Attachment	0 <sup>b</sup>	0.18±0.16 <sup>ab</sup>	0.25±0.02 <sup>a</sup>	-0.02±0.08 <sup>b</sup>	0.32±0.10 <sup>a</sup>
<i>jlpA</i>	Attachment	0 <sup>c</sup>	0.09±0.06 <sup>c</sup>	0.43±0.08 <sup>b</sup>	0.08±0.15 <sup>c</sup>	0.55±0.03 <sup>a</sup>
<i>sodB</i>	Superoxide dismutase	0 <sup>b</sup>	-0.03±0.04 <sup>b</sup>	0.20±0.04 <sup>a</sup>	0.08±0.10 <sup>b</sup>	0.19±0.04 <sup>a</sup>
<i>katA</i>	Catalase/oxidative stress	0 <sup>a</sup>	-0.01±0.05 <sup>a</sup>	0.14±0.12 <sup>a</sup>	-0.23±0.11 <sup>b</sup>	-0.18±0.06 <sup>b</sup>
<i>luxS</i>	Quorum sensing	0 <sup>a</sup>	0.05±0.11 <sup>a</sup>	0.09±0.06 <sup>a</sup>	-0.29±0.11 <sup>b</sup>	-0.23±0.08 <sup>b</sup>
<i>racS</i>	Two-component sensor/ histidine kinase	0 <sup>a</sup>	0.06±0.12 <sup>a</sup>	0.09±0.21 <sup>a</sup>	-0.06±0.07 <sup>a</sup>	0.05±0.30 <sup>a</sup>
<i>racR</i>	Two-component regulator	0 <sup>a</sup>	0.14±0.16 <sup>a</sup>	0.06±0.27 <sup>a</sup>	-0.11±0.08 <sup>a</sup>	-0.02±0.06 <sup>a</sup>

<sup>1</sup> n=6 replicates per treatment. Values (mean ± standard error of the mean) with different superscripts within a row indicates significant change in gene expression (P<0.05).



March 26, 2018

**MEMORANDUM**

**TO:** Dr. Dan Donoghue

**FROM:** Bob Beitle, Acting Biosafety Committee Chair

**RE:** Protocol Renewal

**PROTOCOL #:** 06021

**PROTOCOL TITLE:** Reducing Food Borne Pathogens in Poultry

**APPROVED PROJECT PERIOD:** Start Date February 14, 2006 Expiration Date February 13, 2021

The Institutional Biosafety Committee (IBC) has approved your request, dated January 26, 2018, to renew IBC # 06021, "Reducing Food Borne Pathogens in Poultry".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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*The University of Arkansas is an equal opportunity/affirmative action institution.*

**PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:**

List all personnel (including PI and Co-PI) to be involved in this project:

**Name:** (first and last) - **POSITION** (Title, academic degrees, certifications, and material field of expertise)

**QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE**  
Describe previous work or training with biohazardous and/or recombinant DNA and include Biosafety Levels)

**Example:**

Bob Biohazard - Associate Professor, PhD Microbiology

14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.

Dan J. Donoghue, Ph.D. Professor, Food Safety

over 20 years experience as PI running research laboratory working with BL-2 pathogens.

Ann M. Donoghue, Ph.D. Research Leader, Food safety

over 20 years experience as PI running research laboratory working with BL-2 pathogens.

Komala Arsi, Ph. D, Post-doctoral fellow

Post-doctoral fellow trained under Dr. Donoghue's supervision

Abhinav Upadhyay, Ph.D. Post-doctoral fellow

Post-doctoral fellow trained under Dr. Donoghue's supervision

Indu Upadhyaya, Ph. D. Post-doctoral fellow

Post-doctoral fellow trained under Dr. Donoghue's supervision

Basanta Raj Wagle, Graduate Student

Graduate assistant trained under Dr. Donoghue's supervision

Sandip Shreshta, Graduate Student

Graduate assistant trained under Dr. Donoghue's supervision

Additional Personnel Information (if needed):

## **Chapter 4**

**The natural plant compounds, *trans*-cinnamaldehyde, eugenol or carvacrol reduce  
*Campylobacter jejuni* biofilms on poultry plant processing surfaces**

**RUNNING TITLE: PHYTOCHEMICALS REDUCE *C. JEJUNI* BIOFILM**

**The natural plant compounds, *trans*-cinnamaldehyde, eugenol or carvacrol reduce  
*Campylobacter jejuni* biofilms on poultry plant processing surfaces**

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## ABSTRACT

*Campylobacter jejuni* is the leading cause of human foodborne illness globally and is strongly linked with the consumption of contaminated poultry products. Several studies have shown that *C. jejuni* can form sanitizer tolerant biofilms but limited research has been conducted to develop effective control strategies against *C. jejuni* biofilms. This study investigated the efficacy of three Generally Recognized as Safe (GRAS) status phytochemicals namely, *trans*-cinnamaldehyde (TC), eugenol (EG) or carvacrol (CR) in inhibiting *C. jejuni* biofilm formation and inactivating mature biofilm on common food contact surfaces at 20 or 37°C. For the inhibition study, *C. jejuni* NCTC 11168 was grown either in the presence or absence (control) of sub-inhibitory concentrations of TC (0.01%), EG (0.01%) or CR (0.002%) for 48 h and the effect of phytochemicals on biofilm formation was quantified at 24 h intervals. For the inactivation study, *C. jejuni* biofilms developed at 20 or 37°C for 48 h were exposed to the phytochemicals (0, 0.25, 0.5, or 1%) for 1, 5, or 10 min, and surviving *C. jejuni* in the biofilm were enumerated. In addition, the effect of phytochemicals on biofilm architecture and expression of genes and proteins essential for biofilm formation was evaluated. All the studies were conducted three times with duplicate samples. The data were analyzed by the PROC MIXED procedure of SAS. All phytochemicals reduced *C. jejuni* biofilm formation as well as inactivated mature biofilm on polystyrene and steel surface at both temperatures ( $P < 0.05$ ). The highest dose (1%) of TC, EG and CR rapidly inactivated biofilm within 10 min to the below detection limit ( $> 7$  Log reductions; detection limits 1 Log CFU/mL) at 20°C on steel surface. The lowest dose (0.25%) of all phytochemicals reduced counts significantly ( $> 3$  Log CFU/mL) in the broth medium when treated for 1 min at 20°C on a steel surface. The genes encoding for motility systems (*flaA*, *flaB*, *flgA*) were downregulated by all phytochemicals ( $P < 0.05$ ). In addition, the expression of stress response (*cosR*, *ahpC*) and cell

surface modifying (*waaF*) genes was reduced by 0.01% EG. LC-MS/MS based proteomic analysis revealed that TC (0.01%), EG (0.01%) and CR (0.002%) significantly downregulated the expression of NapA protein (required for signaling pathway during oxidative stress). The expression of DnaK (chaperone protein) and bacterioferritin required for biofilm formation were also reduced by TC and CR. Scanning electron microscopy revealed disruption of biofilm architecture and loss of extracellular polymeric substances after phytochemical treatment. Results suggest that TC, EG, and CR could be used as a natural disinfectant for controlling *C. jejuni* biofilms.

**Keywords:** *C. jejuni*, biofilm, phytochemicals, inhibition, inactivation, gene/protein expression

## INTRODUCTION

*Campylobacter* is one of the major foodborne pathogens that causes gastroenteritis in humans worldwide (Marder et al., 2017). Recent reports have shown that the incidence of *Campylobacter* infections was increased by 10% in 2017 compared to 2014-2016 with an annual incidence of 17.83 per 100,000 people (Marder et al., 2017). Among the major *Campylobacter* species, *Campylobacter jejuni* is responsible for approximately 90% of the reported campylobacteriosis cases in humans (Cody et al., 2013). In addition, *C. jejuni* infections have been associated with the occurrence of Guillain-Barré syndrome and reactive arthritis causing significant economic losses and disease burden globally (Spiller, 2007; Gradel et al., 2009; Hoffmann et al., 2011).

The primary source of human *C. jejuni* infections is the handling and/or consumption of contaminated poultry products (Rosner et al., 2017). The survival of *C. jejuni* in the environment such as water, feed, sewage, and flies play a critical role in *C. jejuni* colonization in the birds by 3<sup>rd</sup> week of age and subsequently contaminating poultry products during processing (Annan-Prah and Janc, 1988; Dhillon et al., 2006; Boysen et al., 2016). In addition, *C. jejuni* survives in the processing plant by forming biofilms, which could further contribute to contamination of poultry meat from the environment.

A biofilm is an assemblage of surface-associated microbial communities embedded within the matrix of an extracellular polymeric substances (EPS) (Donlan, 2002; Donlan and Costerton, 2002). The ability of *C. jejuni* to form biofilm has been demonstrated on different surfaces including plastic, glass and steel under different oxygen concentrations (Trachoo et al., 2002; Reuter et al., 2010; Bronowski et al., 2014; Brown et al., 2014; Bronnec et al., 2016). Moreover, studies have demonstrated that *C. jejuni* biofilm formation can be enhanced by atmospheric

oxygen and chicken meat juice (Reuter et al., 2010; Brown et al., 2014). The formation of bacterial biofilm begins with initial attachment of bacteria to surface. The surface attachment strengthens, and the bacterial community quickly becomes irreversibly attached to the target surface. This is followed by maturation of the biofilm and dispersion of bacteria to new location (Donlan and Costerton, 2002). A number of genes that contribute to biofilm formation has been characterized in *C. jejuni* (Bronowski et al., 2014). Several genes coding for motility complex (Joshua et al., 2006; Kalmokhoff et al., 2006; Kim et al., 2015) are essential for biofilm formation. These include *flaA*, *flaB*, *flaC*, *flaG*, *fliA*, *fliS*, *flgA* and *flhA*. In addition, genes encoding stress response (*spoT*, *csrA*, *ahpC*, *cosR*, *cprS*) (Fields and Thompson, 2008; McLennan et al., 2008; Svensson et al., 2009; Oh and Jeon, 2014; Turonova et al., 2015), bacterial cell surface modifications (*peb4*, *waaF*) (Asakura et al., 2007; Naito et al., 2010) and quorum sensing (*luxS*) (Reeser et al., 2007) are also critical for biofilm formation and maturation in *C. jejuni*.

One of the critical components of bacterial biofilm is the EPS which protects underlying bacterial population from harsh environmental conditions. The impermeability of EPS along with the slower growth rates and metabolism of bacteria in the biofilms makes them more resistant to disinfectants, antimicrobials and antibiotics (Reuter et al., 2010; Borges et al., 2016). In a recent study, *C. jejuni* in biofilms exhibits up to 32 fold higher resistance to gentamicin than in the corresponding planktonic forms (Malik et al., 2017). In addition, biofilm facilitates *C. jejuni* to survive for longer period of time (up to 24 days) as compared to planktonic cells under aerobic conditions or submersed in water (Joshua et al., 2006; Lehtola et al., 2006). Thus, developing appropriate processing plant hygiene and sanitation is critical for controlling *C. jejuni* biofilms.

Current intervention approaches for controlling *C. jejuni* biofilms include use of chemicals (Somers et al., 1994; Trachoo et al., 2002; Melo et al., 2017), biofilm-degrading enzymes (Brown

et al., 2015; Kim et al., 2017) and application of bacteriophage (Siringan et al., 2011). Chemical disinfectants such as chlorine, trisodium phosphate and quaternary ammonium compounds have been extensively investigated for their antibiofilm efficacy against *C. jejuni*, however, these compounds have limited effectiveness in controlling *C. jejuni* biofilm, especially in the presence of organic matter (Trachoo et al., 2002; Northcutt et al., 2005; Oyarzabal, 2005). Moreover, productions of mutagens are of concern (Dore, 2015). The use of biofilm-degrading enzymes for controlling biofilms has been reported in various bacteria including *C. jejuni* (Brown et al., 2015; Kim et al., 2017). However, the efficacy of biofilm-degrading enzyme could be reduced with the production of high quantities of EPS and proteolytic activity of exoenzymes produced by the mature biofilms (Whitchurch et al., 2002). Similarly, treatment of *C. jejuni* biofilms with bacteriophages have limited application due to the emergence of resistance strains (Siringan et al., 2011). Therefore, there is an increased attention towards plant antimicrobials as potential antibiofilm compounds to control *C. jejuni*.

Phytochemicals with significant antimicrobial properties have been used as food preservatives for improving food safety since ancient time. These antimicrobials are secondary metabolites produced by plants and acts as a defense mechanism to protect plants from pathogenic microorganisms (Borges et al., 2016). They have diverse mechanisms of action thereby limiting the chance of resistance development in bacteria (Borges et al., 2016). A variety of phytochemicals have been evaluated for their antibacterial effect against foodborne pathogens and several active components have been identified (Burt, 2004; Holley and Patel, 2005). Among the various active components, *trans*-cinnamaldehyde (TC) is an aldehyde extracted from the barks of cinnamon (*Cinnamomum zeylandicum*) whereas eugenol (EG) and carvacrol (CR) are the active components of clove oil (*Eugenia caryophyllus*) and oregano oil (*Origanum glandulosum*) respectively. All the

aforementioned phytochemicals have broad-spectrum antimicrobial activity against bacteria, virus and fungi, and are classified as Generally Recognized as Safe by the United States Food and Drug Administration (21 Code of Federal Regulation part 172.515) (Adams et al., 2004, 2005; Knowles et al., 2005).

