Efficacy of Natural Compounds with Novel Carrier Systems for Controlling Campylobacter jejuni in Post-harvest Poultry

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Efficacy of Natural Compounds with Novel Carrier Systems for Controlling *Campylobacter jejuni* in Post-harvest Poultry

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

by

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Abstract

*Campylobacter jejuni*, a leading cause of bacterial gastroenteritis worldwide, is strongly associated with the consumption and/or mishandling of raw contaminated poultry products. Thus, interventions aiming to reduce *C. jejuni* counts on poultry products could greatly reduce the incidence of human campylobacteriosis. In the first study, the efficacy of a generally recognized as safe (GRAS) compound, carvacrol (CR; derived from oregano oil), as an antimicrobial wash treatment to reduce *C. jejuni* on chicken skin was evaluated. Three delivery systems of CR: suspension, emulsion and nanoemulsion were used. *C. jejuni* counts were reduced up to 4 log_{10} cfu/sample by 2% dose of CR suspension at 0 h (P < 0.05). Carvacrol emulsion or nanoemulsion did not show any additional reduction in *C. jejuni* counts when compared to suspension.

In the second study, the efficacy of gum arabic (GA) or chitosan (CH) coatings fortified with CR to reduce *C. jejuni* on chicken wingettes was investigated as an additional intervention to increase the antimicrobial activity of CR. Inoculated chicken wingettes (~7.5 log_{10} cfu of *C. jejuni*/sample) were randomly assigned to baseline, control (0%), CR (0.25, 0.5 or 1%), GA (10%), CH (2%) or their combinations. After 1 min of coating, wingettes were air dried (1 h) and sampled at days 0, 1, 3, 5, and 7. All three doses of CR, CH or GA-based coating fortified with CR reduced *C. jejuni* from day 0 through 7 by up to 3.0 log_{10} cfu/sample (P < 0.05). Moreover, the antimicrobial efficacy of GA was improved by CR and the coatings reduced *C. jejuni* by ~1 to 2 log_{10} cfu/sample at day 7. In addition, CH-CR coatings reduced total aerobic counts on majority of storage time when compared with baseline. No significant difference in the color of chicken wingettes was observed between treatments. Exposure of this pathogen to sublethal concentrations of CR, CH or combination significantly modulated select genes encoding for energy taxis, motility, binding and
attachment. The results suggest that GA or CH-based coating with CR could potentially be used as a natural antimicrobial to control *C. jejuni* in post-harvest poultry products.
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Dedication

This work is dedicated to my mother


and my sister


“We must embrace pain and burn it as fuel for our journey”

Kenji Miyazawa (1896 – 1933)
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Chapter 1

Introduction
The implications due to foodborne illness are enormous, comprising serious public health concern as well as significant economic and social burden. The Centers for Disease Control and Prevention (2011) estimated that foodborne diseases account for ~48 million illnesses, 128,000 hospitalizations, and 3000 deaths each year in the United States. Among the different foodborne bacterial pathogens, *Campylobacter* is one of the leading causes of foodborne illnesses, with an estimated 1.3 million illnesses each year in the United States (CDC, 2018). This bacterium is a commensal in the intestinal tract of poultry with the ability to colonize at high numbers in the ceca (Beery et al., 1988), which can contaminate poultry carcasses and raw retail poultry products during processing (Berrang et al., 2001; Miwa et al., 2003). Thus, *Campylobacter* infection in humans is highly associated with consumption/or mishandling of contaminated undercooked poultry products. With increasing consumption of poultry products globally, contaminated poultry products pose a significant threat to public health (Coker et al., 2002; National Chicken Council, 2018). Simulations designed to predict the effect of different mitigation strategies showed that the incidence of human campylobacteriosis associated with consumption of chicken meals could be reduced by 30-fold by introducing a 2-log$_{10}$ reduction of the number of *Campylobacter* on the chicken carcasses (Rosenquist et al., 2003). Hence, reduction or elimination of this pathogen in the poultry/poultry products is an essential step to improve poultry product safety.

With the initiation and implementation of *Campylobacter* monitoring and control for poultry products by United States Department of Agriculture (USDA), it is necessary for poultry processors to employ effective strategies to mitigate *Campylobacter* counts throughout processing. In recent years, the addition of antimicrobials in post-chill decontamination tanks have provided an innovative approach for pathogen reduction during poultry processing (McKee, 2011; Nagel et al., 2013). Most of these antimicrobials are synthetic chemicals such as chlorine-based products,
peracetic acid, acidic calcium sulfate and trisodium phosphate (Sohaib et al., 2016). However, there has been an increase in consumer demand for high quality, minimally processed and wholesome foods with natural ingredients. In response to this changing demand, the poultry processors have been looking for practical and natural ways to ensure safety and quality of poultry products. In this regard, the use of natural plant-derived antimicrobials could be an effective approach for reducing *C. jejuni* and thereby, improving microbial safety of raw poultry and poultry products (Venkitanarayanan et al., 2013).

The use of carvacrol (a main component of oregano oil) as an antimicrobial wash/dip treatment is receiving increased attention owing to the many biological properties which are beneficial for its application in the food industry (Wagner et al., 1986; Ben Arfa et al., 2006; de Silva Lima et al., 2013; Ouiroga et al., 2015; Jung et al., 2018). Furthermore, carvacrol is a generally recognized as safe (GRAS) status compound (Code of Federal Regulations 21 part 172). Previous studies from our laboratory demonstrated that carvacrol can reduce *Campylobacter*, both *in vitro* and *in vivo* (Kollanoor Johny et al., 2010; Arsi et al., 2014). The potential use of carvacrol in the food industry to improve food safety in different products has been extensively studied (Burt et al., 2007; Juenja et al., 2008; Ravishankar et al., 2010; Nair et al., 2015). However, solubility of carvacrol in water is a major issue, thereby, limiting its application in a commercial operation. In addition, high concentrations of carvacrol are needed to obtain a maximum effect which could potentially affect the meat quality and sensory properties of poultry products (Ntzimani et al., 2011; Sánchez-González et al., 2011; Petrou et al., 2012). In this regard, recent studies have been focusing on enhancing solubility and antimicrobial efficacy of essential oils by using novel nanotechnology in food industry (Shrivastava et al., 2009; Kour et al., 2014; Otoni et al., 2014; Landry et al., 2015; Gundewadi et al., 2016, 2018). The smaller size of nano-particles and larger
Recent studies have highlighted the use of coating treatments to improve the shelf-life and microbial safety on poultry products (Ricke and Hanning, 2013). Antimicrobial edible coatings or edible coatings fortified with plant-derived antimicrobial can be used to minimize growth of surface pathogens (Cagri et al., 2004; Upadhyay et al., 2015, Upadhya et al., 2016). Any type of thin material prepared from various substances including protein, polysaccharides and lipid used for enrobing various foods to extend the shelf-life and improve the microbial safety of the food product that may be consumed together with that food is considered as an edible coating (Debeaufort et al., 1998; Dehghani et al., 2018). Since antimicrobial edible coatings are not expected to be removed before consumption, they are considered as food additives and they should be GRAS status compounds as codified by the FDA (FDA, 2018). Gum arabic (Code of Federal Regulations 21 part 184) is a natural gum consisting of the hardened sap of various species of the acacia tree (Anderson et al., 1966; Islam et al., 1997). Chitosan is a linear aminopolysaccharide, composed of β(1-4) linked D-glucosamine and N-acetyl-D-glucosamine, and derived from chitin (a structural component of crustacean shells). Both gum arabic and chitosan possess multiple biological activities including antimicrobial activity against various pathogens (Hudson and Smith, 1998; Dutta et al., 2004; Patel and Goyal, 2015). In addition, the antimicrobial efficacy of edible coating could be enhanced by incorporating antimicrobial essential oils such as carvacrol due to potential synergism against C. jejuni (Elsabee and Abdou, 2013).
Justification

_Campylobacter_ spp. are the most frequent cause of bacterial foodborne infections worldwide. The human infections are often linked with either consumption or mishandling of raw contaminated poultry products. Thus, reducing _Campylobacter_ counts on poultry products would greatly reduce human illnesses. To reach this goal, intervention strategies at each stage (multi-hurdle approach) of poultry production are warranted. As of now, there are no treatments to eliminate _Campylobacter_ from colonizing the gastrointestinal tract of poultry. This stresses the importance of post-harvest interventions. Due to consumer preference for minimally processed and no synthetic chemical treatments, the use of natural antimicrobials with GRAS status is gaining attention for improving safety of poultry products. There is a paucity of literature on the use of natural antimicrobials to reduce _C. jejuni_ on poultry products. Thus, more research is needed to find effective natural treatments to reduce/eliminate _C. jejuni_ on poultry products.
References


Chapter 2

Literature Review
2.1 \textit{Campylobacter}

2.1.1 Historical Perspective

\textit{Campylobacter} was first observed by Theodore Escherich in the stool samples from infants with diarrhea in 1886 (Escherich, 1886), and after two decades, the organism was confirmed as causative agent of septic abortion in cattle and sheep (McFadyean and Stockman, 1909). The type species, \textit{Campylobacter fetus (Vibrio fetus)} remained an organism primarily of veterinary concern for many years (Altekruse et al., 1999). The first human infection was reported by Vinzet and colleagues (1947) from the blood of three pregnant women. King (1957) was the first who studied human strains in depth. She identified \textit{Vibrio} like bacteria requiring high optimum temperature for growth than the classical type and referred them as “related vibrios”. In 1963, Sebald and Véron separated the microaerophilic vibrios from the genus \textit{Vibrio} on the grounds that these organisms differed from the classical cholera and halophilic groups in certain fundamental characters (microaerophilic growth, non-fermentative metabolism and low DNA base composition) and proposed the new genus \textit{Campylobacter} (in Greek, a curved rod). Until 1972, only handful of human cases (12) were reported in literature, the reason behind this paucity of reports was the lack of selective media for isolating this organism (Butzler, 2004). It took a long time to realize the endemic nature of this organism in humans due to its fastidious growth requirements and lack of selective culturing technique (Butzler, 2004). The main hurdle for isolation of \textit{Campylobacter} from people with diarrhea was the overgrowth of other organisms present in the stool. Until 1970’s, human infection with \textit{Campylobacter} was diagnosed through microscopic observation of the causative agents from blood samples of patients with gastroenteritis (Butzler, 2004). A door to the isolation of \textit{Campylobacter} opened by researchers Cooper and Slee (1971) when they observed that an isolate of \textit{Campylobacter} was able to grow in the presence of the antibiotic cephalothin.
when incubated in a microaerophilic atmosphere. After a year, another crucial step for the isolation technique of *Campylobacter* from feces was published by Dekeyser and colleagues (1972). This selective culture technique was based on the fact that *Campylobacter* is small enough to pass through a filter (0.65 μm) that holds back other organisms. In addition, this Belgian group added sheep blood, polymixin-B-sulfate, novobiocin, and actidoine to fluid-thioglycolate agar that made isolation of *Campylobacter* from stool possible. Later, in 1977, Martin Skirrow published a selective culture technique which was less labor intensive and more sensitive than the filtration technique (Skirrow, 1977). The development of selective media followed by extensive research on *Campylobacter* redefined the role of *Campylobacter* in human disease from just an opportunist to its significantly greater role as a frequent cause of gastroenteritis (Blazer et al., 1979).

2.1.2 Microbiology

2.1.2.1 Morphology/Taxonomy

The name *Campylobacter* originated from the two Greek words kampylos (curved) and baktron (rod) which briefly describes the morphology of bacterium (Sebald and Véron, 1963). Currently, there are three closely related genera (*Campylobacter, Arcobacter* and *Sulfurospirillum*) under family *Campylobacteraceae* (Lastovica et al., 2014). There are 24 species and 8 subspecies under the genus *Campylobacter* (Fitzgerald and Nachamkin, 2015). However, *C. jejuni* (subspecies *jejuni*) and *C. coli* are the most frequent cause of human enteritis (WHO, 2018). They are gram negative, non-spore forming, slender, S-shaped, spirally curved rods, measuring 0.2-0.9 μm wide and 0.5-5 μm long. In addition, they have a single polar flagellum (two or three times the length of the cells) which gives them a characteristic corkscrew kind of movement (Smibert, 1978; Debruyne et al., 2008; Fitzgerald and Nachamkin, 2015).
2.1.2.2 *In vitro* Culture Condition

The optimal growth for *Campylobacter* spp. is observed at 42°C under microaerophilic conditions (5% oxygen, 10% carbon dioxide and 85% nitrogen; Park, 2002). Nevertheless, *C. jejuni* displays physiological activity even at 4°C (Hazeleger et al., 1998). They are believed to be sensitive to environmental stressors such as pH, temperature and exposure to high oxygen concentration (Park, 2002), albeit *Campylobacter* can survive in a wide range of environmental conditions and food matrices (Ziprin, 2004). Researchers have proposed that when exposed to extreme conditions, *Campylobacter* can go into a viable but nonculturable (VBNC) state which is characterized by a loss of culturability on routine agar and limiting its detection by conventional plate count techniques (Rollins and Colwell, 1986; Tholozan et al., 1999). The VBNC cells have higher physical and chemical resistance than culturable cells, which might be due to their lower metabolic rate and a cell wall strengthened by increased peptidoglycan cross-linking (Signoretto et al., 2000). The VBNC state of *C. jejuni* cells is still a matter of controversy; some researchers consider this physiological state a degenerative form (Mederma et al., 1991), while others claim that the VBNC state is a dormant state and the organism be able to resurrect under favorable conditions (Stern et al., 1994; Baffone et al., 2006).

2.1.2.3 Biofilm Formation

Even though *Campylobacter* is known to be a fastidious organism under laboratory conditions, research has shown that this organism is ubiquitous and able to survive in a wide range of environmental sources such as water, bovine manure, compost, and in frozen poultry products (Cools et al., 2003; Havelaar et al., 2007; Inglis et al., 2010). Besides the ability of *C. jejuni* to form VBNC state, another possible mechanism which *Campylobacter* may utilize to survive in
such inhospitable conditions is the ability to produce or to become a part of a biofilm (Buswell et al., 1998; Reeser et al., 2007; Ica et al., 2012). A biofilm is a collection of either single or multiple species of surface-associated microbial cells enclosed in a self-produced extracellular polymeric substance (EPS) matrix (Donlan, 2002; Siringan et al., 2011). Biofilms are beneficial for microbes as the bacteria in biofilms are known to be > 1,000-fold more resistant to disinfectants and antimicrobials than their planktonic counterparts, and thus, serve as a mechanism for survival during stress (Fux et al., 2005; Sofos and Geornaras 2010). There is a paucity in the information on *Campylobacter* biofilm formation, and unlike the other foodborne pathogens, the overall significance of biofilms in the physiology and survival is still not well understood (Plummer, 2012). *Campylobacter* biofilm formation ability has been studied both in microaerobic and aerobic condition because oxygen is considered a stress factor for *Campylobacter*. Reuter and colleagues (2010) proposed that biofilm formation was increased in the presence of atmospheric oxygen. Additionally, *Campylobacter* persistence in the environment in the form of mixed-species biofilms is more common than in mono-species biofilm as the mixed-species biofilms are more compact than biofilms with only *C. jejuni* (Ica et al., 2012; Feng et al., 2016; Teh et al., 2016). The formation of biofilms in poultry processing plants are of major concern, as they may aid in the survival of *C. jejuni* on many food contact surfaces including conveyor belts and stainless-steel tables, which may serve as potential sources of contamination and possible transmission of the pathogens through the human food chain (Peyrat et al., 2008; García-Sánchez., 2017; Clarke, 2018).

### 2.1.2.4 Genes Contributing to Virulence and Survival of *C. jejuni* in the Host and Environment

The circular genome of *C. jejuni* NCTC11168 is 1,641,481 base pairs (30.6% G+C) in length, 94.3% of the genome code is predicted to encode 1,654 proteins and 54 stable RNA species,
making it the densest bacterial genome sequenced to date (Parkhill et al., 2000). The average gene length is 948 base pairs (Parkhill et al., 2000). *C. jejuni* has several genes that contribute to its virulence and survival in the host and/or environment. Genes are required for motility (*flaA*, *flaB*, *fliA*, *fliK*, *fliF*, *fliM*, *fliY*, *flgI*, *flgH* *flgE*, *rpoN*), chemotaxis (*tlp1*, *tlp4*, *tlc10*, *acfB*, *cetA*, *cetB*), adhesion (*cadF*, *capA*, *flpA*, *fliP*, *peb3* and 4), invasion (*ciaB ciaC, cial, iamA*), toxin production (*cdtA*, *cdtB* and *cdtC*), multidrug and bile resistance (*cmeA, B and cmeC*), stress response (*spoT, katA, aphC sod, cj0358 and 0020*) quorum sensing (*luxS*) have been described by Bolton (2015).

The effective interventions that downregulate the expression of aforementioned genes could have direct impact on undermining the survival and virulence of *C. jejuni* in poultry, poultry products and environment.