In this study, we investigated the efficacy of TC, EG, and CR in inhibiting the biofilm formation and inactivating mature biofilms of *C. jejuni* at two temperatures (20°C and 37°C) and on two different surfaces (polystyrene plates and stainless steel coupons) commonly encountered in processing plant. The ability of phytochemicals to reduce *C. jejuni* biofilm formation was determined by using the sub-inhibitory concentrations (SICs) of TC, EG and CR whereas the inactivation of mature *C. jejuni* biofilm was investigated by using doses (0.25, 0.5 and 1%) above the minimum bactericidal concentrations. Moreover, the effect of SICs of TC, EG and CR on the transcription of genes and proteins of *C. jejuni* critical for biofilm formation was determined. In addition, the effect of treatments on *C. jejuni* biofilm architecture was visualized using scanning electron microscopy and confocal laser scanning microscopy.

## **MATERIALS AND METHODS**

### ***C. jejuni* strain and culture conditions**

*C. jejuni* NCTC 11168 strain was cultured in 10 mL of *Campylobacter* enrichment broth (CEB; International Diagnostics Group, Bury, Lancashire, UK) and incubated under microaerophilic condition (5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>) at 42°C for 48 h. Following the growth, *C. jejuni* was centrifuged and washed twice with Butterfield's phosphate diluent (BPD, 0.625 mM potassium dihydrogen phosphate, pH 7.2) and resuspended in CEB to use as inoculum.

### ***Preparation of chicken meat juice***

A previously published method was used for the preparation of chicken meat juice (Birk et al., 2004). Briefly, frozen whole chickens were obtained from the University of Arkansas poultry pilot processing plant (Fayetteville, AR) and thawed overnight at 4°C. The meat juice was collected and centrifuged at 4,000 rpm for 20 min to remove debris followed by filter sterilization (0.2µm cellulose acetate membrane; VWR International, USA). Based on published literature (Brown et al., 2014) and growth curve analysis, chicken meat juice was added to broth (CEB) at 5% level and used for biofilm experiments.

### ***Determination of C. jejuni biofilm formation on polystyrene plates***

The biofilm formation of *C. jejuni* on polystyrene plates was determined according to a previously published method (Brown et al., 2014). Briefly, 200 µL of CEB broth containing *C. jejuni* (~ 6.0 Log CFU) was added to 96-well polystyrene plates and incubated for 48 h at 20 or 37°C under aerobic condition to facilitate biofilm formation. The biofilm formation was determined by 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining at 24 h intervals. After staining, TTC solution was removed followed by air-drying and bound TTC dye was dissolved in 20% acetone in ethanol, and the A<sub>500</sub> value of the solution was measured. Similar procedure was used to determine *C. jejuni* biofilm formation in broth containing 5% chicken meat juice.

### ***Biofilm inhibition and inactivation assays on polystyrene microtiter plates***

The ability of TC, EG and CR in inhibiting *C. jejuni* biofilm formation on polystyrene plates was determined according to a previously published method (Reeser et al., 2007; Lu et al., 2012). All phytochemicals were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. Two hundred microliters of culture (~ 6.0 Log CFU) was added to each well of a 96-well polystyrene plate (Corning Coster, Cambridge, Mass., USA), followed by addition of SICs of TC (0.01%), EG

(0.01%) or CR (0.002%) in the broth medium (CEB). The plates were incubated at 20 or 37°C for 48 h. Similar procedure was followed to test the antibiofilm efficacy of phytochemicals in the presence of 5% chicken meat juice. The biofilm formation was determined at 24 h intervals by enumeration of *C. jejuni* in the biofilm. At each time points, the medium was removed and the well was washed gently three times with BPD. The bacteria in the biofilms were removed using cell scraper and plated on *Campylobacter* line agar (CLA; Line, 2001). The number of biofilm associated *C. jejuni* were enumerated after incubation at 42°C for 48 h.

The inactivation of mature *C. jejuni* biofilms by TC, EG and CR was determined as described previously (Kim et al., 2017). Briefly, *C. jejuni* (~ 6.0 Log CFU) was inoculated in sterile 96-well polystyrene plate in the presence or absence of chicken meat juice in broth medium (200 µL) and incubated at 20 or 37°C for 48 h. After mature biofilm was formed, the inactivation was carried out with 200 µL of 0, 0.25, 0.5 or 1% of TC, EG or CR in BPD for 1, 5 or 10 min. The treatment solution was discarded and 200 µL of Dey-Engley neutralizing broth (Difco Laboratories, Sparks, MD, USA) was added. The number of surviving *C. jejuni* in the biofilm was determined as described above.

#### ***Preparation of stainless steel coupons***

A previously described method (Jeong et al., 1994) was used for the preparation of stainless steel coupons (Type 304; diameter 5/8 inch; no. 4 finish). Briefly, steel coupons were cleaned with acetone followed by washing in distilled water and then soaking in 100% ethanol. Finally, steel coupons were rinsed with distilled water, subjected to air dry and autoclaved at 121°C for 15 min.

#### ***Biofilm inhibition and inactivation assays on stainless steel coupons***

To determine the effect of TC, EG and CR in inhibiting biofilm formation and inactivating mature biofilm on stainless steel, a published method was used with slight modifications (Trachoo



et al., 2002). For the inhibition study, steel coupons were incubated with 1mL of *C. jejuni* (~6.0 Log CFU) in 24-well polystyrene plates containing SICs of phytochemicals at 20 or 37°C for 48 h. *C. jejuni* counts in the biofilms on steel coupons were determined after washing three times with BPD at 24 h intervals.

For the inactivation of mature biofilm on steel coupons, mature biofilm was developed on steel coupons placed in 24-well polystyrene plates containing *C. jejuni* (~6.0 Log CFU) at 20 or 37°C for 48 h. After biofilm formation, steel coupons were rinsed three times with BPD and transferred in new polystyrene plates and exposed to 0.25, 0.5 or 1% dose of TC, EG or CR for 1, 5 or 10 min. Following rinsing with BPD, the steel coupons were placed in 50 mL centrifuged tubes containing 3 g sterile glass beads (Diameter 2 mm; Thermo Fisher Scientific, Carlsbad, CA, USA) and 10 mL of Dey-Engley neutralizing broth, and vortexed for 1 min. The solution was serially diluted and plated on CLA. *C. jejuni* counts were enumerated after incubation of plates at 42°C for 48 h. Similar inhibition and inactivation studies were conducted in the presence of 5% chicken meat juice in broth medium.

### ***Microscopic examination of C. jejuni biofilms***

Environmental scanning electron microscopy (ESEM) and confocal laser scanning microscopy (CLSM) were used to visualize the effect of TC, EG and CR on biofilm architecture and the viability of *C. jejuni* in biofilms. Biofilms were developed on stainless steel coupons and Lab-Tek two-chamber (no. 1) borosilicate coverglass system (Nunc, Rochester, NY, USA) for ESEM and CLSM respectively at 37°C for 2 days, and exposed to 0.25 % of TC, EG or CR for 10 min. All the samples were rinsed with BPD before further processing. For ESEM, samples were fixed with 2.5% glutaraldehyde in 0.1 M PIPES buffer for 1 h, as described previously (Brown et al., 2013). After fixation, samples were rinsed three times with PIPES buffer and dehydrated in a

series of ethanol solutions (at 30, 40, 50, 60, 70, 80, 90, and three times at 100%) for at least 10 min for each step. The biofilms were dried and coated with gold using Emitech SC7620 sputter coater (Quorum Technologies, Ltd., East Sussex, UK) for 135 s. The coated biofilm samples were visualized using SE detectors at 10 kV beam (Philips XL30 ESEM, FEI Company, Hillsboro, OR). For the CLSM, the viability of *C. jejuni* in the biofilms were determined using FilmTracer™ Live/Dead Biofilm Viability Kit (Molecular probes, OR) according to a published method (Asakura et al., 2007). SYTO-9 and propidium iodide stains were used for the differential staining of live and dead cells. After staining for 20 min, biofilms were visualized in each chamber using hybrid detector at 63X objective in the Leica SP5 Confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL).

#### ***Gene expression analysis of C. jejuni exposed to phytochemical treatments***

The effect of TC, EG and CR on the expression of *C. jejuni* genes essential for biofilm formation was determined using real-time quantitative PCR (RT-qPCR) (Upadhyay et al., 2017; Wagle et al., 2017). Briefly, *C. jejuni* (~6.0 Log CFU/mL) was incubated in the presence or absence of SICs of TC, EG or CR at 37°C for 12 h. The total RNA was extracted using RNA mini kit (Invitrogen, Carlsbad, CA, USA) and complementary DNAs were prepared using iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., CA, USA). The primers were designed using Primer 3 Software (National Center for Biotechnology Information, Bethesda, MD) and obtained from Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). The specificity of primer was tested using NCBI-Primer BLAST, melt curve analysis and *in silico* PCR (Bikandi et al., 2004). The amplified products were detected by using SYBR Green reagents (Bio-Rad Laboratories, Inc.). The 16s rRNA gene was used as the endogenous control and comparative critical threshold ( $\Delta\Delta Ct$ )

method was employed to analyze relative expressions of candidate genes on Quant Studio 3 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific).

### ***Proteomic analysis of C. jejuni in biofilms exposed to phytochemical treatments***

The effect of TC, EG and CR on the proteome of *C. jejuni* in the biofilms was determined using liquid chromatography with tandem mass spectrometry (LC-MS/MS) as described previously (Miyamoto et al., 2015). Briefly, *C. jejuni* (~6.0 Log CFU/mL) was incubated in the presence or absence of SICs of phytochemicals and allowed to develop biofilms at 37°C for 48 h. Following washing with the buffer, proteins were extracted using B-Per<sup>®</sup> bacterial protein extraction reagent (Thermo Fisher) and subjected to SDS-PAGE (4-12% Bis-Tris protein gel, Thermo Fisher). Each lane of gel was excised and destained with 50% acetonitrile in ammonium bicarbonate (Thermo Fisher) for 45 min and vacuum dried for 10 min. The protein extracts were treated with dithiothreitol (1.5 mg/mL in 25 mM ammonium bicarbonate; Bio-Rad) and reduced with iodoacetamide (37 mg/mL in ammonium bicarbonate; Bio-Rad) for 1 h. Following removal of iodoacetamide, the proteins were digested with trypsin (20 ng per  $\mu$ L in 25 mM ammonium bicarbonate) and incubated overnight at 37°C. The resultant peptides were analyzed using LC-MS/MS and the proteins were identified by matching sequences derived from MS spectra with *C. jejuni* protein sequences available in the online database.

### ***Statistical analyses***

The study was a completely randomized design with duplicate samples and the study was repeated three times. The data for each treatment and control were pooled from three independent trials within the same study before analysis. Bacterial counts were logarithmic transferred to maintain the homogeneity of variance (Byrd et al., 2001) and the data were analyzed by using PROC MIXED procedure in the SAS version 9.3 software (SAS Institute Inc., Cary, NC).

Student's *t* test was used for comparisons between treatment and control in the gene expression and proteomic studies. The treatment means were separated by least-square means analysis and the significance levels was  $P < 0.05$  for statistical difference.

## RESULTS

### *Effect of chicken juice on C. jejuni biofilm formation on polystyrene plates*

Figure 1 shows the *C. jejuni* biofilm formation on polystyrene plates in the presence or absence of 5% chicken meat juice. The presence of chicken juice significantly enhanced *C. jejuni* biofilm formation at both temperatures. Besides TTC staining, enumerated counts in the biofilms resulted in no significant difference in *C. jejuni* counts between broth medium and 5% chicken meat juice at both time points (Data not shown). In addition, the absorbance value which estimates the EPS was significantly higher in the biofilms develop for 48 h at 37°C than at 20°C (Fig. 1), however, *C. jejuni* counts in the biofilms at both temperatures was not significantly different (Data not shown).

### *Effect of sub-inhibitory concentrations of TC, EG and CR on C. jejuni biofilm formation on polystyrene microtiter plates and stainless steel coupons*

Figure 2 shows the effect of TC, EG and CR on *C. jejuni* biofilm formation on polystyrene plates at 20 and 37°C in the presence and absence of chicken juice. In the broth medium, biofilm in the control had ~ 7.3 and 8 Log CFU/mL of *C. jejuni* at 20 (Fig. 2A1) and 37°C (Fig. 2A2), respectively. At 20°C, the SICs of TC, EG and CR significantly reduced *C. jejuni* counts in the biofilm by ~ 0.5 and 0.7 Log CFU/mL respectively at 24 and 48 h. At 37°C, the reduction was ~ 0.56 Log CFU/mL at both time points ( $P < 0.05$ ). Similar results were observed in the presence of chicken meat juice at both temperatures (Fig. 2B1-2) where the three phytochemicals reduced *C. jejuni* biofilm formation by ~0.5 Log CFU/mL. Moreover, CR was the most effective treatment

and reduced *C. jejuni* in the biofilm by ~ 1.5 and ~ 0.75 Log CFU/mL respectively at 20°C and 37°C at the end of 48 h as compared to respective control (Fig. 2B1-2).

The effect of TC, EG and CR in inhibiting *C. jejuni* biofilm formation on stainless steel coupons is shown in Figure 3. All phytochemicals reduced *C. jejuni* in the biofilm by ~ 0.5 Log CFU/mL at 20 (Fig. 3A1) and 37°C (Fig. 3A2) at both time points ( $P < 0.05$ ). Similar reductions were observed when biofilm was developed in the presence of 5% chicken meat juice on steel coupons (Fig. 3B1-2). Although phytochemicals were effective in reducing *C. jejuni* counts as compared to respective controls at 24 and 48 h, the phytochemicals did not inhibit the growth of *C. jejuni* biofilm at 48 h as compared to 24 h.

***Effect of TC, EG and CR on mature C. jejuni biofilms developed on polystyrene microtiter plates and stainless steel coupons***

The effect of TC, EG and CR in inactivating mature *C. jejuni* biofilms on polystyrene plates and steel coupons is shown in Tables 2 and 3 respectively. The number of *C. jejuni* recovered from control (biofilms not subjected to treatments) on polystyrene plates was ~ 7.8 and ~8.4 Log CFU/mL respectively at 20 and 37°C in the broth medium (Table 2). Similar counts were observed in the presence of 5% chicken meat juice on polystyrene plates ( $P > 0.05$ ). At 20°C on polystyrene, 1% TC, EG and CR reduced the counts of *C. jejuni* to below detection limit (reductions  $> 7.0$  Log CFU/mL) in the broth medium within 1 min of exposure time. Lower doses (0.25 and 0.5%) of TC, EG and CR reduced *C. jejuni* in the biofilms in a range from 3.2 to 3.8 Log CFU/mL in 10 min. The phytochemicals were effective in inactivating *C. jejuni* biofilms in the presence of chicken juice as well. For example, in the presence of chicken juice at 20°C, 1% CR reduced *C. jejuni* counts by 6.75 Log CFU/mL (most effective treatment) followed by 0.5% CR or 1% EG (reductions ~ 3.8 Log CFU/mL) in 10 min exposure time. At 37°C in the broth medium, 1% EG

or CR was the most effective in reducing *C. jejuni* counts by ~3.75 Log CFU/mL at the end of 10 min. The reductions were similar in the presence of chicken juice where 1% TC, EG and CR reduced the counts by 1.64, 4.11 and 4.88 Log CFU/mL respectively in 10 min.

On stainless steel coupons, *C. jejuni* counts in the control biofilms developed at 20°C were ~ 6.3 Log CFU/mL. The biofilms developed at 37°C had ~7.8 Log CFU/mL of *C. jejuni* present (Table 3). In the biofilms developed at 20°C in the broth medium, 0.5 and 1% EG or CR reduced the counts of *C. jejuni* to below detection limit as early as 1 min of treatment time. TC (0.5, 1%) treatments reduced the counts to below detection limit within 5 min of treatment. The 0.25% of EG or CR also reduced the counts below detection within 5 min (reductions >6.35 Log CFU/mL) whereas 0.25% TC significantly reduced the counts by ~ 4.5 Log CFU/mL in 10 min exposure time. We observed similar results when the biofilms were developed in the presence of 5% chicken meat juice and exposed to 0.5 or 1% TC, EG and CR for 10 min. In addition, 0.25% of TC, EG and CR reduced *C. jejuni* counts significantly by 2.4, 5.2 and 6.05 Log CFU/mL respectively.

At 37°C in the broth medium, 0.5 or 1% CR or 1% EG was the most effective and reduced the counts below detection limit in 10 min. In addition, TC at 0.25, 0.5 and 1% reduced *C. jejuni* counts by 2.8, 3.7 and 4 Log CFU/mL respectively in 10 min exposure time. Similar results were observed in the presence of chicken juice where 0.5 or 1% of CR and 1% EG were the most effective treatments and reduced *C. jejuni* counts by ~ 7.7 Log CFU/mL. In addition, the antibacterial activities of the TC, EG and CR were significantly increased with an increase in exposure time on steel coupons ( $P < 0.05$ ). For example, at 37°C in the presence of chicken juice, 0.25% CR had significantly a higher reduction in 10 min than in 1 min exposure time (reductions ~ 5.5 versus 3 Log CFU/mL).

### ***Effect of phytochemicals on mature biofilms architecture and viability of C. jejuni in the biofilms***

The effect of TC, EG and CR on the biofilm architecture and viability of *C. jejuni* in the biofilms was visualized using ESEM and CLSM (Fig. 4). The biofilm structure was intact and covered with EPS in the biofilm not exposed to phytochemicals (Fig. 4A), whereas the exposure to 0.25% TC, EG, and CR for 10 min removed majority of biofilm structure (Fig. 4B, 4C and 4D respectively). In addition, confocal microscopy revealed that the majority of *C. jejuni* were live (stained green) in the control biofilms, and dead (stained red) after treatments with TC, EG and CR (Fig. 4).

### ***Effect of phytochemicals on the expression of C. jejuni genes coding for biofilm formation***

Figure 5 shows the effect of TC, EG and CR on the expression of *C. jejuni* genes critical for biofilm formation. Phytochemicals at SICs level significantly modulated the expression of genes encoding for motility, cell surface modifications, stress response and quorum sensing. The SIC of TC significantly downregulated bacterial cell mobility genes *flaA*, *flaB*, and *flgA* by ~ 11.7, 9, 4.3 folds respectively (Fig. 5A). However, quorum sensing gene (*luxS*) responsible for cell to cell communication during biofilm formation was upregulated by ~ 6 fold ( $P < 0.05$ ). The expression of stress response genes (*cosR*, *ahpC*) was not affected by TC treatment ( $P > 0.05$ ). Similar to TC, CR also downregulated motility genes *flaA*, *flaB*, *flgA* and upregulated *luxS* (Fig. 5C). The phytochemical EG downregulated (fold change  $> 2$ ) majority of the tested genes (*flaA*, *flaB*, *flaG*, *flgA*, *waaF*, *cosR*, *ahpC*) critical for *C. jejuni* biofilm formation (Fig. 5B).

### ***Effect of phytochemicals on C. jejuni biofilm proteome***

Overall, 76 proteins were identified in the proteome of *C. jejuni* present in the biofilms. Table 4 shows the differential protein expression of *C. jejuni* in biofilms subjected to

phytochemicals treatment as compared to control. The presence of SIC of TC significantly upregulated three proteins and downregulated three proteins critical for biofilm formation ( $P < 0.05$ ). The upregulated proteins were flagellar protein (FliL), cytochrome c553 (Cyf) and putative peptidyl-prolyl cis-isomerase (Cbf2) whereas periplasmic nitrate reductase (NapA), chaperone (DnaK) and bacterioferritin were downregulated. Similar results were observed with EG, which upregulated cytochrome c553 (Cyf) and downregulated NapA ( $P < 0.05$ ). The SIC of CR significantly upregulated NapB and FliL proteins and downregulated NapA and DnaK proteins.

## DISCUSSION

*Campylobacter* contamination of poultry products represents a major risk factor for human campylobacteriosis. Despite being nutritionally fastidious, there is sufficient evidence that the biofilm formation plays a critical role in the survival of *C. jejuni* in the processing environment and consequently contaminating poultry products (Murphy et al., 2006; García-Sánchez et al., 2017; Castro et al., 2018). Moreover, few studies have utilized the potential of phytochemicals in inhibiting and inactivating *C. jejuni* biofilms developed on various surfaces commonly encountered in the processing plant and at conditions mimicking the processing plant environment.

This study investigated the potential of phytochemicals in reducing *C. jejuni* biofilms on surfaces commonly encountered in processing environment. The antibiofilm potential of phytochemicals was tested in the presence of 5% chicken meat juice to represent the meat environment as it has been shown previously that the presence of meat extracts modulates biofilm formation in *C. jejuni* (Brown et al., 2014). We observed that the presence of 5% chicken meat juice significantly enhanced the biofilm formation on plastic surface as compared to broth medium at both tested temperatures (Fig. 1). Brown et al. (2014) had also reported that chicken and pork



meat juice (5-100%) enhanced *C. jejuni* biofilm formation by increasing attachment of *C. jejuni* to abiotic surfaces.

In order to effectively control *C. jejuni* biofilms in the processing plant, both prevention of biofilm formation and killing of pre-formed mature biofilms are important. Therefore, we tested the efficacy of phytochemicals in reducing biofilm formation as well as inactivating mature *C. jejuni* biofilms. We used the SICs of phytochemicals in the inhibition studies and hypothesized that the SICs of phytochemicals affects the critical genes and proteins required by planktonic cells for biofilm formation. The biofilms were developed for 48 h since our results (data not shown) and literature (Reeser et al., 2007) suggests that *C. jejuni* forms mature biofilm by 48 h. In the present study, the SICs of TC, EG and CR exert significant effect in reducing *C. jejuni* biofilm formation on polystyrene (Fig. 2) and steel coupons (Fig. 3) at both time points (24 and 48 h) and tested temperatures. The SIC of CR was the most effective in inhibiting *C. jejuni* biofilms formation on polystyrene plates at 24 h (Fig. 2) whereas these phytochemicals were not significant different in efficacy among each other on steel coupons (Fig. 3) ( $P>0.05$ ). Similar results were reported with the SICs of TC, EG and CR against *L. monocytogenes* (Upadhyay et al., 2013) where the authors observed significant reductions ( $\sim 1.5$  Log CFU/mL) in counts in the biofilms develop for 48 h at 25 and 37°C. Although the SICs of phytochemicals were effective in reducing *C. jejuni* biofilm formation compared to respective control (Fig 2 and 3), the phytochemicals did not inhibit the growth of *C. jejuni* biofilm at 48 h as compared to 24 h. This could be due to volatile nature of phytochemicals leading to degradation of compounds over time.

Previous studies have shown that phytochemicals at SICs level significantly modulate the expression of genes critical for virulence of various pathogenic bacteria (Qiu et al., 2010; Maisuria et al., 2016) including *C. jejuni* (Castillo et al., 2014; Kovács et al., 2016; Upadhyay et al., 2017;

Wagle et al., 2017). However, the potential mechanism of action of TC, EG and CR against *C. jejuni* genes critical for biofilm formation has not been studied. Therefore, a gene expression study was performed to study the change in gene expression profile of *C. jejuni* in response to TC, EG and CR. A variety of genes critical for *C. jejuni* biofilm formation has been previously characterized (Bronowski et al., 2014). Bacterial genes encoding flagellins (FlaA, FlaB, FlaG) and flagellar biosynthesis protein (FlgA) are necessary at the initial stage of *C. jejuni* biofilm formation (Kalmokhoff et al., 2006; Kim et al., 2015). Previously, proteomic analysis revealed that flagellin proteins (FlaA, FlaB) were expressed at higher levels in *C. jejuni* biofilms than in planktonic cells (Kalmokhoff et al., 2006). Moreover, *C. jejuni flgA* mutants were non-motile leading to reduced biofilm formation on food contact surfaces (Kim et al., 2015). Similarly, cell-binding protein (Peb4) and inner core of lipooligosaccharides (WaaF) protect the bacterial cell during stress and contribute to survival by forming biofilm (Asakura et al., 2007; Naito et al., 2010). It was previously reported that CosR is an essential response regulator in *C. jejuni*, which regulates the transcription of oxidative stress genes (*katA*, *ahpC*) (Hwang et al., 2012; Turonova et al., 2015). In addition, CosR is the key protein in the maturation of biofilm and its overexpression was reported to enhance biofilm formation in *C. jejuni* (Oh and Jeon, 2014). Likewise, quorum sensing or cell-to-cell signaling has been reported to play an important role in the cell attachment to form biofilm. Biofilm formation was significantly reduced in *C. jejuni luxS* mutants compared to wild-type (Reeser et al., 2007). Therefore, we selected all the aforementioned genes critical for *C. jejuni* biofilm formation. We observed that TC, EG and CR at SICs significantly downregulated the expression of select flagellar genes critical for initial attachment during biofilm formation (Fig. 5). However, these phytochemicals differ from one another in reducing expression of quorum sensing and stress response genes. For example, EG significantly downregulated *cosR* and *ahpC*, however,

these genes were not affected by TC and CR. These findings suggest that TC, EG and CR may act through different mechanism(s).