### 2.2 Significance of Poultry/Poultry Products in Human Campylobacteriosis

*Campylobacter* spp. are widely distributed in most warm-blooded animals, including food animals such as poultry, cattle, pigs, sheep and ostriches; and in pets such as dogs and cats (Humphrey et al., 2007). The bacteria have also been found in shellfish (Wilson et al., 1996). *Campylobacter* infection in humans have often been reported with the consumption of raw milk, undercooked poultry meat, contaminated foods, water or handling of animals (CDC, 2018).

Chickens are one of the primary reservoirs of *C. jejuni*. Chickens get colonized at the age of 2-3 week with dose as low as 50 organisms (Achen et al., 1998; Knudsen et al., 2006). The primary site of their colonization is lower gastrointestinal tract, notably ceca where they can grow up to $10^8$ cfu/g of cecal material (Beery et al., 1988; Stern et al., 1988; Achen et al., 1998). It has been found that *Campylobacter* prevalence rate in the United States is up to 93% at the farm level (Stern et al., 2001; McCrea et al., 2006; Sahin et al., 2015), and the rate greatly varies by seasons,
regions and production type. Similarly, in the European Union, the *Campylobacter* prevalence ranged from 0.6 to 13.1% in the northernmost countries such as Finland, Norway and Sweden, and up to 80% in the southernmost countries (Newell and Fearnley, 2003; Skarp et al., 2016).

Despite the use of several processing aids in conventional poultry processing plants to reduce or eliminate foodborne pathogens, Cui and colleagues (2005) reported *Campylobacter* in approximately 76% of the organic and 74% of the conventional chicken samples (sampled between September 2002 to August 2003). Likewise, a recent study conducted by Guyard-Nicodème and colleagues (2015) found that approximately 76% of the French chicken meat was contaminated by *Campylobacter* with the load as high as $5 \log_{10} \text{cfu/g}$. It has been reported that *Campylobacter* is highly prevalent in broiler chickens and often considered as the primary source of food-related transmission of this bacteria to humans (Skarp et al., 2016). Both the CDC and EFSA (European Food Safety Authority) have found that chicken meat may directly account for 16% and 20-30% of human cases respectively (CDC, 2018b; EFSA, 2018).

### 2.3 Human Infections due to *Campylobacter*

#### 2.3.1 Campylobacteriosis in Humans

*Campylobacter* infection in humans (campylobacteriosis) is generally considered as self-limiting illness, with patients showing symptoms of diarrhea, fever, abdominal cramps, nausea and vomiting. The incubation period is usually 2 to 5 days and the symptoms last about a week. The incidence of campylobacteriosis has been rising worldwide in the past decade. The number of cases of *Campylobacter* infections have increased in developed countries such as North America, Europe, and Australia (Kaakoush et al., 2015). *Campylobacter* is the most common diarrheal illness in the United States, and it is estimated that more than 1.3 million people are affected each
year (CDC, 2018a). The average number of outbreaks reported each year from 2004-2009 was 28; 59 from 2010-2012 and 35 from 2013-2015 (CDC, 2018b). Similarly, EFSA estimated that more than 190,000 human cases of campylobacteriosis annually, with the actual number of cases ~9 million (EFSA, 2018). The OzFoodNet Working Group (2015) reported that the most commonly notified infections in Australia were *Campylobacter* (17,733 notifications) followed by *Salmonella* (12,271 notifications) in 2011. Kaakoush and colleagues (2015) reported that there was a significant increase in *Campylobacter* infections in Asia, Africa and Middle East. Despite of the limited surveillance data, *Campylobacter* infections were detected in 21% of hospitalized children with diarrhea in African countries and approximately 5-15% of gastroenteritis cases in Asia and the Middle East in the year 2005-2006 (Kaakoush et al., 2015).

### 2.3.2 Human Infection Associated with *Campylobacter*

In people with low immunity, *Campylobacter* occasionally spreads to the bloodstream and causes life-threatening infections such as Guillain-Barré syndrome and reactive arthritis (CDC, 2018c; WHO, 2018).

#### 2.3.2.1 Guillain-Barré Syndrome

Guillain-Barré syndrome (GBS) was first described in 1916 by three French neurologists Georges Guillian, Jean-Alexandre Barré and André Strohl in two soldiers with acute areflexic paralysis followed by recovery (Guillain, 1916; Hughes et al., 2005). The term GBS defines a clinical entity that is characterized by a rapid onset of progressive limb weakness, tingling in the extremities, loss of tendon reflexes and muscles functions. The underlying mechanism is due to biological mimicry between *Campylobacter* antigens and human gangliosides leading to
production of cross-reactive antibodies, which attack the peripheral nerves resulting in loss of nerve function (Hughes et al., 1997; Olivé et al., 1997; Ang et al., 2004).

Currently GBS is divided into 3 subtypes based upon neurological symptoms related to different immunological mechanisms (Winer, 2014). The 3 subtypes are listed as: acute inflammatory demyelinating polyradiculoneuropathy (AIDP); acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN) (van Doorn et al., 2008). Both AIDP and AMAN are associated with \textit{C. jejuni} infection; however, axonal neuropathy is more common (Rees et al., 1995; Ho et al., 1999; Ogawara et al., 2000; Hadden et al., 2001).

GBS is rare, affecting only about 1 in 100,000 people in the United States (CDC 2018d). Despite the high incidence of \textit{C. jejuni} infections in the general population, the incidence of GBS triggered by \textit{C. jejuni} infection is relatively low. In the United States, it is estimated that 1 in every 1058 \textit{Campylobacter} infections may lead to GBS (Buzby et al., 1997). The two possible reasons for such a low incidence of GBS could be: 1) Only a small proportion of \textit{C. jejuni} strains have ganglioside mimics on their lipooligosaccharides (Nachamkin et al., 2002); 2) Not all the patients exposed to \textit{C. jejuni} generate an antiganglioside response. However, the exact reasons why certain individuals break tolerance and enter into an auto-reactive state is not well understood (Willison et al., 2013).

2.3.2.2 Reactive Arthritis

Reactive arthritis (ReA) is a form of seronegative spondyloarthritis, a painful form of joint inflammation that arises after certain types of gastrointestinal or genitourinary bacterial infections such as \textit{Chlamydia, Salmonella, Shigella, Yersinia} and \textit{Campylobacter} infections (Ahvonen et al., 1969; Granfors et al., 1989; Carter, 2006; Pope et al., 2007). The relationship between
development of ReA and *Campylobacter* infection was first described in late 1970’s (Urman et al., 1977; Berden et al., 1979; Weir et al., 1979). ReA typically develops within 4 weeks of intestinal or urogenital infections. The common symptoms of ReA include pain and inflammation of joint, tendon, skin, mucosa, or eyes (Carter, 2006). The pathophysiology of ReA is still not well understood and is under intensive research; however, it has been hypothesized that molecular mimicry between Human Leukocyte Antigen B27 (HLA-B27) and bacterial molecules has been found in *Yersinia* (Aho et al., 1974), *Shigella* (Van Bohemen et al., 1984) and *Salmonella* (Hermann et al., 1993). This mimicry could induce tolerance and lack of clearance of these organisms (Ferreira et al., 2015). Studies have shown that the incidence of ReA following *Campylobacter* infection vary widely. A population-based study by Hannu and colleagues (2002) in Finland from April 1997 to September 1998 suggested the estimated rate of 4.3 per 10,000 infections, while a systematic literature review in Embase, PubMed, and Scopus databases by Ajene and colleagues (2013) determined it to be 9 per 1000 *Campylobacter* infections. Unlike in other rheumatic conditions, nonsteroidal anti-inflammatory drugs are the first-line treatment for the management of ReA (Carter, 2009). Disease-modifying anti-rheumatic drugs such as Sulfazalasine are effective for the peripheral manifestations, and use of glucocorticoids in ReA is contraindicated, except for an occasional intra-articular injection (García-Kutzbach et al., 2018).

### 2.4 USDA Regulation for Controlling *Campylobacter* in Raw Chicken

The United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) rigorously enforces *Campylobacter* monitoring and control for poultry (USDA FSIS, 2018a). The *Campylobacter* verification sampling is conducted in establishments by FSIS inspection program personnel who will collect samples using routine sampling using a 52-week moving window approach. Performance standards for broiler carcass and chicken parts require
that no more than 8 of 51 samples (15.7%) and 4 of 52 (13%) samples respectively be positive for 
*Campylobacter* (USDA FSIS, 2018a). The minimum number of samples should be no less than 10 for broiler carcass and 13 for chicken parts within 52-week moving window. For example, if a poultry processor tests 26 samples 52-week period and only two samples test positive for *Campylobacter*, then the percent positive is $7.69\% = \frac{2}{26} \times 100$. In this example, the resulting percent positive (7.69%) is less than 13%, maximum acceptable percent positive for chicken parts. As such, the establishment would pass the performance standard. A test is considered positive when any *Campylobacter* organisms are detected in the samples (USDA FSIS, 2018a).

**2.5 Intervention Strategies**

Since the farm is the preliminary site of *Campylobacter* entry and/or contamination, reduction of *Campylobacter*-positive flocks, decreasing prevalence and bacterial counts on raw chicken is the most relevant strategy to reduce the number of human campylobacteriosis cases. Thus, the major intervention strategies should be targeted at farm level (pre-harvest intervention strategies). However, currently in conventional poultry production there are no such treatments which are consistently effective to reduce or eliminate *Campylobacter* from colonizing the birds (Lin, 2009). Due to the lack of effective pre-harvest interventions, the need for post-harvest interventions of pathogen reduction/elimination is of utmost importance (Wagenaar et al., 2015). Quantitative risk assessment models have indicated that a reduction of *C. jejuni* counts on a broiler carcass by $2 \log_{10}$ units could result in a 30-fold reduction of human incidence (Rosenquist et al., 2003). Hence, reduction or elimination of *Campylobacter* in the poultry/poultry products is an essential step to address this food safety issue. The following post-harvest interventions have been studied to eliminate/reduce *Campylobacter* load on chicken carcasses.
2.5.1 Hauling and Transportation

Feces, feathers, and skin are the main sources of *Campylobacter* contamination during transportation and holding. Studies have shown that pathogen colonization increases due to defecation onto crates and birds, and, the persistence of *Campylobacter* on the crates is a potential source of contamination for *Campylobacter* negative flocks as the crates are reused (Stern et al., 1995; Slader et al., 2002; Berrang et al., 2004; Gomółka-Pawlicka et al., 2014). Therefore, several decontamination processes such as washing with hot water (55 to 70°C), forced hot air, high concentrations of disinfectant (e.g., 1,000 ppm of bleach, 2500 ppm peracetic acid) and physical scrubbing or ultrasonic treatment have been advised before reuse (El-Assaad et al., 1995; Ramesh et al., 2004; Allen et al., 2008a; Allen et al., 2008b; Berrang et al., 2011). In addition, Hastings and colleagues (2011) demonstrated that the number of *Campylobacter*-positive swabs from silver ion containing crates were lower when compared with the standard crates obtained after removal of live birds at the processing plant and across the decontamination process (pre-wash to 3 h post-sanitizer).

2.5.2 Preventing Cross-contamination at the Slaughterhouse

Logically it seems ideal to follow certain practices such as, scheduled slaughter (identifying flocks positive for *Campylobacter* before slaughter and subjecting carcasses from these flocks to special treatment) and logistic slaughter (slaughtering positive flocks after negative flocks) to the slaughtering process to prevent or reduce cross-contamination. However, practically, it may not be possible, as a high number of birds must be slaughtered per hour and disinfecting the machinery between every two carcasses or between flocks may not possible (Wagenaar et al., 2006; Umaraw et al., 2017).
2.5.3 Interventions at the Poultry Processing Plant

The slaughter of poultry in commercial slaughterhouses is a highly automated and efficient process. Despite technological advancement, contamination of chicken carcasses during processing is still a major problem (Seliwiorstow et al., 2015; Althaus et al., 2017). Thus, proper decontamination of carcasses at processing facilities should not be neglected. Methods to decontaminate the carcass during processing can be divided into physical, chemical, biological or the combinations of these technologies (Hugas and Tsigarida, 2008). Importantly these methods should be cost-effective, environmentally friendly, and easy to implement into the production scheme and finally not result in negative attributes to the final product (Loretz et al., 2010)

2.5.3.1 Physical Treatments

The objective of these treatments is to remove attached fecal materials and microbial flora off the whole chicken carcass. Physical methods include but not limited to washing with water, chilling and irradiation.

2.5.3.1.1 Washing

Washing with potable water has been used extensively in conventional poultry processing since 1978 and may result in an overall reduction of carcass surface contamination by 90 to 99% (Dickson and Anderson, 1992). In an attempt to comply with zero tolerance requirements for visible contamination on carcasses and to meet microbiological performance standards under the hazard analysis and critical control point (HACCP) final rule (FSIS, 1996), a typical poultry processing plant (150,000 to 200,000 birds per day) uses 3.7 to 7.5 million liters of water every day (Sellers and Kiepper, 2001), and spends $500,000 to $1 million per year (Jackson et al., 1999). Currently, poultry processing plants in the United States utilize warm water to kill bacteria and to
reduce the surface tension of water for effective removal of microflora and fecal matter (Bashor et al., 2004). A study by Li and colleagues (2002) observed that hot water spray (60°C) reduced *Campylobacter* counts by 0.78 log$_{10}$ cfu/carcass compared with cold water (20°C) spray. Similarly, it has been found that carcasses washed with hot water (70°C) for 40 s followed by cold water (12-15°C) spray for 10 s produced 1.6 log$_{10}$ reduction in *Campylobacter* counts with no negative effect on chicken skin (Purnell et al., 2004).

### 2.5.3.1.2 Chilling

Regulations in the United States require poultry carcasses to be cooled (4°C or lower) rapidly (carcasses weighing < 4 lbs, within 4 h of processing; carcasses weight 4-8 lbs, within 6 h; and those weighing >8 lbs, within 8 h of processing unless such poultry is to be frozen or cooked immediately at the official establishment) to prevent outgrowth of potential foodborne pathogens on the products (USDA FSIS, 2014). The most common method of chilling in the United States is the immersion chilling which is a fast chilling technique and relatively cost effective. In this process, carcasses are moved through tanks against water current containing a mixture of ice and cold potable water (Sams, 2000; El-Shibiny et al., 2009). However, the use of other chilling methods (air-dry systems and evaporative air chillers) have been increased as a consequence of limited supply of water world-wide, wastewater discharge, and carcass water content (Huezo et al., 2007). It was reported that feather follicles opened after picking but become closed during chilling allowing *Campylobacter* to entrap within them (Berndtson et al., 1992). Nevertheless, recent study showed that most follicles closed immediately after defeathering (Latt et al., 2018). Various antimicrobial compounds have been used in immersion tanks to ensure the reduction of pathogens to a level safe for humans (USDA FSIS, 2018b).
2.5.3.1.3 Irradiation

It has been proposed that irradiation has the potential to reduce foodborne pathogens in freshly chilled or frozen poultry products without affecting the organoleptic properties. The high energy (1.5 and 4.5 kGy) rays of irradiation directly and indirectly damage the DNA of microbes, effecting their ability to grow or reproduce (CDC, 2001). Xavier and colleagues (2016) demonstrated that cobalt 60 gamma irradiation at doses of 1.5, 3.0 and 4.5 kGy effectively eliminated *Campylobacter* spp. from chilled inoculated chicken hearts. The use of 1.5 to 3 kGy on fresh or frozen raw packaged poultry products was approved by FDA and USDA (Keener et al., 2004). Even though antimicrobial properties of irradiation is well documented, this method is poorly accepted by consumers (MacRitchie et al., 2014; Wagenaar et al., 2015; Xavier et al., 2016).

2.5.3.2 Chemical Treatments

Antimicrobial compounds that are approved by both the FDA and USDA to decontaminate poultry carcasses are classified as processing aids (National Chicken Council, 2013). The USDA FSIS provides a list of processing aids with specific information on acceptable levels that are used in processing plants (USDA FSIS, 2018b). Processing aids which are commonly used by U.S. poultry processors are discussed below.