To determine the effect of TC, EG and CR on proteome of *C. jejuni* present in the biofilms, LS-MS/MS based protein identification and quantification of phytochemical-treated and un-treated *C. jejuni* biofilms was conducted. Periplasmic nitrate reductase (NapABC enzyme) is an enzyme responsible for utilization of nitrate as an energy source for bacterial growth and also protects against oxidative stress (Pittman et al., 2007). Similarly, heat shock protein 70 kD (also known as chaperone DnaK) contributes to motility, stress responses, and pathogenesis in *Escherichia coli* (Arita-Morioka et al., 2015). A loss of this protein lead to reduction in biofilm formation in *Staphylococcus aureus*. Similar findings were observed with *Streptococcus mutans* where it regulates RpoS and CsgD proteins essential for curli-dependent biofilm formation (Rockabrand et al., 1998; Arita-Morioka et al., 2015). In our proteomic analysis, NapA was significantly downregulated in TC, EG and CR-treated biofilms as compared to un-treated *C. jejuni* biofilms (Table 4). The SICs of TC and CR also reduced the expression of DnaK. In addition, we identified few uncharacterized proteins and the specific roles of such proteins need to be explore in future studies. These findings suggest that antibiofilm effect of TC, EG and CR could potentially be mediated through modulation of these proteins critical for *C. jejuni* biofilm formation.

To inactivate mature *C. jejuni* biofilms, we used bactericidal concentrations (0.25, 0.5, and 1%) of phytochemicals and hypothesized that phytochemicals kill biofilm associated *C. jejuni* by potentially disrupting their cell membrane thereby leading to membrane dysfunction, cellular damage and inactivation of biofilms from the surfaces. *C. jejuni* biofilms were developed for 48 h since our TTC staining results suggest that *C. jejuni* biofilm matures by 48 h (Fig. 1). We found that TC, EG and CR were effective in killing *C. jejuni* in the mature biofilms on polystyrene (Table

2) and steel surface (Table 3) at both temperatures. Previously, Lu et al. (2012) had reported inactivation of *C. jejuni* biofilms after 24 h treatment time with 1  $\mu$ M concentration of diallyl sulphide (an antimicrobial agent from *Allium* spp). Antibiofilm efficacy of TC, EG and CR has also been reported against *L. monocytogenes* (Upadhyay et al., 2013) and *E. coli* (Perez-Conesa et al., 2006) suggesting that the phytochemicals exert antibiofilm effect on several pathogens, however, commonalities in their mechanism of action against various pathogens or the presence of a single target across pathogens that the plant compounds affect has not been identified yet. Considering these results, the select phytochemicals could be effective in reducing *C. jejuni* biofilm formation either in monoculture or when present with other biofilm forming foodborne pathogens.

In the inactivation studies, 1% CR was the most effective in killing *C. jejuni* biofilms formed in the presence of chicken meat juice on polystyrene surfaces at both temperatures (Table 2). Similarly, in the presence of chicken meat juice, CR was the most effective followed by EG and TC in inactivating *C. jejuni* biofilm on stainless steel coupons at 37°C in 1 min exposure time (Table 3). In general, we observed an increase in the antibiofilm effect of TC, EG and CR with an increase in their concentrations and more effective killing was found on biofilm developed at 20°C than at 37°C. The increased effectiveness of phytochemicals at 20°C could be due to lower EPS production at 20°C than at 37°C after 48 h as reflected by the absorbance value in TTC staining (Fig. 1). Similar results were reported by Reeser et al. (2007) where the absorbance was 5 times lower at 25°C than at 37°C in the *C. jejuni* biofilms developed for 48 h. In our study, phytochemicals were more effective in reducing biofilms developed on steel surfaces (Table 3) than on polystyrene plates (Table 2) owing to good hydrophobicity of plastic surfaces for interaction with bacteria leading to stronger biofilm formation. Previously, Reeser et al. (2007) had determined that the physiochemical properties of the abiotic surfaces affect the *C. jejuni*

attachment on surfaces to form biofilm and reported a higher degree of *C. jejuni* biofilm on hydrophobic surfaces (polystyrene and polyvinyl chloride) than on hydrophilic surfaces (glass, copper, steel).

To validate the inactivation results, we visualized the architecture of treated biofilms using ESEM and CLSM. We observed that EPS was removed after 10 min of exposure to 0.25% TC, EG or CR (Fig. 4). Since EPS is critical for *C. jejuni* biofilms, loss of EPS could be a potential antibiofilm mechanism of the tested phytochemicals. In addition, predominant *C. jejuni* in the control were live (green) whereas the majority of *C. jejuni* were dead (red) after treatments. Similar results of confocal microscopy were reported previously with TC, EG and CR against *L. monocytogenes* biofilms (Upadhyay et al., 2013).

In conclusion, TC, EG and CR were effective in reducing *C. jejuni* biofilm formation and inactivating mature biofilms on polystyrene plates and stainless steel coupons at 20 and 37°C. In addition, phytochemicals modulated critical genes and proteins required for *C. jejuni* biofilm formation. Since *C. jejuni* can form biofilms in the processing environment leading to contamination of products, phytochemicals such as TC, EG and CR could potentially be used for controlling biofilms thereby reducing the risk of human infections.

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## REFERENCES

- Adams, T. B., S. M. Cohen, J. Doull, V. J. Feron, J. I. Goodman, L. J. Marnett, I. C. Munro, P. S. Portoghese, R. L. Smith, W. J. Waddell, and B. M. Wagner. 2005. The FEMA GRAS assessment of hydroxy-and alkoxy-substituted benzyl derivatives used as flavor ingredients. *Food Chem. Toxicol.* 43: 1241-1271.
- Adams, T. B., S. M. Cohen, J. Doull, V.J. Feron, J. I. Goodman, L. J. Marnett, I. C. Munro, P. S. Portoghese, R. L. Smith, W. J. Waddell, and B. M. Wagner. 2004. The FEMA GRAS assessment of cinnamyl derivatives used as flavor ingredients. *Food Chem. Toxicol.* 42: 157-185.
- Annan-Prah, A., and M. Janc. 1998. The mode of spread of *Campylobacter jejuni/coli* to broiler flocks. *J. Vet. Med.* 35: 11-18.
- Arita-Morioka, K., K. Yamanaka, Y. Mizunoe, T. Ogura, and S. Sugimoto. 2015. Novel strategy for biofilm inhibition by using small molecules targeting molecular chaperone DnaK. *Antimicrob. Agents Chemother.* 59: 633-641.
- Asakura, H., M. Yamasaki, S. Yamamoto, and S. Igimi. 2007. Deletion of *peb4* gene impairs cell adhesion and biofilm formation in *Campylobacter jejuni*. *FEMS Microbiol. Lett.* 275: 278-285.
- Birk, T., H. Ingmer, M. T. Andersen, K. Jørgensen and L. Brøndsted. 2004. Chicken juice, a food-based model system suitable to study survival of *Campylobacter jejuni*. *Lett. Appl. Microbiol.* 38:66-71.
- Borges, A., A. C. Abreu, C. Dias, M. J. Saavedra, F. Borges, and M. Simões. 2016. New perspectives on the use of phytochemicals as an emergent strategy to control bacterial infections including biofilms. *Molecules* 21:877.
- Boysen, L., M. Nauta, and H. Rosenquist. 2016. *Campylobacter* spp. and *Escherichia coli* contamination of broiler carcasses across the slaughter line in Danish slaughterhouses. *Microb. Risk Anal.* 2: 63-67.
- Bronnec, V., H. Turoňová, A. Bouju, S. Cruveiller, R. Rodrigues, K. Demnerova, O. Tresse, N. Haddad, and M. Zagorec. 2016. Adhesion, biofilm formation, and genomic features of *Campylobacter jejuni* Bf, an atypical strain able to grow under aerobic conditions. *Front. Microbiol.* 7: 1002.
- Bronowski, C., C. E. James, and C. Winstanley. 2014. Role of environmental survival in transmission of *Campylobacter jejuni*. *FEMS Microbiol. Lett.* 356: 8-19.
- Brown, H. L., M. Reuter, K. Hanman, R. P. Betts, and A. H. M. van Vliet. 2015. Prevention of biofilm formation and removal of existing biofilms by extracellular DNases of *Campylobacter jejuni*. *PLoS One* 10: e0121680.
- Brown, H. L., M. Reuter, L. J. Salt, K. L. Cross, R. P. Betts, and A. H. M. van Vliet. 2014. Chicken juice enhances surface attachment and biofilm formation of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 80: 7053-7060.

- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods-a review. *Int. J. Food Microbiol.* 94: 223-253.
- Byrd, J. A., B. M. Hargis, D. J. Caldwell, R. H. Bailey, K. L. Herron, J. L. McReynolds, R. L. Brewer, R. C. Anderson, K. M. Bischoff, T. R. Callaway, and L. F. Kubena. 2001. Effect of lactic acid administration in the drinking water during preslaughter feed withdrawal on *Salmonella* and *Campylobacter* contamination of broilers. *Poult. Sci.* 80:278-283.
- Castillo, S., N. Heredia, E. Arechiga-Carvajal, and S. García. 2014. Citrus extracts as inhibitors of quorum sensing, biofilm formation and motility of *Campylobacter jejuni*. *Food Biotechnol.* 28: 106-122.
- Castro, A. G. S. A., E. Dorneles, E. L. S. Santos, T. M. Alves, G. R. Silva, T. C. Figueiredo, D. Assis, A. P. Lage, and S. V. Caçado. 2018. Viability of *Campylobacter* spp. in frozen and chilled broiler carcasses according to real-time PCR with propidium monoazide pretreatment. *Poult. Sci.* 0: 1-6.
- Cody, A. J., N. D. McCarthy, M. J. van Rensburg, T. Isinkaye, S. D. Bentley, J. Parkhill, K. E. Dingle, I. C. J. W. Bowler, K. A. Jolley, and M. C. J. Maiden. 2013. Real-time genomic epidemiological evaluation of human *Campylobacter* isolates by use of whole-genome multilocus sequence typing. *J. Clin. Microbiol.* 51: 2526-2534.
- Dhillon, A. S., H. L. Shivaprasad, D. Schaberg, F. Wier, S. Weber, and D. Bandli. *Campylobacter jejuni* infection in broiler chickens. *Avian Dis.* 50: 55-58.
- Donlan, R. M. 2002. Biofilms: microbial life on surfaces. *Emerg. Infect. Dis.* 8: 881-890.
- Donlan, R. M. and J. W. Costerton. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15: 167-193.
- Dore, M. H. 2015. Threats to Human Health: Use of chlorine, an obsolete treatment technology. Pages 197-212 in *Global Drinking Water Management and Conservation*. Springer Water. Springer, Cham. doi.org/10.1007/978-3-319-11032-5\_9.
- Fields, J. A. and S. A. Thompson. 2008. *Campylobacter jejuni* CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. *J. Bacteriol.* 190: 3411-3416.
- García-Sánchez, L., B. Melero, I. Jaime, M. Hänninen, M. Rossi, and J. Rovira. 2017. *Campylobacter jejuni* survival in a poultry processing plant environment. *Food Microbiol.* 65: 185-192.
- Gradel, K. O., H. L. Nielsen, H. C. Schønheyder, T. Ejlersen, B. Kristensen, and H. Nielsen. 2009. Increased short-and long-term risk of inflammatory bowel disease after salmonella or campylobacter gastroenteritis. *Gastroenterology* 137: 495-501.
- Hoffmann, S., M. B. Batz, and J. G. Morris Jr. 2012. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. *J. Food Prot.* 75: 1292-1302.
- Holley, R. A. and D. Patel. 2005. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiol.* 22: 273-292.



- Hwang, S., Q. Zhang, S. Ryu, and B. Jeon. 2012. Transcriptional regulation of the CmeABC multidrug efflux pump and the KatA catalase by CosR in *Campylobacter jejuni*. *J. Bacteriol.* 194: 6883-6891.
- Jeong, D. K. and J. F. Frank. 1994. Growth of *Listeria monocytogenes* at 10°C in biofilms with microorganisms isolated from meat and dairy processing environments. *J. Food Prot.* 57: 576-586.
- Joshua, G. W. P., C. Guthrie-Irons, A. V. Karlyshev, and B. W. Wren. 2006. Biofilm formation in *Campylobacter jejuni*. *Microbiol.* 152: 387-396.
- Kalmokoff, M., P. Lanthier, T. L. Tremblay, M. Foss, P. C. Lau, G. Sanders, J. Austin, J. Kelly, and C. M. Szymanski. 2006. Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. *J. Bacteriol.* 188: 4312-4320.
- Kim, J. S., C. Park, and Y. J. Kim. 2015. Role of flgA for flagellar biosynthesis and biofilm formation of *Campylobacter jejuni* NCTC11168. *J. Microbiol. Biotechnol.* 25: 1871-1879.
- Kim, S., C. Park, E. Lee, W. Bang, Y. Kim, and J. Kim. 2017. Biofilm formation of *Campylobacter* strains isolated from raw chickens and its reduction with DNase I treatment. *Food Control* 71: 94-100.
- Knowles, J. R., S. Roller, D. B. Murray, and A. S. Naidu. 2005. Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium. *Appl. Environ. Microbiol.* 71: 797-803.
- Kovács, J. K., P. Felső, L. Makszin, Z. Pápai, G. Horváth, H. Ábrahám, T. Palkovics, A. Böszörményi, L. Emödy, and G. Schneider. 2016. Antimicrobial and virulence-modulating effects of clove essential oil on the foodborne pathogen *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 82:6158-6166.
- Lehtola, M. J., T. Pitkänen, L. Miebach, and I. T. Miettinen. 2006. Survival of *Campylobacter jejuni* in potable water biofilms: a comparative study with different detection methods. *Wat. Sci. Tech.* 54: 57-61.
- Lemos, J. A., Y. Luzardo, and R. A. Burne. 2007. Physiologic effects of forced down-regulation of dnaK and groEL expression in *Streptococcus mutans*. *J. Bacteriol.* 189: 1582-1588.
- Line, J. E. 2001. Development of a selective differential agar for isolation and enumeration of *Campylobacter* spp. *J. Food Prot.* 64: 1711-1715.
- Lu, X., D. R. Samuelson, B. A. Rasco, and M. E. Konkel. 2012. Antimicrobial effect of diallyl sulphide on *Campylobacter jejuni* biofilms. *J. Antimicrobial. Chemother.* : 1915-1926.
- Maisuria, V. B., Y. Lopez-de Los Santos, N. Tufenkji, and E. Déziel. 2016. Cranberry-derived proanthocyanidins impair virulence and inhibit quorum sensing of *Pseudomonas aeruginosa*. *Sci. Rep.* 6.
- Malik, H., S. Rajagunalan, M. S. Kumar, J. L. Kataria, P. Anjay, S. Sachan, A. K. Upadhyay, and A. Kumar. 2017. Assessment of Antibiotics Effect on Planktonic and Biofilm Forms of *Campylobacter* Isolates. *Israel J. Vet. Med.* 72: 4.

- Marder, E. P., P. R. Cieslak, A. B. Cronquist, J. Dunn, S. Lathrop, T. Rabatsky-Ehr, P. Ryan, K. Smith, M. Tobin-D'Angelo, D. J. Vugia, and S. Zansky. 2017. Incidence and trends of infections with pathogens transmitted commonly through food and the effect of increasing use of culture-independent diagnostic tests on surveillance-foodborne diseases active surveillance network, 10 US Sites, 2013-2016. *MMWR. Morb. Mortal. Wkly. Rep.* 66: 397-403. doi: 10.15585/mmwr.mm6615a1.
- McLennan, M. K., D. D. Ringoir, E. Frirdich, S. L. Svensson, D. H. Wells, H. Jarrell, C. M. Szymanski, and E. C. Gaynor. 2008. *Campylobacter jejuni* biofilms up-regulated in the absence of the stringent response utilize a calcofluor white-reactive polysaccharide. *J. Bacteriol.* 190: 1097-1107.
- Melo, R. T., E. P. Mendonça, G. P. Monteiro, M. C. Siqueira, C. B. Pereira, P. A. B. M. Peres, H. Fernandez, and D. A. Rossi. 2017. Intrinsic and extrinsic aspects on *Campylobacter jejuni* biofilms. *Front. Microbiol.* 8: 1332.
- Murphy, C., C. Carroll, and K. N. Jordan. 2006. Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol.* 100: 623-632.
- Naito, M., E. Frirdich, J. A. Fields, M. Pryjma, J. Li, A. Cameron, M. Gilbert, S. A. Thompson, and E. C. Gaynor. 2010. Effects of sequential *Campylobacter jejuni* 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. *J. Bacteriol.* 192: 2182-2192.
- Northcutt, J. K., D. P. Smith, M. T. Musgrove, K. D. Ingram, and Arthur Hinton Jr. 2005. Microbiological impact of spray washing broiler carcasses using different chlorine concentrations and water temperatures. *Poult. Sci.* 84: 1648-1652.
- Oh, E. and B. Jeon. 2014. Role of alkyl hydroperoxide reductase (AhpC) in the biofilm formation of *Campylobacter jejuni*. *PLoS One.* 9: e87312.
- Oldfield, N. J., A. P. Moran, L.A. Millar, M. M. Prendergast, and J. M. Ketley. 2002. Characterization of the *Campylobacter jejuni* heptosyltransferase II gene, *waaF*, provides genetic evidence that extracellular polysaccharide is lipid A core independent. *J. Bacteriol.* 184: 2100-2107.
- Oyarzabal, Omar A. 2005. Reduction of *Campylobacter* spp. by commercial antimicrobials applied during the processing of broiler chickens: a review from the United States perspective. *J. Food Prot.* 68: 1752-1760.
- Perez-Conesa, D., L. McLandsborough, and J. Weiss. 2006. Inhibition and inactivation of *Listeria monocytogenes* and *Escherichia coli* O157: H7 colony biofilms by micellar-encapsulated eugenol and carvacrol. *J. Food Prot.* 69: 2947-2954.
- Pittman, M. S., K. T. Elvers, L. Lee, M. A. Jones, R. K. Poole, S. F. Park, and D. J. Kelly. 2007. Growth of *Campylobacter jejuni* on nitrate and nitrite: electron transport to NapA and NrfA via NrfH and distinct roles for NrfA and the globin Cgb in protection against nitrosative stress. *Mol. Microbiol.* 63: 575-590.

- Qiu, J., H. Feng, J. Lu, H. Xiang, D. Wang, J. Dong, X. and Deng. 2010. Eugenol reduces the expression of virulence-related exoproteins in *Staphylococcus aureus*. *Appl. Environ. Microbiol.* 76: 5846-5851.
- Reeser, R. J., R. T. Medler, S. J. Billington, B. H. Jost, and L. A. Joens. 2007. Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. *Appl. Environ. Microbiol.* 73: 1908-1913.
- Reuter, M., A. Mallett, B. M. Pearson, and A. H. M. Vliet. 2010. Biofilm formation by *Campylobacter jejuni* is increased under aerobic conditions. *Appl. Environ. Microbiol.* 76: 2122-2128.
- Rockabrand, D., K. Livers, T. Austin, R. Kaiser, D. Jensen, R. Burgess, and P. Blum. 1998. Roles of DnaK and RpoS in starvation-induced thermotolerance of *Escherichia coli*. *J. Bacteriol.* 180: 846-854.
- Rosner, B. M., A. Schielke, X. Didelot, F. Kops, J. Breidenbach, N. Willrich, G. Götz, T. Alter, K. Stingl, C. Josenhans, and S. Suerbaum. 2017. A combined case-control and molecular source attribution study of human *Campylobacter* infections in Germany, 2011–2014. *Sci. Rep.* 7: 5139.
- Rossi, D. A., Melo, R. T., Mendonça, E. P. & Monteiro, G. P. 2017. Biofilms of *Salmonella* and *Campylobacter* in the Poultry Industry in *Poultry Science* 93-113. InTech Open Science.
- Singh, V. K., M. Syring, A. Singh, K. Singhal, A. Dalecki, and T. Johansson. 2012. An insight into the significance of the DnaK heat shock system in *Staphylococcus aureus*. *Int. J. Med. Microbiol.* 302: 242-252.
- Siringan, P., P. L. Connerton, R. J. H. Payne, and I. F. Connerton. 2011. Bacteriophage-mediated dispersal of *Campylobacter jejuni* biofilms. *Appl. Environ. Microbiol.* 77: 3320-3326.
- Somers, E. B., J. L. Schoeni, and A. C. L. Wong. 1994. Effect of trisodium phosphate on biofilm and planktonic cells of *Campylobacter jejuni*, *Escherichia coli* O157: H7, *Listeria monocytogenes* and *Salmonella typhimurium*. *Int. J. Food Microbiol.* 22: 269-276.
- Spiller, R. C. 2007. Role of infection in irritable bowel syndrome. *J. Gastroenterol.* 42: 41-47.
- Svensson, S. L., L. M. Davis, J. K. MacKichan, B. J. Allan, M. Pajaniappan, S. A. Thompson, and E. C. Gaynor. 2009. The CprS sensor kinase of the zoonotic pathogen *Campylobacter jejuni* influences biofilm formation and is required for optimal chick colonization. *Mol. Microbiol.* 71: 253-272.
- Trachoo, N., J. F. Frank, and N. J. Stern. 2002. Survival of *Campylobacter jejuni* in biofilms isolated from chicken houses. *J. Food Prot.* 65: 1110-1116.
- Turonova, H., R. Briandet, R. Rodrigues, M. Hernould, N. Hayek, A. Stintzi, J. Pazlarova, and O. Tresse. 2015. Biofilm spatial organization by the emerging pathogen *Campylobacter jejuni*: comparison between NCTC 11168 and 81-176 strains under microaerobic and oxygen-enriched conditions. *Front. Microbiol.* 6: 709.

- Upadhyay, A., I. Upadhyaya, A. Kollanoor-Johny, and K. Venkitanarayanan. 2013. Antibiofilm effect of plant derived antimicrobials on *Listeria monocytogenes*. *Food Microbiol.* 36: 79-89.
- Upadhyay, A., K.Arsi, B. R. Wagle, I. Upadhyaya, S. Shrestha, A. M. Donoghue, and D. J. Donoghue. 2017. Trans-Cinnamaldehyde, carvacrol, and eugenol reduce *Campylobacter jejuni* colonization factors and expression of virulence genes in Vitro. *Front. Microbiol.* 8: 713.
- Wagle, B. R., A. Upadhyay, K. Arsi, S. Shrestha, K. Venkitanarayanan, A. M. Donoghue, and D. J. Donoghue. 2017a. Application of  $\beta$ -Resorcylic acid as potential antimicrobial feed additive to reduce *Campylobacter* colonization in broiler chickens. *Front. Microbiol.* 8: 599.
- Wagle, B. R., K. Arsi, A. Upadhyay, S. Shrestha, K. Venkitanarayanan, A. M. Donoghue, and D. J. Donoghue. 2017b.  $\beta$ -Resorcylic Acid, a phytophenolic compound, reduces *Campylobacter jejuni* in postharvest poultry. *J. Food Prot.* 80: 1243-1251.
- Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick. 2002. Extracellular DNA required for bacterial biofilm formation. *Science* 295: 1487-1487.

## Figure Legends

**Figure 1.** *C. jejuni* biofilm formation on polystyrene plates at 20°C and 37°C. Error bars represent SEM (n=6). *C. jejuni* (~6.0 Log CFU/mL) in the presence or absence of 5% chicken meat juice was incubated to form biofilm in sterile 96-well plates. The biofilm formation was determined by TTC staining at 24 h interval. Different letters indicate the statistical difference across time or temperatures (P<0.05).

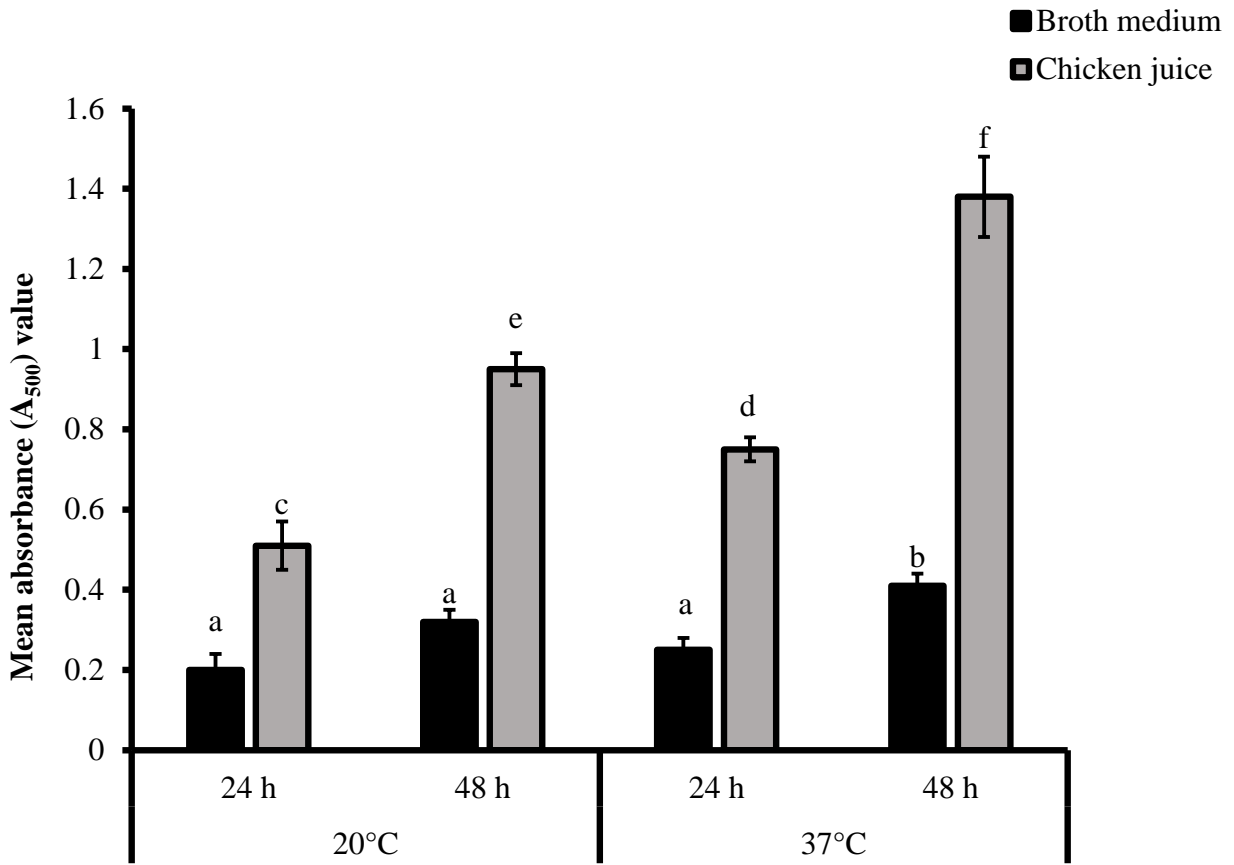
**Figure 2.** Effect of SICs of *trans*-cinnamaldehyde (TC), eugenol (EG), and carvacrol (CR) on *Campylobacter jejuni* NCTC 11168 biofilm formation on polystyrene plates in the broth medium (**Panel 1**) and in the presence of 5% chicken meat juice (**Panel 2**). Error bars represent SEM (n=6). *C. jejuni* (~6.0 Log CFU/mL) in the presence of TC (0.01%), EG (0.01%) or CR (0.002%) was incubated to form biofilm in sterile 96-well plates at 20°C (**A**) or 37°C (**B**). The number of *C. jejuni* in the biofilm was enumerated at 24 or 48 h. All treatments were significantly different from control at both 24 and 48 h (P<0.05).