2.5.3.2.1 Sodium hypochlorite (Chlorine)

Chlorine has been used in poultry processing for more than five decades throughout the processing line to reduce spoilage as well as foodborne pathogens (Keener et al., 2004; Oyarzbal, 2005). The USDA has set the recommended levels; 20 ppm in bird washers, 5 ppm in the recycled water used in the pre-chiller and 50 ppm in the primary chillers (USDA FSIS, 2018b). Earlier studies have shown the potential use of chlorine as an antimicrobial wash/dip, with only 0.1 mg/L
of free chlorine being required to inactive 99% of *C. jejuni* after 5 min of contact (Blaser et al., 1986). The benefits of being readily available at low cost and low concentration required for efficacy has resulted in the widespread use of chlorine in processing plants (Keener et al., 2004). However, recent studies have shown that chlorine has limited efficacy due to its dependence on the pH of water (ideal pH 6 to 6.5), presence of organic matter and exposure time (Keener et al., 2004; Oyarzbal, 2005). It has been found that the washer systems containing 25-35 ppm of total chlorine on average reduced *Campylobacter* population by 0.5 log₁₀ cfu/mL (Bashor et al., 2004). Similar results were reported by Suejee and colleagues (2016) where they detected *Campylobacter* from ground chicken treated with 50 ppm chlorine

### 2.5.3.2.2 Chlorine dioxide

Chlorine dioxide is an antimicrobial compound commonly used as a disinfectant and sanitizer (Keener et al., 2004). It is seven times more effective than chlorine in reducing pathogenic microorganism which allow it to be effective even at lower concentrations without producing corrosive action on processing equipment (Lillard, 1979). Bolder and colleagues (2004) reported a 0.7 log₁₀ reduction of *C. jejuni* with the application of 4.25 ppm chlorine dioxide. In another study, 97% *C. jejuni* cells were damaged when exposed to 20 ppm for 2 min *in vitro* (Smigic et al., 2011). It is allowed to be used at levels not to exceed 3 ppm in water that has direct contact with whole fresh poultry carcasses (USDA FSIS, 2018b).

### 2.5.3.2.3 Trisodium phosphate

Trisodium phosphate (TSP) is an inorganic, white, crystalline material that complies with the specifications of the Food Chemical Codex (Keener et al., 2004). The antibacterial effect of TSP is well known against a wide range of bacteria; however, it is more active against Gram-
negative pathogens such as *Salmonella*, *Campylobacter*, and *E. coli* (Bashor et al., 2004; Keener et al., 2004). Trisodium phosphate has a pH of 11.8 at a recommended concentration (12% w/v) for use during pre-chill and post-chill poultry processing. The proposed mechanism of action of TSP is the high pH of the solution, and surfactant property (Keener et al., 2004). Koolman and co-workers (2014) showed that 12% TSP alone or in combination with organic acids and its salt (citric acid or capric acid sodium salt) resulted in significant reduction (2-3 log_{10} cfu/cm^2) of *C. jejuni* counts on drumsticks. Similar results were observed by Sarjit and colleagues (2015) on chicken meat under simulated commercial water chilling conditions. Although TSP offers significant anti-*Campylobacter* activity, the use of TSP can be costly and affects the water holding capacity of meat (Keener et al., 2004).

### 2.5.3.2.4 Cetylpyridium chloride

Cetylpyridium chloride (CPC) is a cationic quaternary ammonium compound with antimicrobial activity against a wide range of Gram-negative bacteria (Oyarzabal, 2005). The use of CPC as a processing aid was approved by the USDA FSIS with the maximum recommended dose of 0.8% (USDA FSIS, 2018b). It was found that 0.5% (wt/vol) CPC significantly reduced *Campylobacter* by >1.0 log_{10} cfu on chicken breast skin (Arritt et al., 2002). Similarly, Beers and colleagues (2006) reported significant reduction (0.8 to 2.1 log_{10}) of *Campylobacter* on pre-chill carcasses sprayed with a commercial CPC (Cecure®).

### 2.5.3.2.5 Acidified sodium chlorite (ASC)

The use of ASC was approved by the FDA, EPA and the USDA as an antimicrobial spray or dip for poultry, red meats, vegetables, fruits and seafood (Kemp et al., 2001). Acidified sodium chlorite exhibits its antimicrobial property by oxidizing microbial cell components at pH between
2.3 to 3.3 (Keener et al., 2004). It was found that a post-chill application of ASC reduced Campylobacter to < 0.2 log_{10} cfu/mL of carcass rinse (Oyarzabal et al, 2005).

2.5.3.2.6 Organic Acids

Organic acids such as acetic acid, lactic acid, citric acid and succinic acid have been approved as processing aids in poultry processing (USDA FSIS, 2018b). Unlike chlorine, these acids are very stable in the presence of organic materials (Keener et al., 2004). The effectiveness of the organic acids depends upon concentration, temperature and contact time (Dickson and Anderson, 1992). It was found that carcasses sprayed with 4% lactic acid solution reduced 0.4 log_{10} cfu/g of C. jejuni on breast skin whereas 8% solution of lactic acid reduced counts by 1.9 log_{10} cfu/g. However, higher dose of lactic acid adversely affected the meat quality of the carcasses (Burfoot et al., 2015).

2.5.3.2.7 Peracetic acid

Peracetic acid (PAA) is the peroxide of acetic acid (AA) that has higher oxidation potential than chlorine thereby offers greater antimicrobial activity against wide variety of microbes. It is commercially available in the equilibrium mixture of PAA, AA, hydrogen peroxide and water (Kitis, 2004). Studies have shown that its antimicrobial activity is based on the release of active oxygen which disrupts the function of cell membrane, denature proteins and inactive catalase (enzyme required to detoxify free hydroxyl radicals) (Leaper, 1984; Liberti and Notrnicola, 1999; Kitis, 2004). Peracetic acid has been listed as a processing aid to decontaminate poultry carcasses and parts in the US poultry industry since 2001 (FDA, 2018). In a survey of U.S. commercial poultry operations, PAA was the leading chemical intervention (McKee, 2011). In accordance with the FSIS Directive 7120.1, the upper allowable level of PAA in chiller water is 220 ppm and for
post-chill dip application is 2000 ppm (USDA FSIS, 2018b). Park and colleagues (2017) showed that post-chill application of PAA at 1200 ppm reduced *Campylobacter* counts by 1.5 log₁₀ cfu/g when compared to water spray. However, there are significant occupational hazards (irritation of eyes, skin and difficulty in breathing) associated with doses as low as 340 ppm of PAA (Pechacek et al., 2015).

### 2.6 Natural Approach to Reduce Foodborne Pathogens on Poultry Products

Most of the processing aids that were discussed above are synthetic or inorganic chemicals. The emerging problems regarding the negative impact exerted by some synthetic preservatives on the health of consumers versus the benefits imparted by natural antimicrobials has increased consumer preference for minimally processed or less chemically treated foods which has turned more attention to plant derived antimicrobials as an alternative. Finding healing power in plants is an ancient idea. In recent years, research has focused to explore the potential of plant-derived antimicrobials as safe and effective antimicrobial treatments for improving food safety (Burt, 2004; Wagle et al., 2017; Mendonca et al., 2018). Plants represent vast resources of antimicrobial compounds. The antimicrobial compounds in plant materials are commonly found in the essential oil fraction of different parts (leaves, flowers, buds, bulbs, seeds, or other parts) of plants (Gutierrez et al., 2008). These antimicrobial compounds contain different chemical groups in their structure such as alkaloids, terpenoids, polyphenols and sulfur-containing compounds. Thus, these antimicrobials have different mechanism of actions against microbes, which makes them less likely to develop resistance by bacteria (Cowan, 1999; Burt, 2004; Savoia, 2012). Several studies have demonstrated the potential use of plant-derived antimicrobials for controlling *C. jejuni* on poultry products (Fisher and Phillips, 2006; Riedel et al., 2009; Wagle et al., 2017). A study conducted by Fisher and Phillips (2006) found potential use of lemon, orange and bergamot
essential oils and their components against major foodborne pathogens including *C. jejuni* in food systems. Riedel and colleagues (2009) observed 3 log\textsubscript{10} cfu/mL reduction in *C. jejuni* counts after washing chicken skin with 1.6% grape fruit extract for 1 min. Previously, we observed that chicken skin or meat washed with β-resorcylic acid derived from angiosperms significantly reduced (2 log\textsubscript{10} cfu) *C. jejuni* counts. Moreover, β-resorcylic acid downregulated genes responsible for virulence and survival of *C. jejuni* in the chicken meat juice (Wagle et al., 2017).

2.6.1 Carvacrol

### 2.6.1 Properties of Carvacrol

Carvacrol (also known as cymophenol) is a monoterpeneoid phenol, which is the major component of essential oils obtain from oregano (*Origanum vulgare*), thyme (*Thymus vulgais*), pepperwort (*Lepidium flavum*) and wild bergamot (Lawrence, 1984; Prudent et al., 1995; Russo et al., 1998; Alagawany et al., 2015). Also, carvacrol has been produced by chemical and biotechnological synthesis via metabolic engineered microorganisms (More et al., 2007). It is a GRAS status compound (Code of Federal Regulations 21 part 172) and has been used as a flavoring agent in sweets, beverages and chewing gum (Nostro et al., 2012; USFDA, 2018). Since ancient time, plants containing carvacrol have been used for medicinal purpose (Nostro et al., 2012). It is reported to have a wide variety of biological properties including anti-inflammatory (da Silva Lima et al., 2013), antioxidant (Quiroga et al., 2015), anti-cancer (Jung et al., 2018), acetylcholinesterase inhibitor (a therapeutic strategy to treat Alzheimer’s disease; Jukic et al., 2007), analgesic (Wagner et al., 1986), antihepatotoxic (Uyanoglu et al., 2008), antiparasitic (Force et al., 2000), insecticidal (Karpouhtsis et al., 1998) and antimicrobial (Preuss et al., 2005; Ben Arfa et al., 2006). Studies have shown that carvacrol exert significant antimicrobial effect
against various foodborne pathogens both in vitro and in vivo (Kim et al., 1995; Pol et al 1999; Du et al., 2008). Kollanoor Johny and colleagues (2010) found that carvacrol significantly reduced Salmonella Enteritidis and C. jejuni counts in the chicken cecal contents (in vitro). Previous study from our lab also showed that in-feed supplementation of carvacrol alone or in combination with thymol significantly reduced C. jejuni counts in 10-day old broiler chickens (Arsi et al., 2014).

2.6.2 Proposed Mechanisms of Action of Carvacrol

The antimicrobial action of carvacrol has been attributed to its considerable effects on the structural and functional properties of cytoplasmic membrane of microbes. Since carvacrol is a hydrophobic compound, it interacts with the lipid layer of the cytoplasmic membrane and itself aligns between the fatty acid chains causing the expansion and destabilization of the membrane structure leading to disruption of cell permeability (Lambert et al., 2001). It has been demonstrated that carvacrol reduces ATP synthesis or increases ATP hydrolysis inside the bacterial cell resulting in rapid depletion of intracellular ATP pool (Ultee et al., 1999). Moreover, it was hypothesized that carvacrol acts as a trans-membrane carrier of H⁺ and K⁺: the undissociated molecule diffuses inside the cell where it donates a H⁺ in exchange of K⁺, then the undissociated ion carrying K⁺ return via cell membrane into the external environment (Ultee et al., 2002). In the external environment, it releases K⁺ and accept H⁺, and then reenters the cell in the same way. The repetition of this cycle leads to the change in pH gradient across the bacterial cell, thereby leading to bacterial cell damage (Ultee et al., 2002).

2.6.3 Carvacrol in the Food Industry

Over the last decade, the potential use of carvacrol in the food industry to improve food safety has been extensively studied. Since carvacrol has multiple health benefits, the incorporation
of this compound in food products could improve food safety as well as product quality. Burt and co-workers (2007) found that carvacrol vapor as low as 20% (v/v) in ethanol significantly reduced *Salmonella* Enteritidis on raw chicken breast. They also observed that all viable cells ($5 \times 10^3$ cfu) were eliminated by vapor concentration above 40% at 37°C. Ravishankar and colleagues (2010) showed that 1% carvacrol dip treatment of artificially contaminated celery for 10 min reduced antibiotic-resistant *S. enterica* counts to below detection ($<1 \log_{10}$ cfu) on day 3. In addition, they observed 5-log reduction of *S. enterica* on oysters by day 3. Recently, Nair and colleagues (2015) reported that carvacrol (0.5, 1, or 2%) reduced *Salmonella* counts in turkey breast cutlets. They also found that the combined application of carvacrol (0.25, 0.5, and 1%) treatment for 30 s and modified atmospheric packaging (95% carbon dioxide and 5% oxygen) resulted in significant reduction ($1 – 2 \log_{10}$ cfu/g) of both *Salmonella* and *Campylobacter* counts on turkey breast cutlets. It has been found that carvacrol from 0.5 – 1% facilitate thermal destruction of *E. Coli* O157:H7 in raw ground beef (Juenja et al., 2008).

### 2.6.1.4 Nanoemulsion Dip Treatments

Many essential oils including carvacrol are inhibitory to foodborne pathogens; however, high concentrations are needed to observe maximum effect which may potentially affect organoleptic properties of foods (Nychas, 1995, Burt, 2004). Moreover, essential oils are poorly soluble in water limiting their application on a commercial scale. Thus, studies using novel technology to increase the water solubility and antimicrobial activity of essential oils are warranted. One such technology could be the use of nanotechnology, which has been a promising strategy to improve the solubility, stability, delivery, antimicrobial efficacy, and masking the disagreeable taste of hydrophobic phytochemicals. The American National Institute of Occupational Safety and Health (NIOSH) defined nanotechnology as a “system of innovative
methods to control and manipulate matter at near atomic scale to produce new materials, structures, and devices” (NIOSH, 2007). The term nanoemulsions also refers to a colloidal particulate system in which oil/water/oil dispersion are stabilized by surfactant molecules resulting in a droplet size range from 20 to 600 nm (Jaiswal et al., 2015). The smaller size and greater surface area is associated with the increased solubility and stability in water. In addition, smaller size enhances permeation capacity, thereby, improving antimicrobial efficacy (Gelperina et al., 2005). Recent studies and reviews on the use of nanotechnology in the food industry have identified the possible applications of nanotechnologies to improve food safety (Shrivastava et al., 2009; Kour et al., 2014; Otoni et al., 2014; Landry et al., 2015; Gundewadi et al., 2016, 2018).

2.6.2 Antimicrobial Edible Films and Coatings

In recent years, much attention has been focused on research to replace conventional packaging materials by biodegradable materials. Edible films and coatings are thin layers that are coated on the surface of food products. They are not expected to be removed before consumption and are prepared from various food grade substances including proteins, polysaccharides and lipids. The concept of edible films and coatings has emerged due to the fact that they can serve as a barrier to moisture, gas, and flavors thereby limiting losses of volatile flavor compounds, minimizing deterioration, and extending the shelf-life of food products (Kurt et al., 2017).

The application of edible coatings on poultry products is a part of a multi-hurdle intervention approach for improving food safety (Gennadios et al., 1997; Biladeau et al., 2009; Ravishankar et al., 2009; Janes et al., 2012; Fernández-Pan et al., 2014). Currently, researchers are focusing on enhancing the functionality of the coating treatments by integrating antimicrobial substances such as essential oils to deliver another level of food safety (Fernández-Pan et al., 2014;
Noori et al., 2018). The gradual release of the antimicrobial agents from the edible films and coatings leads to continuous protection of foods from contamination and limits the effect on sensory quality (Sánchez-Ortega et al., 2014).

2.6.2.1 Gum arabic

2.6.2.1 Overview

Gum arabic, or acacia gum is a natural gum consisting of the hardened sap of various species of the acacia tree and is composed of a highly branched arrangement of galactose, arabinose, rhamnose, and glucuronic acids (Anderson et al., 1966; Islam et al., 1997). In traditional medicine, it has been used for the treatment of chronic kidney diseases in Middle Eastern countries (Islam et al., 1997; Nasir et al., 2012).

2.6.2.2 Gum arabic in the Food Industry

The FDA (Code of Federal Regulations 21 part 184) has designated gum arabic as a GRAS multipurpose food ingredient for beverages, chewing gum, confectionaries, dairy products, nuts and nut products with the percent ranges from 1-85% (FDA, 2018). Gum arabic is an excellent source of dietary fiber, which is fermentable in the colon to release short-chain fatty acids and has low energy value (1.7 calories/g) which makes it suitable for food-fortification (Foodnavigator-usa, 2008; Patel and Goyal, 2015). Gum arabic has diverse functions such as enhancement of vegetable shelf-life, anti-obesity effects, antimicrobial effects, anti-inflammatory and anticoagulation effects (Patel and Goyal, 2015). It is a well known emulsifier for use in the essential oil and flavor industries (Montenegro et al., 2012). Different post-harvest studies using gum arabic on different food matrices have revealed improved shelf-life and food safety. It has been reported that apples coated with gum arabic improved the shelf-life and quality (El-Anany et
al., 2009). Similarly, Ali and co-workers (2010) found significant improvement (P < 0.05) on shelf-life and post-harvest quality on tomatoes coated with 10% gum arabic when stored at 20°C and 80-90% relative humidity for 20 days. Recently, it has been observed that Salmonella Enteritidis counts were significantly reduced (P < 0.05) on shell eggs when coated with either 10% gum arabic alone or in combinations with different phytochemicals (carvacrol, eugenol, or β-resorcylic acid) for 7 days (Upadhyaya et al., 2016).