**Figure 3.** Effect of SICs of *trans*-cinnamaldehyde (TC), eugenol (EG), and carvacrol (CR) on *Campylobacter jejuni* NCTC 11168 biofilm formation on stainless steel coupons in the broth medium (**Panel 1**) and in the presence of 5% chicken meat juice (**Panel 2**). Error bars represent SEM (n=6). *C. jejuni* (~6.0 Log CFU/mL) in the presence of TC (0.01%), EG (0.01%) or CR (0.002%) was incubated to form biofilm on steel coupons at 20°C (**A**) or 37°C (**B**). The number of *C. jejuni* in the biofilm was enumerated at 24 or 48 h. All treatments were significantly different from control at both 24 and 48 h (P<0.05).

**Figure 4.** Environmental scanning electron microscopy (ESEM) and confocal laser scanning micrographs (CLSM) of *Campylobacter jejuni* NCTC 11168 biofilm before treatment with phytochemicals (controls; **A**) and after treatment with 0.25% of (**B**) *trans*-cinnamaldehyde, (**C**) eugenol, or (**D**) carvacrol. Biofilms were formed at 37°C for 2 days and were exposed to phytochemicals for 10 min followed by gentle washing. For the ESEM, the treated biofilms were fixed with 2.5% glutaraldehyde and dehydrated in a series of ethanol concentration (30-100%). Dried biofilms were coated with gold using Emitech SC7620 sputter coater and visualized using 10kV beam in ESEM. For the CLSM, the treated biofilms were stained with 0.01 mM SYTO (green dye) and 0.06 mM propidium iodide (red) for 20 min, and visualized at 63X objective in Leica SP5 confocal microscope.

**Figure 5.** Effect of SICs of *trans*-cinnamaldehyde (**A**), eugenol (**B**), and carvacrol (**C**) on the expression of *Campylobacter jejuni* (NCTC 11168) genes critical for biofilm formation. Error bars represent SEM (n=6). *C. jejuni* (~6.0 Log CFU/mL) in the presence of TC (0.01%), EG (0.01%) or CR (0.002%) was incubated at 37°C for 12 h followed by RNA extraction and cDNA synthesis. RT-qPCR was conducted with 16S-rRNA serving as endogenous control. \*indicates significant change in the expression of genes at P<0.05.

Figure 1



*C. jejuni* biofilm formation at different time and temperatures

Figure 2

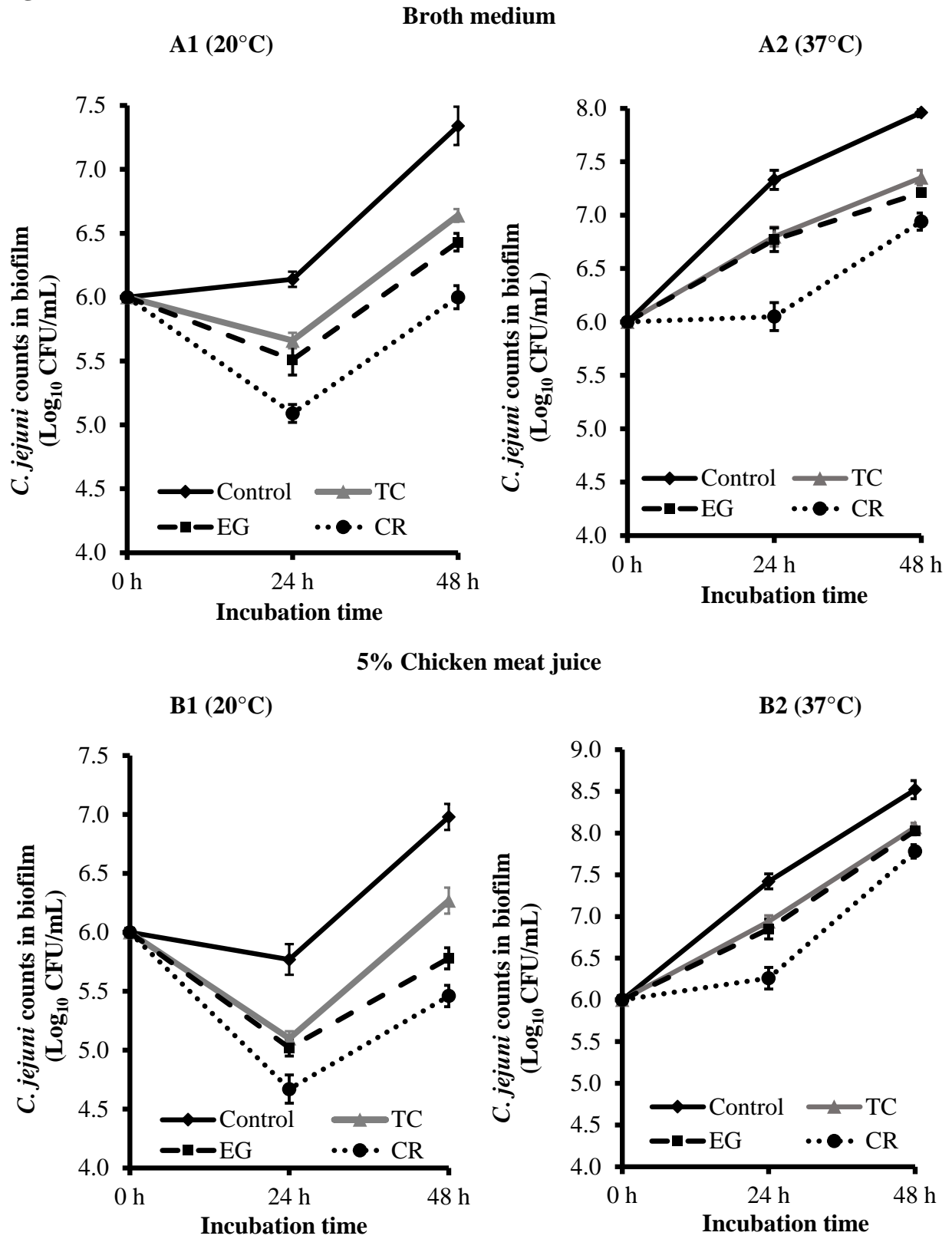
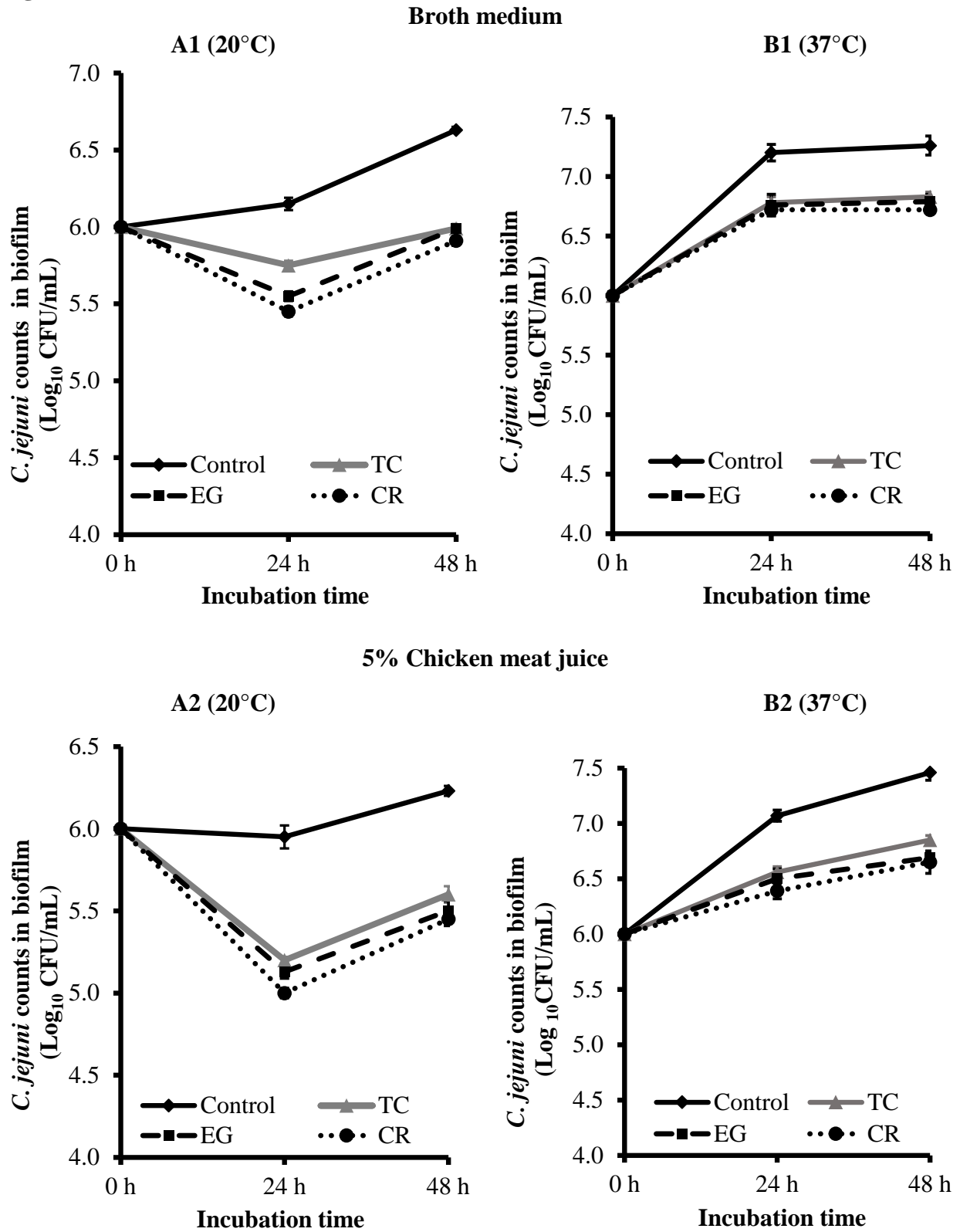