2.6.2.2 Chitosan

2.6.2.2.1 Overview

Chitosan is a linear aminopolysaccharide, composed of β (1-4) linked D-glucosamine and N-acety-D-glucosamine, and derived from chitin (a structural component of crustacean shells). It has excellent properties such as bio-compatibility, biodegradability, non-toxicity, antimicrobial/antifungal, and adsorption (Hudson and Smith, 1998; Dutta et al., 2004). It is estimated that 6-8 million metric tons of chitin is produced globally each year by seafood processing (Yan and Chen, 2015). Because of high production of chitosan from the seafood industry, commercial application of chitin derivatives for coating treatments could be an economically feasible solution for the management of seafood by-products (Shahidi and Synowiecki, 1991).

2.6.2.2.2 Proposed Mechanisms of Action of Chitosan

The exact mechanisms of the antimicrobial activity of chitosan are still unknown. However, it is known that the antimicrobial activity of this compound is governed by several factors such as, microbial cell age, positive charge density, molecular weight, chelating capacity, hydrophilic capacity, ionic strength in medium, pH, temperature and reactive time (Kong et al.,
It has been suggested that the positively charged amino group of chitosan disrupts the negatively charged cell membrane leading to leakage of cellular contents (Young et al., 1982; Ganan et al., 2009) and chelation of trace minerals thereby inhibiting the microbial growth (Cuero et al., 1991). In addition, it also disrupts protein synthesis by binding to the host DNA (Sudarshan et al., 1992).

### 2.6.2.2.3 Importance of Chitosan in Poultry/Poultry Product Safety

There has been a growing interest in recent times to develop effective food safety intervention strategies using chitosan. A post-harvest study conducted by Menconi and colleagues (2013) demonstrated that *Salmonella* Typhimurium inoculated chicken skin when dipped in 0.5% chitosan solution for 30 s significantly reduced the counts at 24 h in comparison to the samples dipped in phosphate-buffered saline. Moreover, 0.5% chitosan significantly reduced spoilage bacteria, thus highlighting the role for chitosan in enhancing food safety and improving shelf-life of poultry products. Likewise, a significant reduction (up to 4 log_{10} cfu/cm^2) was observed in ready-to-eat turkey deli meat coated with either chitosan-lauric arginate easter or chitosan-nisin (Guo et al., 2014). Arkoun and colleagues (2018) reported that chitosan-based edible films extended the shelf-life of chicken breast by one week. Similarly, Souza and co-workers (2018) showed that chitosan incorporated with ginger essential oil improved the shelf-life of fresh poultry meat. Our previous studies have shown that in-feed supplementation of 0.5% medium molecular weight chitosan was effective (P < 0.05) in reducing *C. jejuni* counts in 14-day old broiler chickens, and also down-regulated the expression of chicken colonization genes as compared to control (Arambel et al., 2015). In addition, in our post-harvest studies, we observed significant reduction (P < 0.05) of *C. jejuni* counts on chicken wingettes coated with 2% medium molecular weight chitosan at day 3, 5 and 7 of refrigerated samples. While fortifying the chitosan with caprylic acid...
or lactic acid bacteria, the significant reduction was observed at day 0, 1, 3, 5, and 7 (Woo-Ming, 2015).

2.7 Hypothesis and Objectives of the Dissertation:

Based on published literature and preliminary data, it was hypothesized that natural compounds (plant/animal-derived) exert significant antimicrobial effect against \textit{C. jejuni} in post-harvest poultry. In addition, those compounds modulate expression of virulence genes of \textit{C. jejuni}.

The specific objectives of this dissertation were:

1. To investigate the efficacy of carvacrol suspension, emulsion and nanoemulsion as an antimicrobial wash treatment to reduce \textit{C. jejuni} counts on chicken skin.

2. To investigate the efficacy of gum arabic or chitosan-based coating fortified with carvacrol to reduce \textit{C. jejuni} counts on chicken wingettes.
2.8 References


Chapter 3

Carvacrol antimicrobial wash treatments reduce *Campylobacter jejuni* and aerobic bacteria on broiler chicken skin.
Carvacrol antimicrobial wash treatments reduce *Campylobacter jejuni* and aerobic bacteria on broiler chicken skin

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1. Abstract

Campylobacter jejuni, a major cause of human gastroenteritis worldwide, is often associated with the consumption of contaminated poultry products. With increasing consumer preference to natural and minimally processed foods, interventions utilizing natural antimicrobials for controlling C. jejuni on poultry products is gaining popularity. This study investigated the efficacy of the generally recognized as safe compound, carvacrol (CR) as a wash treatment in reducing C. jejuni and aerobic bacteria on chicken skin. Two separate studies, each with two trials were conducted. In the first study, the efficacy of CR suspension (0, 0.25, 0.5, 1 and 2%) was investigated, whereas in the second, the efficacy of CR as emulsion and nanoemulsion was studied. In both studies, skin samples were inoculated with 50 μL (~8 log_{10} cfu/sample) of a cocktail of four wild strains of C. jejuni. After 30 min of attachment, inoculated skin samples were washed with the respective treatments for 1 min, subjected to drip dry for 2 min and processed at 0, 8, 24 h post treatment for enumeration of C. jejuni and aerobic bacterial counts (n=5/treatment/time point). In addition, the effect of treatments on the color of chicken skin was evaluated. The data were analyzed by ANOVA using PROC MIXED procedure of SAS 9.3. All the tested doses of CR suspension consistently reduced C. jejuni counts across all time points. The 2% CR wash was the most effective treatment and reduced C. jejuni counts by ~4 log_{10} cfu/sample (P < 0.05). In addition, 1% and 2% CR significantly reduced aerobic counts (up to 2 log_{10} cfu/sample) at all time points. The results from the second study suggest that anti-Campylobacter efficacy of CR emulsion or nanoemulsion treatments were not improved compared to CR suspension. Several CR suspension treatments were more effective than corresponding emulsion and nanoemulsion treatments. No significant difference in colors of skin samples between treatments was observed.
(P > 0.05). The results suggest that CR could potentially be used as an antimicrobial wash treatment in post-harvest poultry.

Key words: *Campylobacter jejuni*, carvacrol, emulsion, nanoemulsion, chicken skin
2. Introduction

*Campylobacter* infection in humans is one of the most common bacterial foodborne diseases worldwide (WHO, 2017). In the United States, *Campylobacter* causes an estimated 1.3 million illnesses each year. However, the actual figure might be higher as many cases go undiagnosed or unreported (CDC, 2017). *Campylobacter jejuni* accounts for 90% of human campylobacteriosis cases characterized by vomiting, bloody diarrhea, abdominal cramps and fever (WHO, 2017). In certain cases, campylobacteriosis leads to serious sequelae such as, reactive arthritis and Guillain-Barré syndrome (Spiller, 2007; Gradel et al., 2009) that could lead to death. It was estimated that the annual cost associated with campylobacteriosis is approximately 1.9 billion dollars (Hoffmann et al., 2015). Surveys of raw agricultural products support epidemiologic evidence implicating undercooked and contaminated chicken meat as one of the primary sources for human *C. jejuni* infection (Kramer et al., 2000). Poultry are the reservoir for *C. jejuni* wherein the pathogen colonizes at high level (10⁸ cfu/g of cecal material) in the lower gastrointestinal tract (Blaser et al., 1983; Evans, 1991; Wagenaar et al., 2015) leading to product contamination during slaughter and poultry processing.

Commercial poultry processors have implemented various Hazard Analysis and Critical Control Points and good manufacturing practices to lower the pathogen load on carcasses, however, potential for contamination exists in the farm to fork supply chain (Elvers et al., 2011; Alonso-Hernando et al., 2013). *Campylobacter* spp. was isolated from up to 76% of retail organic chicken meat and 74% of conventional chickens collected from retail stores in Maryland between September 2002 and August 2003 (Cui et al., 2005). A more recent study by Williams and Omar (2012) observed that the average prevalence of *Campylobacter* spp. in skinless boneless retail meat from 2005 through 2011 in Alabama, USA was 41%. With a growing population and increasing
consumer awareness, the consumption of poultry meat has tripled over the last 5 decades. (USDA-FAS, 2018). As a result of the high consumption rate of poultry meat and greater prevalence of *C. jejuni* on retail poultry products, the risk of *C. jejuni* infections in humans is substantial (Nauta et al., 2007; Marder et al., 2017).

A number of studies have been conducted for controlling *C. jejuni* in poultry with varied degree of success (Byrd et al., 2001; Cole et al., 2006; Arsi et al., 2014; Guyard-Nicodeme et al., 2015; Shrestha et al., 2017; Wagle et al., 2017; Huneau-Salaün et al., 2018). As part of multi-hurdle approach to control foodborne pathogens, research is being focused on developing safer and effective post-harvest interventions for controlling *C. jejuni* on poultry products. Various interventions including physical methods such as freezing, chilling, hot, steam and electrolyzed water treatments, ultrasound, and irradiation, have been tested to reduce the pathogen counts on carcasses (Patterson, 1995; Park et al., 2002; Corry et al., 2007; James et al., 2007; Musavian et al., 2014). In addition, chemicals such as organic acids, chlorine or phosphate-based compounds have been used as chemical decontaminant with pathogen reduction ranging from 1 to 2.2 log_{10} (Zhao and Doyle, 2006; Bauermeister et al., 2008; Riedel et al., 2009; Birk et al., 2010; Loretz et al., 2010; Thormar et al., 2011). However, potential concerns with regards to change in the meat quality, reduced consumer acceptance and cost of treatments has made most of the aforementioned treatments less applicable for decontaminating poultry carcasses at industrial setting (Dawson et al., 1963; Cox et al., 1974; Bilgili et al., 1998; Whyte et al., 2003). To address this, researchers are focusing on using natural plant-derived antimicrobials for carcass decontamination (Smith-Palmer et al., 1998).

The antimicrobial properties of various essential oils in the food system is well documented in the literature (Chouliara et al., 2007; Sharma et al., 2017). Carvacrol [2-Methyl-5-(1-
methylethyl) phenol] is a monoterpenic phenol biosynthesized from \( \gamma \)-terpinene. This phenolic compound is the major component of the essential oil fraction of oregano (Arrebola et al., 1994). Carvacrol (CR) is currently listed as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (Code of Federal Regulations 21 Part 172). Carvacrol exhibits a plethora of biological activities including broad spectrum antimicrobial action (Ultee et al., 2000; Xu et al., 2008; Kollanoor Johny et al., 2010), antioxidant (Aeschbach et al., 1994; Ramos et al., 2014), anticancer (Arunasree, 2010; Mehdi et al., 2011; Luo et al., 2016) and antifungal properties (Lima et al., 2013; Chavan and Tupe, 2014). Previous studies from our lab and collaborators have demonstrated the efficacy of CR against \textit{C. jejuni} in vitro studies on cecal contents (Kollanoor Johny et al., 2010) as well as \textit{in vivo} studies in 10-day old broiler chickens (Arsi et al., 2014); however, CR as an antimicrobial wash treatment for reducing \textit{C. jejuni} in post-harvest poultry has not been investigated.

Despite multiple benefits, the use of essential oils as a natural antimicrobial in food systems is limited primarily due to flavor concern and need for high concentrations to exert their antimicrobial effect. Moreover, CR has low water solubility. Albeit, the total surface area, solubility, and the antimicrobial property of CR could be enhanced by using emulsion/nanoemulsion technology (Landry et al., 2014, 2015; Yadav et al., 2014). We hypothesized that CR could reduce both \textit{C. jejuni} and aerobic counts on poultry products and could be used as an effective antimicrobial treatment to improve food safety. In addition, we also hypothesized that CR emulsion and nanoemulsion would result in greater bacterial reductions than CR suspension alone.
The present study investigated the efficacy of CR (suspension, emulsion, nanoemulsion) as an antimicrobial wash to reduce C. jejuni and aerobic bacteria on chicken skin. In addition, the effect of aforementioned treatments on the color of chicken skin was investigated.

3. Materials and Methods

Bacterial Strains and Culture Conditions

Four wild-type strains (S1, S3, S4 and S4) of C. jejuni were used for this study. The C. jejuni inoculum for each trial was prepared as described by Shrestha and colleagues (2017). Briefly, one loopful of glycerol stock of each strain was inoculated into separate 15 mL tube containing 5 mL of sterile Campylobacter Enrichment Broth (CEB; catalogue no. 7526A, Neogen Corp, Lansing, MI) followed by incubation at 42°C under microaerophilic atmosphere (5% O2, 10% CO2, and 85% N2) for 48 h. After incubation, C. jejuni strains were sub-cultured for 24 h and centrifuged at 3500 × g for 10 min. The cell pellet was resuspended in 20 mL of Butterfield’s Phosphate Diluent (BPD; 0.625 mM potassium dihydrogen phosphate, pH 6.67) and used as inoculum.

Study 1: Evaluation of Antimicrobial Activity of CR Suspension on Chicken Skin

Preparation of CR Suspension Treatments. For the preparation of CR suspension, 2% (vol/vol) stock suspension of CR (catalogue no. W224502, Sigma-Aldrich Co., St. Louis, MO) was made in sterile BPD followed by stirring at 300 rpm for 15 min. Two-fold concentrations of the stock solution were made in BPD to obtain 0.25, 0.5 and 1% CR suspension. The pH of all the treatment solutions were ~6.6.

Preparation, inoculation and treatments of chicken skin samples. Chicken thighs were procured form the University of Arkansas Poultry Pilot Processing Plant (Fayetteville, AR). The skin was removed from the muscle tissue, cut into pieces (4 cm × 4 cm) and stored at -20°C until
the day of experiment. A total of 150 skin samples were used for two trials. For each trial, 75 skin samples were randomly allocated to 5 treatments (0, 0.25, 0.5, 1 and 2% CR; n=5 samples per treatment per time point). Each skin sample was inoculated with 50 µL (~8 log10 cfu/sample) of a cocktail of four wild strains of *C. jejuni* and allowed to adhere for 30 min. Inoculated skin samples were washed with 25 mL of respective treatment solutions for 1 min, drip dried for 2 min and were processed either immediately (0 h) or after 8 or 24 h of storage at 4°C.

**Microbial Analysis.** For the sample processing, each skin sample was transferred in 10 mL of Dey-Engley neutralizing broth (catalogue no. C7371, Hardy diagnostic, Santa Maria) and vortexed at 700 rpm for 15 s. The sample was 10-fold diluted in BPD and plated using the spread plate technique onto *Campylobacter* Line agar (CLA; Line, 2001) for *C. jejuni* enumeration and tryptic soy agar (TSA; catalogue no. C7121, Hardy diagnostics, Santa Maria, CA) for aerobic bacteria enumeration. The inoculated CLA or TSA plates were incubated at 42°C for 48 h under microaerophilic atmosphere or at 37°C for 24 h under aerobic condition respectively. Bacterial colonies were expressed in log10 cfu/sample.

**Study 2: Comparative Antimicrobial Evaluation of CR Suspension, Emulsion and Nanoemulsion on Chicken Skin**

**Preparation of CR emulsion and nanoemulsion.** All the compounds used for the preparation of emulsion and nanoemulsion were designated as GRAS by the FDA. Emulsion was prepared by using spontaneous emulsification procedure as described previously (Ostertag et al., 2012) with minor modifications. Briefly, an organic phase was made by adding CR (30 mL) and Tween-80 (37.5 mL) (catalogue no. 01516, Chem-impex int’l inc., Wood Dale, IL) followed by stirring using a magnetic stirrer at 750 rpm for 30 min. Sodium phosphate buffer (5mM; pH 7.0; Sigma-Aldrich) was added into organic phase at the rate of 4 mL/min with continuous stirring for
1 h. Emulsion solutions were stored at 4°C until needed. The final concentration of CR in the emulsion was 10% which was then diluted in BPD to prepare 0.25, 0.5, 1 and 2% CR emulsion.

The preparation of nanoemulsion was based on the optimized system as described by Chang et al. (2013) and Abd-Elsalam and Khoklov (2015) with some modification. Briefly, a coarse emulsion of CR (15 mL), Tween-80 (30 mL) and buffer (255 mL) was prepared as described above and mixed for 30 min. The mixture was then sonicated using an ultrasonicator (Qsonica Q700, Newtown, CT, USA) for 10 min. The stock solution was 5% CR, which was further diluted in BPD to obtain 0.25, 0.5, 1 and 2% CR nanoemulsion.

Characterization of emulsion and nanoemulsion. The droplet size, zeta potential and polydispersity index (PDI) of emulsion and nanoemulsion was measured using previously published method (Zainol et al., 2012; Abd-Elsalam and Khoklov, 2015). Briefly, 10 µL of emulsion or nanoemulsion was diluted with 1 mL of deionized water at room temperature. Three replicates of independent batches were analyzed by a dynamic light scattering method using Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, WR, UK). The thermodynamic stability of freshly prepared emulsion and nanoemulsion was determined using standard published method (Shafiq and Shakeel, 2010; Abd-Elsalam and Khoklov, 2015). Briefly, the solutions were centrifuged at 3500 × g for 20 min to evaluate the phase separation. In addition, four cycles of heating and cooling were performed between 4°C and 40°C to study the effect of temperature on the stability of emulsion and nanoemulsion.