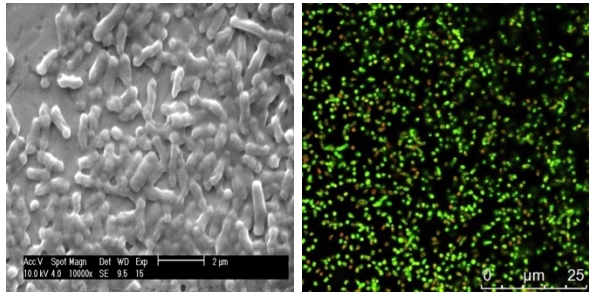
Figure 3



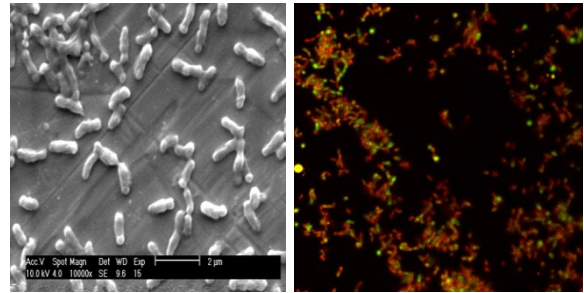


**Figure 4**

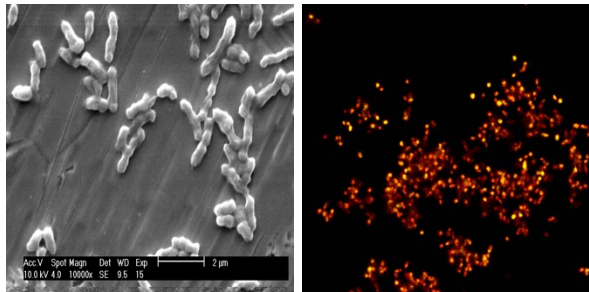
**A**



**B**



**C**



**D**

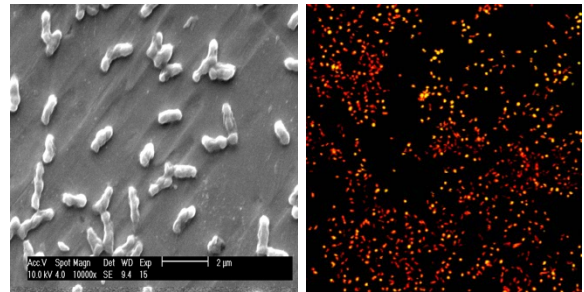
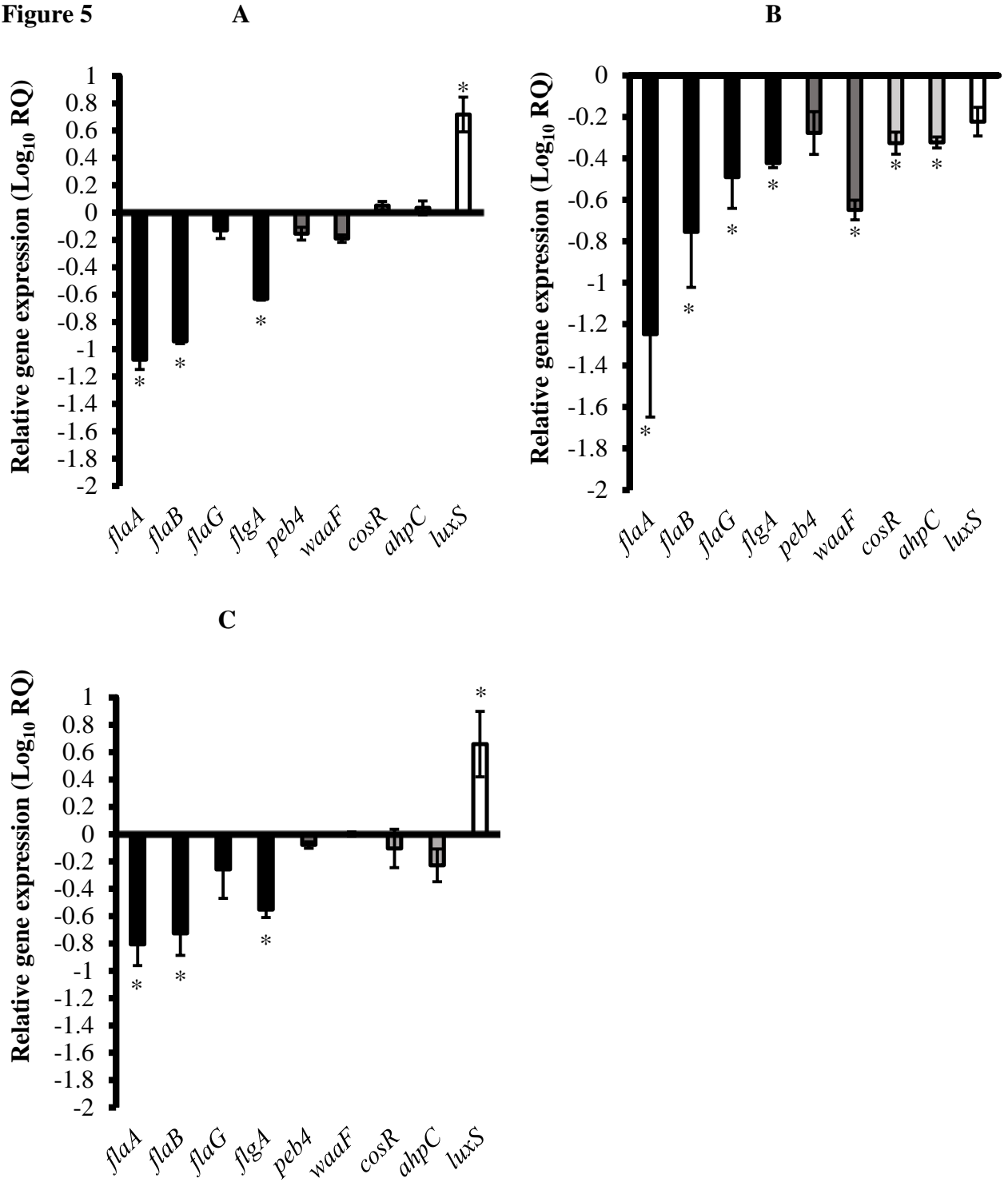


Figure 5



**Table 1:** Primers used for gene expression analysis using real-time quantitative PCR.

Gene with Accession no.	Primer	Sequence (5'-3')
<b>16S-rRNA</b>	Forward	5'-ATAAGCACCGGCTAACTCCG-3'
(NC_002163.1)	Reverse	5'-TTACGCCCAGTGATTCCGAG-3'
<i>flaA</i> (NC_002163.1)	Forward	5'-AGCGTTTGCAAAACCTGTGG-3'
(NC_002163.1)	Reverse	5'-ATGAGTAGCGCAGGAAGTGG-3'
<i>flaB</i> (NC_002163.1)	Forward	5'-AGCGTTTGCAAAACCTGTGG-3'
(NC_002163.1)	Reverse	5'-ATGAGTAGCGCAGGAAGTGG-3'
<i>flaG</i> (NC_002163.1)	Forward	5'-AGAACAAGTGAGACACAGGCT-3'
(NC_002163.1)	Reverse	5'-TTGCTGTCCATCATCGCCTT-3'
<i>flgA</i> (NC_002163.1)	Forward	5'-TTTGCACGAATCCTTTCGCC-3'
(NC_002163.1)	Reverse	5'-TCGGGGTTTTAAGCGAAGCA-3'
<i>peb4</i> (NC_002163.1)	Forward	5'-AAGGTGGTGAGCTTGGTTGG-3'
(NC_002163.1)	Reverse	5'-TTAAGCGCGAAAGCAGCATC-3'
<i>waaF</i> (NC_002163.1)	Forward	5'-CCTGGTGCAAGCTTTGGAAG-3'
(NC_002163.1)	Reverse	5'-TTGTTTCGGCTTTTCCTGCAC-3'
<i>cosR</i> (NC_002163.1)	Forward	5'-TCAGGTTCTTCCCAGATGGC-3'
(NC_002163.1)	Reverse	5'-CGCACTTAGCAAGACATTCGG-3'
<i>ahpC</i> (NC_002163.1)	Forward	5'-AGTTCGCCATGCTGTGGTTA-3'
(NC_002163.1)	Reverse	5'-CCTGCAGGACAAACTTCACC-3'
<i>luxS</i> (NC_002163.1)	Forward	5'-AGTGTTGCAAAAGCTTGGGA-3'
(NC_002163.1)	Reverse	5'-GCATTGCACAAGTTCCGCAT-3'

**Table 2:** Effect of 0.25, 0.5, and 1% concentrations of *trans*-cinnamaldehyde (TC), eugenol (EG), and carvacrol (CR) on mature *Campylobacter jejuni* NCTC 11168 biofilm formed on polystyrene microtiter plates at 20°C or 37°C in the broth medium and in the presence of chicken meat juice (n=6). The biofilms were exposed to phytochemical treatment for 1, 5 or 10 min. Values (Log CFU/mL) presented as mean ± standard error of the mean.

Treatments	20°C						37°C						
	Broth medium			Chicken juice			Broth medium			Chicken juice			
	1 min	5 min	10 min	1 min	5 min	10 min	1 min	5 min	10 min	1 min	5 min	10 min	
<b>Control</b>	8.10±0.10 <sup>a*</sup>	7.79±0.08 <sup>a</sup>	7.78±0.08 <sup>a</sup>	7.81±0.05 <sup>a</sup>	7.81±0.05 <sup>a</sup>	7.81±0.05 <sup>a</sup>	8.35±0.11 <sup>a</sup>	8.44±0.10 <sup>a</sup>	8.39±0.09 <sup>a</sup>	8.59±0.04 <sup>a</sup>	8.66±0.03 <sup>a</sup>	8.63±0.04 <sup>a</sup>	
<b>TC (%)</b>	<b>0.25</b>	6.80±0.09 <sup>b</sup>	5.63±0.08 <sup>b</sup>	4.54±0.07 <sup>b</sup>	6.69±0.11 <sup>b</sup>	6.15±0.04 <sup>b</sup>	5.14±0.04 <sup>b</sup>	7.80±0.05 <sup>b</sup>	7.78±0.05 <sup>b</sup>	7.54±0.04 <sup>b</sup>	8.38±0.06 <sup>ab</sup>	8.26±0.03 <sup>b</sup>	7.98±0.04 <sup>b</sup>
	<b>0.5</b>	6.41±0.07 <sup>c</sup>	4.32±0.09 <sup>c</sup>	4.00±0.06 <sup>cd</sup>	6.45±0.14 <sup>b</sup>	5.19±0.06 <sup>c</sup>	4.89±0.03 <sup>bc</sup>	7.62±0.05 <sup>bc</sup>	7.53±0.04 <sup>b</sup>	7.39±0.07 <sup>b</sup>	7.67±0.04 <sup>d</sup>	7.68±0.06 <sup>c</sup>	7.60±0.14 <sup>c</sup>
	<b>1</b>	ND <sup>e</sup>	ND <sup>d</sup>	ND <sup>e</sup>	5.27±0.06 <sup>cd</sup>	4.89±0.06 <sup>c</sup>	4.59±0.07 <sup>c</sup>	7.68±0.12 <sup>b</sup>	5.79±0.09 <sup>d</sup>	5.61±0.07 <sup>d</sup>	7.52±0.03 <sup>d</sup>	7.36±0.05 <sup>c</sup>	6.99±0.19 <sup>d</sup>
<b>EG (%)</b>	<b>0.25</b>	6.58±0.05 <sup>bc</sup>	5.40±0.09 <sup>b</sup>	4.52±0.06 <sup>b</sup>	6.68±0.09 <sup>b</sup>	6.20±0.05 <sup>b</sup>	5.00±0.07 <sup>bc</sup>	7.49±0.06 <sup>bc</sup>	6.47±0.49 <sup>c</sup>	6.34±0.10 <sup>c</sup>	8.09±0.08 <sup>bc</sup>	7.66±0.16 <sup>c</sup>	6.75±0.16 <sup>de</sup>
	<b>0.5</b>	6.09±0.07 <sup>d</sup>	4.34±0.05 <sup>c</sup>	3.91±0.09 <sup>d</sup>	6.38±0.04 <sup>b</sup>	5.11±0.05 <sup>c</sup>	4.82±0.02 <sup>c</sup>	7.57±0.11 <sup>bc</sup>	5.59±0.10 <sup>d</sup>	4.95±0.16 <sup>e</sup>	7.84±0.25 <sup>cd</sup>	6.16±0.04 <sup>e</sup>	5.72±0.07 <sup>f</sup>
	<b>1</b>	ND <sup>e</sup>	ND <sup>d</sup>	ND <sup>e</sup>	5.01±0.06 <sup>de</sup>	4.89±0.03 <sup>c</sup>	3.93±0.08 <sup>d</sup>	7.31±0.08 <sup>c</sup>	5.16±0.21 <sup>e</sup>	4.55±0.27 <sup>fg</sup>	6.67±0.38 <sup>e</sup>	5.18±0.11 <sup>f</sup>	4.52±0.20 <sup>g</sup>
<b>CR (%)</b>	<b>0.25</b>	6.37±0.07 <sup>cd</sup>	5.25±0.11 <sup>b</sup>	4.33±0.07 <sup>bc</sup>	5.44±0.01 <sup>c</sup>	5.16±0.04 <sup>c</sup>	4.80±0.08 <sup>c</sup>	6.94±0.17 <sup>d</sup>	6.30±0.11 <sup>c</sup>	6.07±0.05 <sup>c</sup>	6.91±0.16 <sup>e</sup>	6.76±0.05 <sup>d</sup>	6.46±0.09 <sup>e</sup>
	<b>0.5</b>	6.14±0.07 <sup>cd</sup>	4.26±0.09 <sup>c</sup>	3.98±0.07 <sup>cd</sup>	4.73±0.09 <sup>e</sup>	4.49±0.05 <sup>d</sup>	4.03±0.11 <sup>d</sup>	5.87±0.15 <sup>e</sup>	5.55±0.05 <sup>d</sup>	4.89±0.09 <sup>ef</sup>	5.41±0.12 <sup>f</sup>	5.18±0.05 <sup>f</sup>	4.50±0.09 <sup>g</sup>
	<b>1</b>	ND <sup>e</sup>	ND <sup>d</sup>	ND <sup>e</sup>	3.30±0.32 <sup>f</sup>	3.11±0.07 <sup>e</sup>	1.12±0.26 <sup>e</sup>	5.76±0.09 <sup>e</sup>	5.07±0.25 <sup>e</sup>	4.46±0.09 <sup>g</sup>	4.90±0.07 <sup>g</sup>	4.77±0.13 <sup>g</sup>	3.75±0.13 <sup>h</sup>

\*The different superscripts within columns differ significantly at P<0.05. ND: not detectable below 1 Log CFU/mL.