Preparation, inoculation and treatments of chicken skin samples. A total of 450 skin samples were used for two replicate trials. For each trial, 225 skin samples were randomly divided into 15 treatment groups consisting of baseline, BPD, Tween-80 and four doses (0.25, 0.5, 1 and
2%) of CR suspension, emulsion and nanoemulsion respectively (n=5 samples per treatment per time point). Chicken skin samples were prepared and inoculated followed by wash treatments as described in Study 1. The sample processing and enumeration of C. jejuni and total aerobic bacteria were conducted according to the procedure described above. Additionally, C. jejuni counts in the wash solution (post-treatment) was determined by plating 250 µL wash treatment on CLA plates followed by incubation as described previously.

**Determination of Color**

The color measurements of skin samples treated with CR suspension, emulsion or nanoemulsion were carried out using a chroma meter (CR-300, Konica Minolta Sensing Inc., Japan) and this was used to objectively measure International Commission on Illumination (CIE) L*, a*, b* values (L* measure relative lightness, a* relative redness and b* relative yellowness). Two replicate trials were conducted. For each trial, 150 samples were randomly divided into 15 treatment groups (as described above) for two time points (0 and 24 h). The instrument was calibrated against white tile before measurements were recorded. Three readings were taken on the lateral side of each sample.

**Statistical Analysis**

For the microbial analysis, C. jejuni and aerobic bacteria counts (cfu/sample) were transformed to log10 cfu/sample to maintain the homogeneity of variance (Byrd et al., 2001). For the color analysis, data from two trials were pooled for each treatment before analysis. The data were analyzed using ANOVA with the PROC MIXED procedure in the SAS statistical software, version 9.3 (SAS Institute Inc., Cary, NC). Means were partitioned by LSMEANS analysis, and a P value of < 0.05 was required for statistical significance.
4. Results

Properties and stability of CR emulsion and nanoemulsion

The average particle size (Z-average), PDI and zeta-potential for emulsion were \( \sim 505.6 \pm 101 \), \( 1\pm 0.12 \) and \(-24.9 \pm 11.3 \text{ mV} \) respectively, whereas for nanoemulsion the values for average particle size (Z-average), PDI and zeta-potential were \( \sim 260.3 \pm 25 \text{ nm} \), \( 0.039 \pm 0.01 \) and \(-18.7 \pm 5.54 \text{ mV} \) respectively. The distribution was unimodal. The solutions were stable after centrifugation at \( 3500 \times g \) for 20 min, and resisted four cycle of heating and cooling.

Antimicrobial efficacy of CR suspension against C. jejuni and aerobic bacteria on chicken skin

The effect of CR on C. jejuni. Figure 1 shows the effect of CR suspension in reducing \textit{C. jejuni} on inoculated chicken skin stored at \( 4^\circ \text{C} \) for three different time points (0, 8 and 24 h). The \textit{C. jejuni} counts present on the control (skin washed with BPD) was \( \sim 5.5 \) and \( \sim 6 \log_{10} \text{ cfu/sample} \) for the trial 1 and 2 respectively. All the tested doses of CR suspension (0.25, 0.5, 1 and 2\%) significantly reduced \textit{C. jejuni} counts (> 1.4 \log_{10} \text{ cfu/sample}) compared to the controls. Two percent CR suspension reduced \textit{C. jejuni} by 3.22 \log_{10} \text{ cfu/sample} at 0 h in trial 1 and \sim 4.0 \log_{10} \text{ cfu/sample} at 0 h in trial 2 when compared with the controls. All the doses of CR suspensions (0.25, 0.5, 1 or 2\%) were equally effective in reducing \textit{C. jejuni} counts when compared among each other at all storage time points in trial 1. Similar patterns were observed in trial 2 at 24 h, however, 2\% CR suspension was more effective in reducing \textit{C. jejuni} counts than 0.25 or 1\% dose at 0 h.

The effect of CR on aerobic bacteria. Figure 2 shows the efficacy of CR in reducing aerobic bacterial counts on the chicken skin. The aerobic bacterial counts recovered from the controls ranged from \( \sim 3.5 \) to 5 or from 4 to 4.5 \log_{10} \text{ cfu/sample} for trial 1 and 2 respectively. The 1 and 2\% CR suspension consistently reduced aerobic bacterial counts (> 0.93 \log_{10} \text{ cfu/sample})
Comparative antimicrobial efficacy of CR suspension, emulsion and nanoemulsion against C. jejuni and aerobic bacteria on chicken skin

The effect of CR on C. jejuni. The effect of CR suspension, emulsion and nanoemulsion on the survival of C. jejuni on chicken skin is presented in Table 1. C. jejuni recovered from baseline (skin samples not subjected to treatments) was \( \approx 7 \log_{10} \text{cfu/sample} \). Washing of the skin samples in either BPD (control for CR suspension) or Tween-80 (control for CR emulsion or nanoemulsion) reduced C. jejuni counts by \( \approx 1 \) to \( \approx 1.5 \log_{10} \text{cfu/sample} \) when compared with the baseline across all time points in both trials (\( P < 0.05 \)). All doses of CR (0.25, 0.5, 1 or 2%) suspension significantly reduced C. jejuni counts when compared with its control (BPD) in both trials. The 2% CR suspension treatment was the most effective and reduced C. jejuni counts by at least \( \approx 3 \log_{10} \text{cfu/sample} \) at 0 h in both trials. Most of the tested doses of CR emulsion and nanoemulsion significantly reduced C. jejuni counts at 0, 8 and 24 h of storage when compared to Tween-80 (control) in both trials with the exception of 0.5% CR emulsion or nanoemulsion in trial 1, and 0.25 % CR nanoemulsion in trial 2. When the CR suspension was compared with the respective doses of either emulsion or nanoemulsion, no consistent difference in efficacy among the three different forms of CR was observed (except 1% CR suspension versus 1% CR nanoemulsion). When the CR emulsion versus nanoemulsion were compared within time points and doses, the 0.25 and 0.5% CR emulsions produced efficacy similar to the respective doses of nanoemulsion in both trials.
**The effect of CR on aerobic bacteria.** The total aerobic bacterial counts (Table 2) recovered from baseline was ~5.5 and 4.3 log_{10} cfu/sample in trial 1 and 2 respectively. Washing of skin sample with either BPD or 4% Tween-80 did not reduce aerobic counts in either trial when compared to the baseline (P > 0.05). Most of the CR suspension treatments significantly reduced aerobic counts as compared to the BPD controls in both trials (except for CR 0.25% at 0 and 24 h in trial 2). The 1% CR emulsion or 2% CR nanoemulsion in trial 1 and only 0.5% emulsion in trial 2 significantly reduced aerobic counts as compared to the baseline (P < 0.05), however, when compared with the 4% Tween-80, none of the treatments of either emulsion of nanoemulsion consistently reduced total aerobic counts in both trials (P > 0.05). When the CR suspension was compared with the respective doses of either emulsion or nanoemulsion, there was no consistent difference in efficacy among the three different forms of CR. The efficacy of CR emulsion and nanoemulsion were similar in majority of time points in both trials.

**Survival of C. jejuni in CR wash treatments**

Table 3 shows the survival of *C. jejuni* in the various wash treatments. The number of *C. jejuni* surviving in BPD wash treatments ranged from ~5.75 to 6.35 log_{10} cfu/mL in trial 1 and from ~6.13 to 6.50 log_{10} cfu/mL in trial 2. In case of the 4% Tween-80 solution, the number of *C. jejuni* was similar when compared with BPD (P > 0.05). However, *C. jejuni* counts were reduced to below detection limit (0.6 log_{10} cfu/mL) in all CR wash treatments.

**Effect of CR treatments on the color of skin**

Table 4 shows the effect of CR suspension, emulsion or nanoemulsion on the color of skin stored at 4°C for 0 and 24 h. Samples washed with control (BPD) showed lightness (L*), redness (a*) and yellowness (b*) values of 74.80 ± 1.54, 2.27 ± 0.57 and 10.80 ± 0.70 respectively for 0 h.
and 77.8 ± 0.76, 1.68 ± 0.49 and 10.08 ± 0.50 respectively for 24 h. None of the suspensions, emulsions or nanoemulsions of CR significantly changed the L*, a* and b* of samples at 0 h or at 24 h of storage.

5. Discussion

With increasing consumer demand for natural, antibiotic free and minimally processed foods, the use of GRAS status plant-derived compounds is gaining more attention as safe and effective antimicrobials for decontamination of poultry carcass. The rough surface of chicken skin facilitates attachment and survival of *C. jejuni* during the multiple stages of processing (Wempe et al., 1983; Corry and Atabay, 2001; Stern et al., 2001; Tan et al., 2014). Therefore, we used chicken skin as a model to represent the whole carcass for testing the efficacy of CR against *C. jejuni*.

Currently, peracetic acid (1000 ppm) and chlorine (50 ppm) are the most frequently used chemicals for carcass decontamination by poultry processors (USDA FSIS, 2017), however, the aforementioned chemicals results in minimal reduction of *C. jejuni* (Bauermeister et al., 2008; Nagel et al., 2013). In our preliminary trial, chlorine (50 ppm) and peracetic acid (1000 ppm) wash treatments reduced *C. jejuni* on chicken skin by ~0.34 and 1.75 log_{10} cfu/sample respectively (data not shown). In the present study, we observed that CR as an antimicrobial wash treatment significantly reduced *C. jejuni* counts on chicken skin. The reduction was up to 4 log_{10} cfu/sample for 2% CR compared with the samples washed in BPD controls (Figure 1). Similar results were reported previously against various pathogens on other foods. Ultee and coworkers (2000) reported that CR showed a dose dependent inhibition of *Bacillus cereus* in artificially inoculated cooked rice. Antibiotic-resistant *Salmonella enterica* was significantly reduced on celery (below detection limit; detection limit < 1 log_{10}) on day 0 and oysters (~5 log_{10})
on day 3 washed in 1% CR for 10 min or 1 h (Ravishankar et al., 2010). Upadhyay and coworkers (2014) showed that dipping (for 1 and 3 min) of cantaloupes rind plugs in 2% CR alone or in combination with 2% hydrogen peroxide significantly reduced *Listeria monocytogenes* on cantaloupes by > 2.0 to 2.5 log₁₀. The group also observed that CR with hydrogen peroxide reduced *L. monocytogenes* to undetectable level (detection limit < 1 log₁₀/cm²) with the 10 min dipping time at 25°C. Recently, a study demonstrated that three common foodborne pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Typhimurium) were significantly reduced on beef when immersed in 0.3 or 0.5% CR with teriyaki sauce after 1, 3, and 7 days stored at 4°C (Moon et al., 2017). The reduction of *C. jejuni* on inoculated chicken skin by CR shows its potential to be used as a natural safe chemical decontamination of chicken carcasses in processing plants.

Since meat and meat products are very perishable foods and require protection from microbial spoilage, the meat industry is constantly looking for intervention strategies to increase the shelf-life and safety of meat products. The use of a GRAS compound that reduces both foodborne pathogens and spoilage organisms could be a good option to improve safety and quality of food products. Many studies have been conducted using oregano oil to control spoilage microorganisms thereby increasing shelf-life of perishable foods (Chouliara et al., 2007; Mexis et al., 2009; Karabagias et al., 2011). Since most of the meat spoilage bacteria are either aerobic or facultative anaerobic (Gill and Greer, 1993), we evaluated the efficacy of CR against total aerobic bacteria. Data from our study showed that 1% and 2% CR suspension significantly reduced (P < 0.05) aerobic bacterial counts when compared to the controls (Figure 2). The maximum reduction (~2.5 log₁₀ cfu/sample) was obtained with 2% CR. Thus, CR could be used to inhibit the growth of meat spoilage organisms and thereby increasing the shelf-life of poultry products.
Although the majority of the essential oils are categorized as GRAS (Kabara, 1991), their use in the food products are greatly limited due to their flavor consideration and limited solubility in water. Recently, several studies have shown that formulation of emulsion and nanoemulsion significantly improved the water solubility and alter the antimicrobial efficacy of essential oils against various microorganisms (Donsì et al., 2011, 2012; Ghosh et al., 2013; Bhargava et al., 2015; Speranza et al., 2015). The oil in water nanoemulsion consists of a fine dispersion of ultra-small oil particles with diameter smaller than 400 nm (Arbor et al., 2008), whereas, emulsion (macro-emulsions) is characterized by particle size of 0.5 to 100 µm (Windhab et al., 2005). The decrease in oil droplet size in a colloidal solution leads to an increase in the total surface area for interaction between essential oils and bacteria thereby potentially enhancing the antimicrobial efficacy of essential oils (Solans et al., 2005; Weiss et al., 2009). Previous studies have explored the antimicrobial efficacy of CR emulsion and/or nanoemulsion against *Salmonella Enteritidis*, *Escherichia coli* O157: H7 (Landry et al., 2014, 2015) and *Lactobacillus plantarum* (Char et al., 2016). However, studies investigating CR emulsion/nanoemulsion efficacy against *C. jejuni* have not been conducted. Stable formulation of CR emulsion [surfactant-to-oil ratio (SOR)=1.25] and nanoemulsion (SOR=2) were selected from various SOR based on our preliminary experiments (Data not shown). The droplet size in CR nanoemulsion was more uniform than emulsion based on PDI (0.039 ± 0.01 vs 1 ± 0.12). However, the emulsion or nanoemulsion formulations did not enhance the antimicrobial efficacy of CR against *C. jejuni* on chicken skin samples versus the suspension alone (Table 1). In fact, for several CR suspension treatments, the anti-*Campylobacter* efficacy was found to be better in suspension than emulsion or nanoemulsion formulations. One potential possibility is the formation of transient, small droplets of CR (with increased surface area and efficacy) in the CR suspension while the treatments are vigorously shaken at washing step. This
could increase anti-\textit{Campylobacter} efficacy of CR suspension. \textit{C. jejuni} was not detected in the CR wash treatments (Table 3) indicating that the wash treatments would not lead to environmental contamination with \textit{C. jejuni}.

The type of microorganisms that colonize fresh meat products depend highly on the characteristics of meat, processing methods and storage conditions (Huis in’t Veld, 1996). In addition to the better anti-\textit{Campylobacter} activity of CR suspension over emulsion or nanoemulsion, we observed the effects of CR suspension is promising against aerobic bacteria when compared with other two forms of CR (Table 2).

The color of poultry carcasses and poultry meat products is one of the important attributes of product quality because it is directly related to the purchasing decision of consumers. The lightness value, L*, represents the darkest black at L* = 0, and the brightest white at L* = 100. The -a* to +a* and -b* to +b* represent green-red and blue-yellow respectively (Pathare et al., 2013). We did not observe a significant change (P > 0.05) in the color values (L*, a* and b*) either at 0 or 24 h after washing with CR suspension, emulsion or nanoemulsion treatments when compared with the controls (BPD or Tween-80) or baseline (Table 4). These results indicate that CR treatments could be used as a potential wash treatment to reduce the load of \textit{C. jejuni} and meat spoilage bacteria without changing chicken skin color.

In conclusion, CR suspension, emulsion and nanoemulsion as a wash treatment produced a consistent reduction in \textit{C. jejuni} counts on chicken skin. The reduction (~4 log\textsubscript{10} cfu/sample) by the highest dose of CR suspension is promising since a 2-log\textsubscript{10} reduction of \textit{Campylobacter} on the poultry carcass could reduce the risk of human campylobacteriosis by up to 30-fold (Rosenquist et al., 2003). In addition, CR suspension reduced aerobic bacteria counts without changing the color of chicken skin. Thus, CR treatments could be a good alternatives of conventional chemicals.
to reduce the *Campylobacter* and meat spoilage bacteria on the chicken carcasses; however, follow-up investigations testing the effects of CR should be conducted in an industrial setting prior to implementing these treatments in the poultry processing plant.
6. Acknowledgement

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8. References


Figure 1: Evaluating the efficacy of carvacrol suspension (0, 0.25, 0.5, 1, and 2% CR) as a wash treatment against *C. jejuni* on chicken skin. Inoculated chicken skin samples (~8 log₁₀ cfu/sample) were washed 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-c” designates the statistical difference among the treatments within the same time points (P < 0.05). Values (log₁₀ cfu/sample) presented as mean ± standard error of mean.
Figure 2: Evaluating the efficacy of carvacrol suspension (0, 0.25, 0.5, 1, and 2% CR) as a wash treatment against total aerobic bacteria on chicken skin at 0, 8 or 24 h of refrigerated storage. In each trial, “a-c” designates the statistical difference among the treatments within the same time points (P < 0.05). Values (log₁₀ cfu/sample) presented as mean ± standard error of mean.)
Table 1: Effect of carvacrol suspension, emulsion and nanoemulsion on *Campylobacter jejuni* survival on chicken skin\(^1,2\)

<table>
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<td>0.25%</td>
<td>5.08 ± 0.15(^ef)</td>
<td>4.50 ± 0.13(^g)</td>
<td>3.80 ± 0.07(^gh)</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>4.86 ± 0.12(^fg)</td>
<td>4.88 ± 0.23(^efg)</td>
<td>3.42 ± 0.08(^hi)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>4.40 ± 0.22(^g)</td>
<td>4.77 ± 0.39(^fg)</td>
<td>3.16 ± 0.27(^ij)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>3.49 ± 0.54(^h)</td>
<td>3.69 ± 0.40(^h)</td>
<td>2.80 ± 0.27(^j)</td>
</tr>
<tr>
<td></td>
<td>0.25%</td>
<td>5.53 ± 0.10(^de)</td>
<td>4.93 ± 0.12(^defg)</td>
<td>4.99 ± 0.08(^de)</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>5.23 ± 0.07(^ef)</td>
<td>5.71 ± 0.09(^bc)</td>
<td>4.68 ± 0.09(^ef)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>5.07 ± 0.08(^ef)</td>
<td>5.46 ± 0.22(^cd)</td>
<td>4.38 ± 0.08(^f)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>4.43 ± 0.10(^g)</td>
<td>5.45 ± 0.17(^cd)</td>
<td>3.22 ± 0.33(^i)</td>
</tr>
<tr>
<td></td>
<td>0.25%</td>
<td>5.96 ± 0.08(^cd)</td>
<td>5.29 ± 0.12(^def)</td>
<td>5.36 ± 0.17(^cd)</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>5.24 ± 0.08(^ef)</td>
<td>5.63 ± 0.16(^bc)</td>
<td>4.93 ± 0.15(^de)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>5.25 ± 0.04(^ef)</td>
<td>5.48 ± 0.19(^cd)</td>
<td>3.90 ± 0.17(^g)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>4.95 ± 0.13(^f)</td>
<td>5.42 ± 0.17(^de)</td>
<td>3.91 ± 0.12(^g)</td>
</tr>
<tr>
<td>_baseline</td>
<td>Baseline</td>
<td>7.10 ± 0.07(^a)</td>
<td>7.31 ± 0.06(^a)</td>
<td>7.08 ± 0.02(^a)</td>
</tr>
<tr>
<td>Controls</td>
<td>BPD</td>
<td>5.91 ± 0.09(^b)</td>
<td>5.86 ± 0.08(^b)</td>
<td>5.74 ± 0.08(^b)</td>
</tr>
<tr>
<td></td>
<td>4% Tween-80</td>
<td>6.03 ± 0.07(^b)</td>
<td>5.76 ± 0.09(^b)</td>
<td>5.70 ± 0.10(^b)</td>
</tr>
<tr>
<td></td>
<td>0.25%</td>
<td>4.98 ± 0.09(^cd)</td>
<td>4.56 ± 0.14(^e)</td>
<td>4.26 ± 0.30(^f)</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>4.17 ± 0.21(^f)</td>
<td>3.82 ± 0.25(^fs)</td>
<td>4.18 ± 0.24(^fg)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>4.15 ± 0.18(^f)</td>
<td>3.57 ± 0.26(^g)</td>
<td>2.69 ± 0.28(^i)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>2.46 ± 0.53(^g)</td>
<td>3.53 ± 0.09(^g)</td>
<td>3.33 ± 0.23(^h)</td>
</tr>
<tr>
<td></td>
<td>0.25%</td>
<td>5.00 ± 0.17(^cd)</td>
<td>4.98 ± 0.15(^ed)</td>
<td>4.94 ± 0.06(^ed)</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>4.70 ± 0.18(^de)</td>
<td>4.50 ± 0.06(^e)</td>
<td>4.26 ± 0.18(^ef)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>4.26 ± 0.11(^ef)</td>
<td>4.00 ± 0.12(^f)</td>
<td>3.93 ± 0.24(^fg)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>4.26 ± 0.11(^f)</td>
<td>4.02 ± 0.21(^f)</td>
<td>3.96 ± 0.26(^fg)</td>
</tr>
<tr>
<td></td>
<td>0.25%</td>
<td>5.49 ± 0.10(^bc)</td>
<td>5.15 ± 0.13(^c)</td>
<td>5.14 ± 0.10(^f)</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>5.00 ± 0.15(^cd)</td>
<td>4.54 ± 0.12(^e)</td>
<td>4.39 ± 0.18(^ef)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>4.79 ± 0.07(^de)</td>
<td>4.67 ± 0.06(^de)</td>
<td>4.51 ± 0.10(^de)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>4.30 ± 0.12(^ef)</td>
<td>3.83 ± 0.14(^fg)</td>
<td>3.70 ± 0.08(^gh)</td>
</tr>
</tbody>
</table>

\(^1\)n = 5 replicates per treatment per time point per trial. Values (log\(_{10}\) CFU/sample) presented as mean ± standard error of mean.

\(^2\)C. jejuni counts within column in the same trial with no common superscript differ significantly (P < 0.05).
Table 2: Effect of carvacrol suspension, emulsion and nanoemulsion against aerobic bacteria counts on chicken skin\(^1,2\)

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatments</th>
<th>0 h</th>
<th>8 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5.76 ± 0.07(^a)</td>
<td>5.43 ± 0.19(^a)</td>
<td>5.15 ± 0.06(^a)</td>
</tr>
<tr>
<td>Baseline</td>
<td>BPD</td>
<td>5.78 ± 0.09(^a)</td>
<td>4.86 ± 0.15(^ab)</td>
<td>4.90 ± 0.22(^ab)</td>
</tr>
<tr>
<td>Controls</td>
<td>4% Tween-80</td>
<td>5.58 ± 0.09(^ab)</td>
<td>4.67 ± 0.11(^bcd)</td>
<td>4.87 ± 0.26(^ab)</td>
</tr>
<tr>
<td>1% Suspension</td>
<td>0.25%</td>
<td>4.88 ± 0.15(^cde)</td>
<td>3.93 ± 0.19(^fg)</td>
<td>3.19 ± 0.30(^e)</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>4.66 ± 0.22(^c)</td>
<td>3.54 ± 0.46(^fg)</td>
<td>3.66 ± 0.23(^de)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>4.67 ± 0.36(^de)</td>
<td>3.52 ± 0.22(^fg)</td>
<td>3.47 ± 0.21(^e)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>3.95 ± 0.15(^f)</td>
<td>3.43 ± 0.41(^g)</td>
<td>4.11 ± 0.22(^ed)</td>
</tr>
<tr>
<td>1% Emulsion</td>
<td>0.25%</td>
<td>5.61 ± 0.11(^ab)</td>
<td>4.04 ± 0.3(^defg)</td>
<td>4.28 ± 0.10(^c)</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>5.33 ± 0.08(^abc)</td>
<td>4.24 ± 0.28(^bcdef)</td>
<td>4.85 ± 0.22(^ab)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>4.89 ± 0.29(^cde)</td>
<td>4.12 ± 0.32(^cddefg)</td>
<td>4.37 ± 0.33(^bc)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>4.57 ± 0.26(^e)</td>
<td>4.87 ± 0.15(^a)</td>
<td>4.55 ± 0.09(^bc)</td>
</tr>
<tr>
<td>1% Nanoemulsion</td>
<td>0.25%</td>
<td>5.57 ± 0.06(^ab)</td>
<td>4.71 ± 0.44(^abcd)</td>
<td>5.23 ± 0.07(^a)</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>5.76 ± 0.07(^a)</td>
<td>4.40 ± 0.15(^bcde)</td>
<td>4.19 ± 0.11(^cd)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>5.01 ± 0.2(^cde)</td>
<td>4.78 ± 0.22(^abc)</td>
<td>4.40 ± 0.03(^bc)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>5.16 ± 0.11(^bcd)</td>
<td>4.62 ± 0.19(^bcde)</td>
<td>4.45 ± 0.15(^bc)</td>
</tr>
<tr>
<td>2% Baseline</td>
<td>BPD</td>
<td>4.23 ± 0.08(^ab)</td>
<td>4.31 ± 0.06(^a)</td>
<td>4.29 ± 0.11(^a)</td>
</tr>
<tr>
<td>Controls</td>
<td>4% Tween-80</td>
<td>3.89 ± 0.09(^bcde)</td>
<td>3.85 ± 0.11(^abc)</td>
<td>4.14 ± 0.20(^ab)</td>
</tr>
<tr>
<td>0.25% Suspension</td>
<td>4%</td>
<td>3.78 ± 0.08(^bcde)</td>
<td>4.16 ± 0.24(^ab)</td>
<td>4.02 ± 0.08(^abc)</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>4.10 ± 0.20(^b)</td>
<td>3.14 ± 0.19(^de)</td>
<td>3.71 ± 0.24(^abc)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>2.65 ± 0.18(^gh)</td>
<td>2.34 ± 0.16(^f)</td>
<td>2.91 ± 0.29(^de)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>2.18 ± 0.12(^h)</td>
<td>2.46 ± 0.14(^f)</td>
<td>2.31 ± 0.2(^f)</td>
</tr>
<tr>
<td>2% Emulsion</td>
<td>0.25%</td>
<td>3.93 ± 0.22(^bcd)</td>
<td>3.54 ± 0.11(^ed)</td>
<td>3.52 ± 0.12(^e)</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>3.50 ± 0.17(^de)</td>
<td>3.00 ± 0.14(^e)</td>
<td>2.91 ± 0.26(^de)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>3.40 ± 0.07(^ef)</td>
<td>3.97 ± 0.17(^abc)</td>
<td>3.61 ± 0.06(^bc)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>4.24 ± 0.34(^ab)</td>
<td>3.98 ± 0.19(^abc)</td>
<td>3.44 ± 0.30(^ed)</td>
</tr>
<tr>
<td>2% Nanoemulsion</td>
<td>0.25%</td>
<td>4.05 ± 0.23(^bc)</td>
<td>4.03 ± 0.27(^abc)</td>
<td>4.16 ± 0.16(^ab)</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>3.73 ± 0.10(^bcde)</td>
<td>3.67 ± 0.08(^bc)</td>
<td>3.76 ± 0.09(^abc)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>3.56 ± 0.02(^cde)</td>
<td>3.92 ± 0.22(^abc)</td>
<td>3.92 ± 0.25(^abc)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>4.71 ± 0.23(^a)</td>
<td>3.86 ± 0.15(^abc)</td>
<td>3.88 ± 0.28(^abc)</td>
</tr>
</tbody>
</table>

\(^{1}\)n = 5 replicates per treatment per time point per trial. Values (log_{10} cfu/sample) presented as mean ± standard error of mean.

\(^{2}\)Aerobic bacteria counts within column in the same trial with no common superscript differ significantly (P < 0.05).
Table 3: Effect of different doses of carvacrol on survival of *Campylobacter jejuni* in wash treatments¹,²

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatments</th>
<th>0 h</th>
<th>8 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BPD</td>
<td>6.11 ± 0.09</td>
<td>5.75 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>4% Tween-80</td>
<td>5.98 ± 0.09</td>
<td>5.80 ± 0.11</td>
<td>6.40 ± 0.26</td>
</tr>
<tr>
<td>Controls</td>
<td>0.25%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1 Emulsion</td>
<td>0.25%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>2%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2 Emulsion</td>
<td>0.25%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>0.25%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

¹ n = 5 replicates per treatment per time point per trial. Values (log₁₀ cfu/mL) presented as mean ± standard error of mean. Within the same trial at the same time, BPD and 4% Tween-80 were not significantly different (P > 0.05).

² ND = below detection limit, detection limit ≥ 0.6 log₁₀ cfu/mL
Table 4: Color values of chicken skin samples treated with carvacrola

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Treatments</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>74.78 ± 0.60</td>
<td>2.06 ± 0.16</td>
<td>10.18 ± 0.40</td>
</tr>
<tr>
<td>Controls</td>
<td>BPD</td>
<td>74.80 ± 1.54</td>
<td>2.27 ± 0.57</td>
<td>10.80 ± 0.70</td>
</tr>
<tr>
<td></td>
<td>4% Tween-80</td>
<td>75.22 ± 0.64</td>
<td>2.02 ± 0.14</td>
<td>10.40 ± 0.31</td>
</tr>
<tr>
<td>Suspension</td>
<td>0.25%</td>
<td>76.10 ± 0.46</td>
<td>2.44 ± 0.41</td>
<td>10.18 ± 1.03</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>76.86 ± 0.81</td>
<td>2.43 ± 0.30</td>
<td>9.85 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>74.28 ± 0.51</td>
<td>2.74 ± 0.26</td>
<td>10.93 ± 0.71</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>73.81 ± 0.82</td>
<td>2.09 ± 0.33</td>
<td>10.70 ± 0.54</td>
</tr>
<tr>
<td>Emulsion</td>
<td>0.25%</td>
<td>75.06 ± 1.10</td>
<td>1.83 ± 0.09</td>
<td>9.70 ± 1.03</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>75.13 ± 1.94</td>
<td>1.81 ± 0.46</td>
<td>9.17 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>73.99 ± 1.55</td>
<td>1.88 ± 0.21</td>
<td>9.61 ± 0.77</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>73.73 ± 1.40</td>
<td>2.24 ± 0.34</td>
<td>10.43 ± 0.97</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>0.25%</td>
<td>75.40 ± 1.30</td>
<td>1.70 ± 0.31</td>
<td>9.69 ± 1.03</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>74.95 ± 1.40</td>
<td>2.34 ± 0.82</td>
<td>9.88 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>74.95 ± 0.98</td>
<td>1.76 ± 0.19</td>
<td>9.35 ± 0.61</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>75.16 ± 0.96</td>
<td>1.72 ± 0.23</td>
<td>11.00 ± 0.78</td>
</tr>
<tr>
<td>Baseline</td>
<td>76.80 ± 0.39</td>
<td>1.69 ± 0.11</td>
<td>9.30 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>BPD</td>
<td>77.80 ± 0.76</td>
<td>1.68 ± 0.49</td>
<td>10.08 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>4% Tween-80</td>
<td>76.76 ± 0.73</td>
<td>1.78 ± 0.16</td>
<td>9.46 ± 0.30</td>
</tr>
<tr>
<td>Suspension</td>
<td>0.25%</td>
<td>78.93 ± 0.67</td>
<td>2.43 ± 0.35</td>
<td>10.31 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>79.14 ± 0.85</td>
<td>2.36 ± 0.59</td>
<td>10.48 ± 1.15</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>78.83 ± 0.50</td>
<td>1.36 ± 0.50</td>
<td>9.66 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>78.89 ± 0.38</td>
<td>1.33 ± 0.84</td>
<td>10.68 ± 0.39</td>
</tr>
<tr>
<td>Emulsion</td>
<td>0.25%</td>
<td>77.68 ± 1.37</td>
<td>1.26 ± 0.50</td>
<td>9.45 ± 0.90</td>
</tr>
<tr>
<td></td>
<td>0.50%</td>
<td>77.51 ± 2.57</td>
<td>1.47 ± 0.39</td>
<td>9.00 ± 1.87</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>77.65 ± 0.98</td>
<td>1.75 ± 0.39</td>
<td>9.88 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>76.60 ± 0.56</td>
<td>1.79 ± 0.17</td>
<td>10.44 ± 0.65</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>0.25%</td>
<td>76.05 ± 1.12</td>
<td>1.41 ± 0.30</td>
<td>9.23 ± 1.04</td>
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<tr>
<td></td>
<td>0.50%</td>
<td>76.96 ± 1.98</td>
<td>1.90 ± 0.41</td>
<td>8.93 ± 0.66</td>
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<tr>
<td></td>
<td>1%</td>
<td>77.48 ± 1.31</td>
<td>1.57 ± 0.42</td>
<td>9.97 ± 0.98</td>
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<td></td>
<td>2%</td>
<td>76.02 ± 0.86</td>
<td>1.48 ± 0.46</td>
<td>9.39 ± 1.04</td>
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a n= 5 replicates per treatment per time. Values (means ± standard error of the mean). Within the same column at the same time, none of the treatments were significantly different with the controls (BPD or 4% Tween-80) or baseline (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.
PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:

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

<table>
<thead>
<tr>
<th>Name: (first and last)</th>
<th>POSITION (Title, academic degrees, certifications, and material field of expertise)</th>
<th>QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE</th>
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</thead>
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<tr>
<td>Example:</td>
<td></td>
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</tr>
<tr>
<td>Bob Biohazard -</td>
<td>Associate Professor, PhD Microbiology</td>
<td>14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.</td>
</tr>
<tr>
<td>Dan J. Donoghue, Ph.D.</td>
<td>Professor, Food Safety</td>
<td>over 20 years experience as PI running research laboratory working with BL-2 pathogens.</td>
</tr>
<tr>
<td>Ann M. Donoghue, Ph.D.</td>
<td>Research Leader, Food safety</td>
<td>over 20 years experience as PI running research laboratory working with BL-2 pathogens.</td>
</tr>
<tr>
<td>Komala Arsi, Ph.D.</td>
<td>Post-doctoral fellow</td>
<td>Post-doctoral fellow trained under Dr. Donoghue's supervision</td>
</tr>
<tr>
<td>Abhinav Upadhyay, Ph.D.</td>
<td>Post-doctoral fellow</td>
<td>Post-doctoral fellow trained under Dr. Donoghue's supervision</td>
</tr>
<tr>
<td>Indu Upadhyaya, Ph.D.</td>
<td>Post-doctoral fellow</td>
<td>Post-doctoral fellow trained under Dr. Donoghue's supervision</td>
</tr>
<tr>
<td>Basanta Raj Wagle, Ph.D.</td>
<td>Program Associate</td>
<td>Program Associate under Dr. Donoghue's supervision</td>
</tr>
<tr>
<td>Sandip Shrestha</td>
<td></td>
<td>Graduate assistant trained under Dr. Donoghue's supervision</td>
</tr>
</tbody>
</table>

Additional Personnel Information (if needed):

Page 6 of 12
Edible coatings fortified with carvacrol reduce *Campylobacter jejuni* on chicken wingettes and modulate expression of select virulence genes
Title: Edible coatings fortified with carvacrol reduce *Campylobacter jejuni* on chicken wingettes and modulate expression of select virulence genes

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**Written for Food Control**

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1. Abstract

*Campylobacter jejuni* is a leading cause of foodborne disease in humans, and associated primarily with consumption of contaminated poultry and poultry products. Intervention strategies aimed at reducing *C. jejuni* contamination on poultry products could significantly reduce *C. jejuni* infection in humans. Antimicrobial edible coatings are increasingly being used by food producers to prevent food contamination against pathogens. This study evaluated the efficacy of gum arabic (GA) and chitosan (CH) fortified with carvacrol (CR) as an antimicrobial coating treatment for reducing *C. jejuni* on chicken wingettes. Aforementioned compounds are generally recognized as safe (GRAS) status compounds obtained from gum arabic tree, crustaceans and oregano oil respectively. A total of four separate trials were conducted in which wingettes were randomly assigned to baseline (no coating), saline control (wingettes washed with saline), GA (10%), CH (2%), CR (0.25, 0.5 or 1%) or their combinations. Each wingette was inoculated with a cocktail of four wild-type strains of *C. jejuni* (~7.5 log_{10} cfu/sample). Following 1 min of coating in aforementioned treatments, wingettes were air dried (1 h) and sampled at 0, 1, 3, 5 and 7 days of refrigerated storage for *C. jejuni* and total aerobic counts (n = 5 wingettes/treatment/day). In addition, the effect of treatments on wingette color was measured using a Minolta colorimeter. Furthermore, the effect of treatments on the expression of *C. jejuni* survival/virulence genes was evaluated using real-time quantitative PCR. Results showed that all three doses of CR, CH or GA-based coating fortified with CR reduced *C. jejuni* from day 0 through 7 by up to 3.0 log_{10} cfu/sample (P < 0.05). The antimicrobial efficacy of GA was improved by CR and the coatings reduced *C. jejuni* by ~1 to 2 log_{10} cfu/sample at day 7. In addition, CH-CR coatings reduced total aerobic counts when compared with non-coated samples for a majority of the storage times. No significant difference in the color of chicken wingettes was observed between treatments.
Exposure of pathogen to sublethal concentrations of CR, CH or combination significantly modulated select genes encoding for energy taxis \((cetB)\), motility \((motA)\), binding \((cadF)\) and attachment \((jlpA)\). The results suggest that GA or CH-based coating with CR could potentially be used as a natural antimicrobial to control \(C.\ jejuni\) in post-harvest poultry products.

**Key words:** Campylobacter, Carvacrol, Gum Arabic, Chitosan, Poultry, Antibiotic alternative
2. Introduction

Campylobacter jejuni infection in humans continues to be a significant public health problem throughout the world (WHO, 2018). In the United States, Campylobacter causes 1.3 million illnesses each year and is often associated with consumption of contaminated chicken meat (CDC, 2018). Epidemiological studies have shown that up to 70% to 80% of retail raw chicken meat in the United States is contaminated with Campylobacter (Cui et al., 2005). Recently, the incidence of Campylobacter in the United States surpassed that of Salmonella (17.43 vs 16.66 per 100,000 people; Marder et al., 2017). The high level (10⁷/g of cecal content) of Campylobacter in the ceca of market age birds (Beery et al., 1988) leads to potential carcass contamination at processing plants thereby posing a serious public health threat. The concerns are further raised due to the low infective dose (∼500 cells; Black et al., 1988) required to cause infection in humans and potentially fatal sequelae such as Guillain-Barré syndrome (Rhodes and Tattersfield, 1982).

Conventional poultry processing constitutes several steps (scalding, picking, evisceration, chilling) that reduces but does not eliminate Campylobacter contamination on carcasses (Elvers et al., 2011; Alonso-Hernando et al., 2013). In addition, poultry processors in the United States heavily rely on the use of inorganic/synthetic chemicals, such as peracetic acid, trisodium phosphate and chlorine-based compounds to reduce poultry carcass contamination (Keener et al., 2004; Oyarzabal, 2005; McKee, 2011). However, with increasing consumer demand for safe, natural and minimally processed foods, the use of natural, plant-derived antimicrobials with generally recognized as safe (GRAS) status is gaining attention for improving safety of poultry products. These compounds are naturally derived metabolites and/or by-products from various plants sources and have been used as the chief source of antimicrobials in human medicine for thousands of years (Solecki, 1975; Fabricant and Farnsworth, 2001). The antimicrobial activity of
several plant-derived compounds has been documented (Nychas, 1995; Savoia, 2012). Carvacrol (CR; 5-isopropyl-2-methylphenol) is a polyphenolic compound which is present in the essential oil fractions of oregano (60 to 74% carvacrol) and thyme (45% carvacrol) (Lagouri et al., 1993). Studies have shown that this compound has significant antibacterial properties against a wide range of foodborne pathogens including Salmonella spp. (Kim et al., 1995; Kollanoor Johny et al., 2010; Mattson et al., 2011), Campylobacter jejuni (Ravishankar et al., 2008), Listeria monocytogenes (Upadhyay et al., 2015), E. coli O157:H7 (Du et al., 2008), and Bacillus cereus (Ultee et al., 2002). Additionally, recent studies have shown that CR can potentially reduce carcinogenesis, suggesting that CR could be used to reduce the proliferation of cancer cells (Özkan et al., 2011; Jayakumar et al., 2012; Suntres et al., 2015). Carvacrol is currently listed as GRAS by the U.S. Food and Drug Administration (Code of Federal Regulations 21 part 172).

Over the last decade, significant research on the use of antimicrobial films or coating materials for improving microbiological safety and shelf-life of food products has been undertaken (Dutta et al., 2009; Valencia-Chamorro et al., 2011). The application of antimicrobial edible coatings onto the surface of raw poultry carcass could be an alternative to reduce foodborne pathogens including Campylobacter on poultry products. Antimicrobial edible coatings, due to their presence on products, reduces the chance of cross-contamination during storage and handling. Gum arabic (GA) is obtained from the gum arabic tree (Acacia senegal or Senegalia senegal) and is composed of a highly branched arrangement of simple sugars galactose, arabinose, rhamnose, and glucuronic acids (Anderson and Stoddart, 1966; Street and Anderson, 1983; Phillips, 1998; Nussinovitch, 2009). The mixture of polysaccharides and glycoproteins gives GA the properties of a glue and binder which is edible for humans. Since it is safe for human consumption, it is one of the most commonly used natural coatings
for various food surfaces and has been used in variety of food preparations such as soft drink syrup, hard gummy candies, marshmallows and nougats (Foodnavigator-usa, 2008; Dauqan and Abdullah, 2013; Patel and Goyal, 2015; FDA, 2018). Pharmaceutical drugs and cosmetics also use the gum as a binder, emulsifying agent and a thickening agent (Smolinske, 1992). In addition, wine makers have used GA as a wine fining agent (Vivas et al., 2001). Recently, the use of GA as a coating to improve shelf-life as well as safety of different food products has been studied. Ali and colleagues (2010) found that 10% GA coating enhanced shelf-life and improved post-harvest quality of tomatoes. Upadhyaya and co-workers (2016) found that 10% GA fortified with different phytochemicals (carvacrol, eugenol or β-resorcylic acid) significantly reduced *Salmonella* Enteritidis counts on shell eggs. Similarly, chitosan (CH), a polysaccharide obtained from crustaceans, is another compound that has been extensively studied as an antimicrobial coating on food products and employed as an effective antimicrobial for reducing various foodborne pathogens including *Listeria monocytogenes* (Upadhyay et al., 2015), *Salmonella* Typhimurium (Menconi et al., 2013) and *Campylobacter jejuni* (Woo-Ming, 2015). The CH-based coatings are non-toxic, non-polluting, biodegradable, edible, and are easy to use in industry setting (Kong et al., 2010; Aider, 2010; Sánchez-González et al., 2011; Elsabee and Abdou, 2013). Both GA and CH are classified as GRAS by the U.S. FDA for use in foods (Code of Federal Regulations 21 part 184, 170).

The aim of the present study was to investigate the anti-*Campylobacter* effect of 10% GA or 2% medium molecular weight (MMW) CH coatings fortified with CR (0.25, 0.5 and 1%) on inoculated chicken wingettes. In addition, the effect of the aforementioned treatments on the color of chicken wingettes was evaluated. The effect of select treatments on the expression of
genes critical for the survival of *C. jejuni* in the environment and virulence was also investigated.

3. Material and Methods

3.1. *C. jejuni* inoculum preparation

Four wild-type strains (S-1, S-3, S-4, and S-8) of *C. jejuni*, previously isolated by our laboratory were used in this experiment. *C. jejuni* inoculum was prepared as described by Shrestha and others (Shrestha et al., 2017). Briefly, one loopful of glycerol stock of the wild-type strain *C. jejuni* was inoculated into 5 mL of *Campylobacter* Enrichment Broth (CEB; catalogue no. 7526A, Neogen Corp, Lansing, MI) and incubated at 42°C in a microaerophilic atmosphere (5% O₂, 10% CO₂, and 85% N₂) for 48 h. Each strain was sub-cultured again at the same temperature and atmospheric conditions for 24 h. Sub-cultured *C. jejuni* was centrifuged at 3500 × g for 10 min, the supernatant discarded and the cell pellet from each strain was mixed and resuspended in required volume of Butterfield’s Phosphate Diluent (BPD; 0.625 mM potassium dihydrogen phosphate, pH 6.67). The resulting suspension was used as inoculum (final bacterial concentration was ∼8.5 to 9.0 log₁₀ cfu/mL). The bacterial count in the four-strain cocktail was confirmed by plating 100 μL of culture suspension and its 10-fold dilution on *Campylobacter* line agar (CLA; Line, 2001) followed by incubation at 42°C in a microaerophilic atmosphere for 48 h.

3.2. Wingette sample preparation

Chicken wings were procured from the University of Arkansas Poultry Processing Plant (Fayetteville, AR). The middle portion (wingette) of the whole wing was separated by cutting on shoulder and elbow joints. Separated wingettes were stored at -20°C until the day of experiment.
3.3. CR suspension/wash treatment preparation

Carvacrol (catalogue no. W224502, Sigma-Aldrich Co., St. Louis, MO) was suspended in appropriate volume of sterile BPD solution to obtain 0.25, 0.5, 1% CR suspension in BPD. For example, 2 mL of CR was suspended in 198 mL of sterile BPD to get 1% CR suspension. The suspension was stirred at 300 rpm for 15 min before loading to sterile Whirl-Pak™ bag (catalogue no. 018126C, Nasco, Fisher Scientific, Suwanee, GA).

3.4. Coating treatment preparation

The GA (catalogue no. G9752, Sigma-Aldrich CO., St. Louis, MO) coating solution was prepared based on previously published article (Upadhyaya et al., 2016) with slight modification. In brief, 10 g of GA were added to 100 mL of sterile BPD. The solution was then stirred for 1 h at room temperature. Similarly, 2% MMW CH (190 – 310 kDa) (catalogue no. 448877, Sigma-Aldrich, St. Louis, MO) solution was prepared by using the method developed by Woo-Ming (2015). Briefly, 2 g of MMW CH powder was dissolved in 100 mL of 50 mM acetic acid (catalogue no. A38C212, Fisher Scientific, Fair Lawn, New Jersey) solution by stirring overnight at room temperature. To these coating solutions, required amounts of CR were added and mixed continuously for 12 h at room temperature to prepare 0.25, 0.5, and 1% coating treatments with CR.

3.5. Evaluation of antimicrobial activity of coating treatments on chicken wingettes

Coatings of the chicken wingettes was based on the protocol by Woo-Ming (2015). A total of two trials were conducted in the coating experiment with GA. In each trial, 225 thawed wingettes were individually inoculated with 50 µL (~7.5 log\textsubscript{10} cfu/sample) \textit{C. jejuni} and were allowed to adhere for 30 min. Wingettes were randomly divided into nine different treatment
groups which included baseline, BPD (wash control), 10% GA (coating control), CR (0.25, 0.5 and 1%) or CR (0.25, 0.5 and 1%) + 10% GA. Each wingette was individually placed in a sample bag containing 10 mL of the respective treatment for coating/dipping. Working with one treatment group at a time, wingettes were vigorously shaken/massaged for 1 min to obtain a complete coating. After coating/dipping, wingettes were removed from bags and allowed to dry for 1 h (30 min on each side). Wingettes were divided into sampling times (n = 5/treatment/time) at days 0, 1, 3, 5 or 7. To process the day 0 samples, microbial analysis was done immediately after drying while other wingettes were vacuum sealed by a commercially available vacuum sealer (Ziploc® V201, Lake Barrington, IL) and store at 4°C until the day of sampling. The antimicrobial effect of CH coating with CR was evaluated as described above. In each trial, the treatments were baseline, BPD (wash/dip control), 50 mM acetic acid (CH control), 2% CH (coating control), CR (0.25%, 0.5% and 1%) or CR (0.25%, 0.5% and 1%) + 2% CH. For each treatment, 5 wingettes were tested per time point (n = 5/treatment/time).

3.6. Microbial analysis

For all experiments, the samples were individually removed from vacuum packaging and aseptically transferred to a stomacher® 400 classic bag (catalogue no. BA6041, Steward Ltd., Worthing, West Sussex, UK) containing 30 mL of Dey-Engley neutralizing broth (catalogue no. C7371, Hardy diagnostic, Santa Maria) followed by blending for 30 s at 250 rpm (Stomacher® 400 Circulator, Steward Ltd., Worthing, West Sussex, UK). For all samples ten-fold dilutions were prepared from initial dilution in sterile BPD. Each dilution was surface plated onto CLA followed by incubation at 42°C microaerobically for 48 h. For aerobic bacterial enumeration each dilution was plated onto tryptic soy agar (TSA; catalogue no. DF0369176, Becton, Dickinson and
Company, Sparks, MD) followed by incubation at 37°C for 24 h. Bacterial colonies were counted and expressed as cfu/sample.

3.7. Color analysis

Color of wingettes was measured as described by Wagle et al. (2017b). International Commission on Illumination (CIE) L* (lightness), a* (redness), and b* (yellowness) values for each wingette were evaluated using a Minolta colorimeter (CR-300, Konica Minolta Sensing Inc., Japan). The instrument was calibrated against a white tile before the measurements. Three readings were taken on the lateral surface of each wingette, averaged and analyzed.

3.8. RNA extraction, cDNA synthesis, and real-time quantitative PCR

The effect of sub-inhibitory concentrations of CR (0.002%), CH (0.0125%) or CR (0.002%)+CH (0.0125%) combination on expression of selected virulence genes of C. jejuni was determined using a previously published method (Wagle et al., 2017b) with slight modification. Briefly, frozen whole chicken carcasses were obtained from the University of Arkansas poultry pilot processing plant (Fayetteville, AR) and thawed at 4°C for 12 h. The meat exudate was collected into sterile centrifuged tubes followed by centrifugation at 3,000 × g for 20 min. The debris were removed, and the juice was filter sterilized with different size filters (0.8, 0.45 and finally through 0.2-µm cellulose acetate membrane (catalogue no. 28151261 (0.8-µm), 10035088 (0.4-µm), 14224474 (0.2-µm), VWR International, West Chester, PA). C. jejuni S-8 was incubated in chicken juice with or without sub-inhibitory concentrations of CR, CH or CR+CH at room temperature for approximately 1 h under aerobic condition. Total RNA was extracted using RNA mini kit (catalogue no. 12183018A, Invitrogen, Carlsbad, CA, USA). DNase treatment (catalogue no. 18068015, Thermo Fisher Scientific, Carlsbad, CA, USA) was done followed by the
complementary DNA (cDNA) preparation using iScript cDNA synthesis kit (catalogue no. 1708890, Bio-Rad Laboratories, Inc., Hercules, CA). All the primers in this study (Table 1) were designed from published Gene Bank C. jejuni sequences using Primer 3 software (National Center for Biotechnology Information) and obtained from Integrated DNA Technologies. The cDNA was used as the template for PCR reaction and the amplified product was detected by SYBR Green reagent (catalogue no. 1708880, iQ SYBR Green Supermix, Bio-Rad). Data were normalized to endogenous control (16S rRNA) and expression of candidate genes were analyzed using comparative critical threshold method on Quant Studio 3 real-time PCR system (Applied Biosystem, Thermo Fisher).