**Table 3:** Effect of 0.25, 0.5, and 1% concentrations of *trans*-cinnamaldehyde (TC), eugenol (EG), and carvacrol (CR) on mature *Campylobacter jejuni* NCTC 11168 biofilms formed on stainless steel coupons at 20°C or 37°C in the broth medium and in the presence of chicken meat juice (n=6). The biofilms were exposed to phytochemical treatments for 1, 5 or 10 min. Values (Log CFU/mL) presented as mean ± standard error of the mean.

Treatments	20°C						37°C						
	Broth medium			Chicken juice			Broth medium			Chicken juice			
	1 min	5 min	10 min	1 min	5 min	10 min	1 min	5 min	10 min	1 min	5 min	10 min	
<b>Control</b>	6.38±0.11 <sup>a*</sup>	6.24±0.09 <sup>a</sup>	6.35±0.05 <sup>a</sup>	6.08±0.07 <sup>a</sup>	6.00±0.04 <sup>a</sup>	6.05±0.03 <sup>a</sup>	7.91±0.10 <sup>a</sup>	7.84±0.03 <sup>a</sup>	7.88±0.05 <sup>a</sup>	7.79±0.08 <sup>a</sup>	7.89±0.09 <sup>a</sup>	7.75±0.07 <sup>a</sup>	
<b>TC (%)</b>	<b>0.25</b>	3.62±0.08 <sup>b</sup>	3.34±0.06 <sup>b</sup>	1.85±0.08 <sup>b</sup>	4.80±0.07 <sup>b</sup>	4.23±0.04 <sup>b</sup>	3.65±0.06 <sup>b</sup>	6.11±0.08 <sup>b</sup>	5.70±0.10 <sup>b</sup>	5.05±0.08 <sup>b</sup>	6.43±0.04 <sup>b</sup>	6.12±0.05 <sup>b</sup>	5.80±0.07 <sup>b</sup>
	<b>0.5</b>	1.77±0.04 <sup>c</sup>	ND <sup>c</sup>	ND <sup>c</sup>	2.11±0.12 <sup>c</sup>	1.56±0.22 <sup>c</sup>	ND <sup>c</sup>	5.21±0.05 <sup>c</sup>	5.00±0.07 <sup>c</sup>	4.15±0.07 <sup>cd</sup>	5.96±0.08 <sup>c</sup>	5.71±0.04 <sup>c</sup>	5.31±0.03 <sup>c</sup>
	<b>1</b>	ND <sup>d</sup>	ND <sup>c</sup>	ND <sup>c</sup>	1.10±0.20 <sup>d</sup>	ND <sup>e</sup>	ND <sup>c</sup>	5.01±0.06 <sup>c</sup>	4.30±0.08 <sup>d</sup>	3.85±0.07 <sup>d</sup>	5.55±0.07 <sup>d</sup>	5.29±0.08 <sup>d</sup>	4.94±0.12 <sup>d</sup>
<b>EG (%)</b>	<b>0.25</b>	1.59±0.09 <sup>c</sup>	ND <sup>c</sup>	ND <sup>c</sup>	2.00±0.11 <sup>c</sup>	1.02±0.17 <sup>d</sup>	ND <sup>c</sup>	5.76±0.03 <sup>b</sup>	4.95±0.08 <sup>c</sup>	4.33±0.06 <sup>c</sup>	6.19±0.09 <sup>bc</sup>	5.58±0.07 <sup>cd</sup>	5.13±0.09 <sup>cd</sup>
	<b>0.5</b>	ND <sup>d</sup>	ND <sup>c</sup>	ND <sup>c</sup>	1.03±0.13 <sup>d</sup>	ND <sup>e</sup>	ND <sup>c</sup>	4.13±0.09 <sup>d</sup>	3.06±0.09 <sup>e</sup>	1.10±0.06 <sup>f</sup>	5.33±0.05 <sup>d</sup>	3.26±0.07 <sup>e</sup>	1.88±0.15 <sup>e</sup>
	<b>1</b>	ND <sup>d</sup>	ND <sup>c</sup>	ND <sup>c</sup>	ND <sup>e</sup>	ND <sup>e</sup>	ND <sup>c</sup>	ND <sup>f</sup>	ND <sup>f</sup>	ND <sup>g</sup>	4.14±0.15 <sup>f</sup>	3.20±0.05 <sup>e</sup>	ND <sup>f</sup>
<b>CR (%)</b>	<b>0.25</b>	ND <sup>d</sup>	ND <sup>c</sup>	ND <sup>c</sup>	1.80±0.26 <sup>c</sup>	1.21±0.19 <sup>d</sup>	ND <sup>c</sup>	3.21±0.04 <sup>e</sup>	2.94±0.10 <sup>e</sup>	1.95±0.10 <sup>e</sup>	4.71±0.06 <sup>e</sup>	3.39±0.27 <sup>e</sup>	2.25±0.16 <sup>e</sup>
	<b>0.5</b>	ND <sup>d</sup>	ND <sup>c</sup>	ND <sup>c</sup>	ND <sup>e</sup>	ND <sup>e</sup>	ND <sup>c</sup>	ND <sup>f</sup>	ND <sup>f</sup>	ND <sup>g</sup>	2.58±0.06 <sup>g</sup>	1.66±0.36 <sup>f</sup>	ND <sup>f</sup>
	<b>1</b>	ND <sup>d</sup>	ND <sup>c</sup>	ND <sup>c</sup>	ND <sup>e</sup>	ND <sup>e</sup>	ND <sup>c</sup>	ND <sup>f</sup>	ND <sup>f</sup>	ND <sup>g</sup>	ND <sup>h</sup>	ND <sup>g</sup>	ND <sup>f</sup>

\* The different superscripts within columns differ significantly at P<0.05. ND: not detectable below 1 Log CFU/mL.

**Table 4:** List of identified proteins significantly altered in *C. jejuni* NCTC 11168 biofilms treated with TC, EG or CR as compared to untreated biofilms.

<b>Proteins name and function(s)</b>	<b>Alternative ID</b>	<b>Molecular weight (kDa)</b>	<b>P-Value</b>
<b>Upregulated with TC</b>			
Putative peptidyl-prolyl cis-trans isomerase	Cbf2	30	0.039
Cytochrome c553	Cyf	11	0.002
Flagellar protein FliL	FliL	20	0.047
<b>Downregulated with TC</b>			
Periplasmic nitrate reductase	NapA	105	0.002
Chaperone protein DnaK	DnaK	67	0.016
Bacterioferritin, putative	CJJ81176_1519	17	0.016
<b>Upregulated with EG</b>			
Cytochrome c553	Cyf	11	0.025
Uncharacterized protein	CJJ81176_0474	8	0.035
<b>Downregulated with EG</b>			
Periplasmic nitrate reductase	NapA	105	0.024
Uncharacterized protein	CJJ81176_0974	16	0.013
<b>Upregulated with CR</b>			
Periplasmic nitrate reductase, electron transfer subunit	NapB	19	0.047
Flagellar protein FliL	FliL	20	0.001
Uncharacterized protein	CJJ81176_1382	27	0.025
<b>Downregulated with CR</b>			
Periplasmic nitrate reductase	NapA	105	0.016
Chaperone protein DnaK	DnaK	67	0.016



March 26, 2018

**MEMORANDUM**

**TO:** Dr. Dan Donoghue

**FROM:** Bob Beitle, Acting Biosafety Committee Chair

**RE:** Protocol Renewal

**PROTOCOL #:** 06021

**PROTOCOL TITLE:** Reducing Food Borne Pathogens in Poultry

**APPROVED PROJECT PERIOD:** Start Date February 14, 2006 Expiration Date February 13, 2021

The Institutional Biosafety Committee (IBC) has approved your request, dated January 26, 2018, to renew IBC # 06021, "Reducing Food Borne Pathogens in Poultry".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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*The University of Arkansas is an equal opportunity/affirmative action institution.*

**PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:**

List all personnel (including PI and Co-PI) to be involved in this project:

**Name:** (first and last) - **POSITION** (Title, academic degrees, certifications, and material field of expertise)

**QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE**  
Describe previous work or training with biohazardous and/or recombinant DNA and include Biosafety Levels)

**Example:**

Bob Biohazard - Associate Professor, PhD Microbiology

14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.

Dan J. Donoghue, Ph.D. Professor, Food Safety

over 20 years experience as PI running research laboratory working with BL-2 pathogens.

Ann M. Donoghue, Ph.D. Research Leader, Food safety

over 20 years experience as PI running research laboratory working with BL-2 pathogens.

Komala Arsi, Ph. D, Post-doctoral fellow

Post-doctoral fellow trained under Dr. Donoghue's supervision

Abhinav Upadhyay, Ph.D. Post-doctoral fellow

Post-doctoral fellow trained under Dr. Donoghue's supervision

Indu Upadhyaya, Ph. D. Post-doctoral fellow

Post-doctoral fellow trained under Dr. Donoghue's supervision

Basanta Raj Wagle, Graduate Student

Graduate assistant trained under Dr. Donoghue's supervision

Sandip Shreshta, Graduate Student

Graduate assistant trained under Dr. Donoghue's supervision

Additional Personnel Information (if needed):



## CONCLUSIONS

*Campylobacter jejuni* is a major foodborne pathogen causing bacterial illness in humans in the United States and across the world. *C. jejuni* is frequently associated with gastroenteritis, reactive arthritis and Guillain-Barré syndrome. The primary source of human *Campylobacter* infection is the consumption and handling of poultry products. The high level of *Campylobacter* in the ceca of birds ( $\sim 10^8$  CFU/g) and low infective dose ( $\sim 500$  CFU) poses a serious public health concern if carcasses are not properly decontaminated. However, limited success has been reported in the interventions targeting the colonization of *C. jejuni* in birds. Such results highlight the need for novel postharvest interventions to reduce contamination of poultry meat and meat products.

In this project we evaluated the antimicrobial efficacy of eugenol as an antimicrobial wash treatment during poultry processing (Study 1) and as a coating treatment after processing in reducing *C. jejuni* counts on poultry products (Study 2). We also evaluated the antibiofilm efficacy of phytochemicals in reducing *C. jejuni* biofilm formation and inactivating pre-formed mature *C. jejuni* biofilms on polystyrene plates and steel coupons, conditions mimicking the processing plant environment (Study 3). In the first study, the antimicrobial efficacy of EG was studied in suspension, emulsion and nanoemulsion delivery systems. EG suspension reduced *C. jejuni* counts with the greatest reduction of  $>2.0$  Log CFU/sample for the 2% dose of EG ( $P < 0.05$ ). Eugenol emulsions or nanoemulsions did not provide any additional *Campylobacter* reduction when compared with suspension alone.

In the second study, the efficacy of pectin or chitosan coatings fortified with eugenol to reduce *C. jejuni* on chicken wingettes was investigated. Inoculated wingettes were randomly assigned to controls, eugenol (0.5, 1 or 2%), pectin (3%), chitosan (2%) or their combinations. Following 1 min of coating, wingettes were air dried (1 h) and sampled on d 0, 1, 3, 5, and 7. The

incorporation of 0.5, 1 or 2% eugenol in the pectin improved coating efficacy against *C. jejuni* whereas the efficacy of chitosan coating was improved by 2% eugenol treatment ( $P < 0.05$ ). In addition, exposure of *C. jejuni* to eugenol, chitosan or combination significantly modulated select genes encoding for motility, quorum sensing and stress response.

In the third study, the efficacy of eugenol, *trans*-cinnamaldehyde and carvacrol in inhibiting *C. jejuni* biofilm formation and inactivating mature biofilm was evaluated. For the inhibition study, *C. jejuni* was grown either in the presence or absence of sub-inhibitory concentrations of phytochemicals and biofilm formation was quantified at 24 h intervals by enumeration. For the inactivation study, mature *C. jejuni* biofilms were exposed to the phytochemicals (0, 0.25, 0.5, or 1%) for 1, 5, or 10 min, and surviving *C. jejuni* in the biofilm were enumerated. All phytochemicals reduced *C. jejuni* biofilm formation as well as inactivated mature biofilm at both temperatures ( $P < 0.05$ ). Moreover, scanning electron microscopy revealed disruption of biofilm architecture and loss of extracellular polymeric substances after treatment.

In conclusion, eugenol as an antimicrobial dip on chicken skin and the coating treatments on chicken wingettes was effective in reducing *C. jejuni*. In addition, eugenol also acts as an antibiofilm agent to reduce *C. jejuni* on abiotic surfaces. Since a 2-Log reduction of *C. jejuni* from poultry carcass translates into more than 90% reduction in the risk of human *Campylobacter* infections, the aforementioned treatments represent a safe, effective and natural approach that could improve poultry product safety.