3.9. Statistical analysis

The bacterial counts were log10 transformed (log10 cfu/sample) for analysis to achieve homogeneity of variance (Byrd et al., 2001). For the gene expression analysis, data were pooled and expressed as log10 of relative quantification (RQ) and were analyzed using ANOVA with the PROC MIXED procedure in SAS statistical software, version 9.3 (SAS Institute Inc., Cary, NC). Means were partitioned by LSMEANS analysis, and a P < 0.05 was required for statistical significance.

4. Results

4.1. Efficacy of GA-based or CH-based coating treatments (with or without CR) in reducing C. jejuni on chicken wingettes

Table 2 shows the effect of 10% GA coating alone or fortified with CR (0.25, 0.5 or 1%) in reducing C. jejuni on chicken wingettes. The C. jejuni counts recovered from the baseline group (inoculated wingettes group not subjected to any treatment) ranged from ~6.3 to 7.0 log10
cfu/sample. In both trials, samples washed with the BPD control showed significant reduction (~1 log_{10} cfu/sample) of *C. jejuni* counts from day 0 through day 7, when compared to the baseline group. As BPD has no anti-*Campylobacter* property, the observed reduction was probably due to washing away of loosely attached *C. jejuni* cells by the solution. Coating with the 10% GA showed consistent reduction (P < 0.05) in *C. jejuni* counts when compared to the baseline group, however, did not show significant difference with the BPD control in both the trials. All the tested doses of CR (0.25, 0.5 or 1%) significantly reduced *C. jejuni* counts from day 0 to day 7 in both the trials when compared with the BPD control. There was significant difference in anti-*Campylobacter* efficacy between 0.25% CR and 1% CR in trial 1, however, the results were not consistent between trials. The combination groups of 10% GA with different doses of CR (0.25, 0.5 or 1%) consistently reduced *C. jejuni* counts at all days in both trials when compared to the 10% GA control (P < 0.05). For example, addition of 1% CR in GA produced additional reduction in *C. jejuni* counts by ~1.8 log_{10} cfu/sample (trial 1) and ~1.2 log_{10} cfu/sample (trial 2) at day 0 as compared to the GA alone. The difference in antimicrobial efficacy persisted during the storage period between GA and combination treatments. The anti-*Campylobacter* efficacy of GA-CR coating treatments was similar on majority of storage time points in both trials when compared with the respective doses of CR alone.

In an attempt to test the effect of different edible coatings fortified with CR, further testing was done with 2% CH as a coating material (Table 3). As shown in Table 3, the number of *C. jejuni* recovered from the baseline group ranged from ~6.1 to 6.7 log_{10} cfu/sample in trial 1 and ~6.6 to 7.3 log_{10} cfu/sample in trial 2. The BPD control significantly (P < 0.05) reduced the *C. jejuni* counts by ~1 log_{10} cfu/sample at days 3 and 5 in trial 1, whereas in trial 2, the counts were significantly reduced throughout the sampling days when compared to the baseline. The number
of *C. jejuni* recovered from skin samples treated with the 50 mM acetic acid solution (with adjusted pH ~6.4) was not significantly different when compared with the *C. jejuni* counts recovered from samples washed with the BPD (except at day 1 in trial 2). The 2% CH coating consistently reduce *C. jejuni* counts in both trials as compared to baseline counts. When compared with its control (50 mM acetic acid solution), the 2% CH consistently reduced *C. jejuni* counts by ~1 to 1.5 log$_{10}$ cfu/sample, except at day 0 in trial 2 (P < 0.05). As observed in table 2, all the tested doses of CR (0.25, 0.5 and 1%) significantly reduced *C. jejuni* counts from day 0 through 7 in both trials when compared with the BPD control (P < 0.05). The combination treatments were more effective than CH alone at select time points, however, a consistent improvement in the antimicrobial efficacy was not observed. For example, all the combination treatments were more effective than CH at day 1 in trial 1 and at day 0 in trial 2. However, by day 7, the combination treatments and CH coating were similar in their efficacy in reducing *C. jejuni*. A similar pattern was observed when the combination treatments were compared with CR. Only select combination treatments showed increased efficacy as compared to CR alone at various stages during refrigerated storage. For example, the combination of lowest dose (0.25%) of CR and CH increased antimicrobial efficacy by ~0.5 log as compared with the 0.25% CR at days 3 and 7 in trial 1 and days 1 and 7 in trial 2. The 0.5% CR+CH combination was more effective than 0.5% CR treatment at days 1, 5 and 7 in trial 1 and at days 1 and 5 in trial 2. The combination of highest dose (1%) of CR with CH had increased efficacy at days 1 and 3 in trial 1; however, had decreased efficacy at day 3 in trial 2. The combination treatment was similar to corresponding CR treatments on rest of the storage days in both trials.
4.2. Efficacy of GA-based or CH-based coating treatments (with or without CR) in reducing aerobic bacterial counts on chicken wingettes

Table 4 shows the effect of GA (10%), CR (0.25, 0.5 or 1%) and their combinations on the total aerobic counts on chicken wingettes. The total aerobic counts recovered from the baseline group was ~5.34 log_{10} cfu/sample and ~4.24 log_{10} cfu/sample at day 0 in trial 1 and 2 respectively. By the end of the storage, we observed that the aerobic counts in baseline group increased by ~3.33 log_{10} cfu/sample in trial 1 and ~4.46 log_{10} cfu/sample in trial 2. The BPD washing did not significantly reduce aerobic bacterial load except for minimal reductions of ~0.5 log_{10} cfu/sample at days 5 and 7 in trial 1 when compared to the baseline. The 10% GA coating did not exert any antimicrobial effect on the aerobic bacteria. The total aerobic counts recovered from the samples coated with 10% GA was similar to baseline, except for minimal reduction at day 7 in trial 1. Among the 3 CR treatments, only 1% CR wash treatment consistently reduced total aerobic counts by ~0.5 to 1 log_{10} cfu/sample as compared to the BPD control in both trials and all sampling days (except day 5 in trial 1). The combination treatments of GA and CR did not differ in their efficacy in reducing total aerobic counts when compared to either GA or CR on majority of sampling days.

The effect of CH (2%), CR (0.25, 0.5 and 1%) and their combinations on the total aerobic counts on chicken wingettes is presented in Table 5. As observed in Table 4, the total aerobic counts in baseline group increased from day 0 through day 7 by ~2 to 3 log_{10} cfu in both trials. Unlike the 10% GA, the 2% CH showed significant reduction on the total aerobic counts at all sampling days except day 3 in trial 1 when compared to either baseline or BPD control. In trial 2, the reduction of aerobic bacteria with 2% CH was consistent throughout all sampling days. When compared to the 50 mM acetic acid solution, the 2% CH significantly reduced the total aerobic counts at days 0, 5 and 7 in trial 1, and days 1 and 3 in trial 2. The combination treatments of CR
and CH produced microbial reductions on majority of sampling days (except day 3 in trial 1; days 0 and 7 in trial 2) when compared with the BPD and baseline. However, the combination treatments were not significantly more effective than the respective doses of CR or CH alone for majority of the timepoints.

4.3. The effect of treatments on the color of chicken wingettes

The treatment of chicken wingettes with GA (10%), 50 mM acetic acid (pH ~6.5), CH (2%), CR (0.25, 0.5 or 1%), or combinations did not produce significant changes on the color values (L*, a*, b*) of wingettes when compared with either BPD control or baseline group (Tables 6a, 6b and 6c) within the same sampling day. The refrigerated storage had no significant effect on the lightness of chicken wingettes. At the end of 7 days of storage, the redness of wingettes did not significantly differ from day 0 and day 3 in all treatments except baseline and 1% CR. Majority of treatments did not affect yellowness (b*) during refrigerated storage. However, the combination treatments of 0.5, 1% CR with CH and 1% CR with GA decreased the yellowness of wingettes during 7 days of refrigerated storage.


The sub-inhibitory concentration of CR and CH was determined using growth curves (data not shown). Figure 1 shows the effect of sub-inhibitory concentration of CR, CH, and their combination on the expression of selected *C. jejuni* genes required for survival and virulence in the host. The presence of CR at the sub-inhibitory concentration significantly up-regulated energy taxis gene, *cetB*. However, CH and CR+CH combination significantly down-regulated the expression of *cetB*. The expression of motility gene *motA* and fibronectin binding protein gene
"cadF" was significantly downregulated by CR+CH combination treatment but not by CR and CH treatments alone. The gene "jlpA" that helps in bacterial attachment was also reduced in expression by CR+CH combination and CH treatment. The expression of motility gene ("motB"), invasion antigen protein gene ("ciaB"), flagella biosynthesis RNA polymerase sigma protein gene ("fliA") and regulatory protein gene ("racS") were not affected by any of the treatment groups when compared to the control. The acetic acid treatment did not affect the expression of tested genes (P > 0.05).

5. Discussion

In this study, we evaluated the potential of GA or CH-based edible coatings fortified with CR to reduce *C. jejuni* on chicken wingettes. The wingettes were used as a model to represent the treatment of a whole carcass. The increased contact time between coating and the product facilitates in reducing pathogen survival and also prevents subsequent contamination during transport and post-coating handling. Both GA and CH have been extensively studied as coatings on food products such as fresh strawberries, longan fruit, tomato, skinless frankfurters, and shell eggs to improve food safety (El Ghaouth et al., 1991; Jiang and Li, 2001; Ali et al., 2010; Upadhyay et al., 2015; Upadhyaya et al., 2016). GA dissolves in water at neutral pH while CH needs an acidified solution. Therefore, aqueous solution of acetic acid at 50 mM concentration was used to dissolve the CH powder. The gum arabic coating significantly reduced *C. jejuni* counts when compared to non-coated wingettes (baseline). However, GA efficacy was similar to BPD wash treatment suggesting that reductions observed in GA treatment were probably due to removal of loosely attached *C. jejuni* cells (Table 2). Similar results were previously reported where 10% GA coating did not inhibit mycelia growth of *Colletotrichum musae* and *Colletotrichum gloeosporioides* in artificially inoculated bananas and papayas (Maqbool et al., 2011). Likewise, Jiang and colleagues (2013) observed that *Pseudomonas* spp., yeasts and molds counts were not
significantly reduced by the 10% GA coating on mushrooms. Upadhyaya et al. (2016) also observed that the presence of GA coating did not exert any antimicrobial effect on Salmonella present on eggs as compared to controls. In contrast to GA, coatings with the 2% CH showed significant antimicrobial activity against C. jejuni on wingettes (Table 3). Olaimat and colleagues (2014) observed that 2% CH and 0.2% κ-carrageenan combination coating containing mustard extract significantly reduced (up to 2.78 log_{10} cfu/g) C. jejuni counts on chicken breast. In the present study, coating materials fortified with select concentrations of CR significantly improved the antimicrobial activity of coating agents. Moreover, the antimicrobial efficacy was maintained through 7 days of refrigerated storage. Similar results have been observed in previous studies where the addition of CR to GA (Upadhyaya et al., 2016) or to CH (Upadhyay et al., 2015) significantly improved the antimicrobial efficacy of coating material against S. Enteritidis on shell eggs and L. monocytogenes on frankfurters, respectively. The presence of CR wash treatments also reduced C. jejuni counts on wingettes and the efficacy of several CR wash treatments was similar to the coating treatments with GA or CH. However, since wash treatments do not exert any antimicrobial effect after initial treatment, protection from subsequent contamination event during handling or storage is a potential concern.

The presence of aerobic bacteria such as Pseudomonas spp., in refrigerated chicken products can reduce shelf–life and decrease food safety (Huis in't Veld, 1996; De Ledesma et al., 1996; Kim and Marshall, 2000). In this study, we observed that direct application of CR or CR+CH coatings inhibited the growth of total aerobic counts when compared with either non-coated or wingettes washed with BPD control. Previously, Siripatrawan and Noipha (2012) observed that 2% CH reduced the count of total aerobic bacteria, yeast, molds and lactic acid bacteria from day 12 through 20 on pork sausages stored at 4°C. Aşık and Candoğan, (2014) found that 3% CH alone
or in combination with different concentrations (0.5, 1 and 1.5%) of garlic oil significantly reduced the total aerobic counts on shrimp and improved the shelf-life of products by at least 2 days. In agreement with these studies, a recent study conducted by Jasour and co-workers (2015) reported that CH-based coatings supplemented with lactoperoxidase extended the shelf-life of trout fillets by at least 4 days. Chicken skin harbors different microflora, including psychotropic bacteria that contributes to spoilage (Cox et al., 1998; Mead, 2004). We observed an increase in the total aerobic counts throughout the sampling days in all treatments, which could be due to the growth of those psychrophiles attached to the skin surfaces of wingettes.

Since color of the poultry product is one of the main attributes of the product which may influence the purchasing decision for consumers, we evaluated the effect of CR and combination treatments on the color of chicken wingettes. Among L*, a* and b* values, L* is the most important since consumers can easily detect the changes in lightness of the products which could affect their purchasing decisions (Guidi and Castigliego, 2010). We observed that there were no significant differences in color values (L*, a*, b*) of chicken wingettes between treatments and controls within the sampling day (Tables 6a, b, c). Jeong et al. (2011) observed that normal color values (L*, a*, b*) of chicken wings cooled by different chilling methods (water, air or evaporative air) ranged from 67 to 69 for lightness (L*), 2.7 to 4.1 for redness (a*) and 4.6 to 7 for yellowness (b*). In this study, we observed color values for L*, a* and b* ranged from 71.19 to 73.6, 2.56 to 4.5 and 7.96 to 11.97 respectively. The slight increase in L* and b* values could be due to variation in samples and storage procedures. Previous studies have shown that pH of the product is one of the factors that determines the color of the product (Livingston and Brown, 1981; Yang and Chen, 1993; Allen et al., 1997). In this study, the pH of treatment solutions, including controls was ~6.5 to ~6.7. This could be one of the reasons why a change in color of wingettes within the same
sampling day was not observed. Moreover, we observed that refrigerated storage did not affect the lightness of samples. The redness was also not significantly affected in the majority of the treatments when compared with day 0 versus day 7. However, a slight decrease in the yellowness of the samples washed with the coating combinations of either GA with 1% CR or CH with 0.5 and 1% CR was observed. Ahn and Lee (2004) observed that the lightness and redness for aerobically and vacuum packaged turkey breast meat did not significantly change during 15 days of storage. However, they reported an increase in yellowness in aerobically-packaged samples.

The sub-inhibitory concentration of a compound refers to the maximum concentration that does not inhibit pathogen growth. Our previous studies (Arambel et al., 2015; Upadhyay et al., 2017; Wagle et al., 2017a, b) and other researchers (Castillo et al., 2014; Oh and Jeon, 2015; Kovács et al., 2016) have reported that sub-inhibitory concentrations of natural compounds including phytochemicals modulate the expression of virulent genes in major pathogens including C. jejuni thereby potentially changing their pathophysiology and survival efficacy in the environment. To delineate the potential mechanism of action of the tested natural compounds against C. jejuni, we evaluated the effect of sub-inhibitory concentrations of CR (0.002%), CH (0.0125%), and CR+CH combination on the expression of C. jejuni genes critical for survival and virulence. Since 10% GA did not reduce C. jejuni counts on chicken wingettes when compared to the BPD control, we did not include GA as a treatment in gene expression study. To represent the chicken meat environment, gene expression analysis was carried out in chicken meat juice (5% vol/vol) since chicken meat juice is known to enhance surface attachment of C. jejuni (Brown et al., 2014), thereby, enhancing their survival in the poultry products (Birk et al., 2004).

Several recent studies have identified genes responsible for C. jejuni virulence, colonization in the chicken gut, and infection in humans (Dasti et al., 2010; Hermans et al., 2011).
C. jejuni genes motA, motB, fliA are essential for motility (Ketley, 1997; Young et al., 2007). The cetA and cetB genes are responsible for energy taxis (Hendrixson et al., 2001). In addition, cadF and jlpA are required for cell surface attachment (Jin et al., 2003; Hermans et al., 2011). In this study, we observed that the CR treatment did not significant change the expression of a majority of test genes except cetB. In contrast, CH down-regulated the expression of cetB and jlpA suggesting that the two compounds could be acting by different mechanisms. The CR+CH combination significantly downregulated the expression of motA, cadF, jlpA and cetB, indicating that the combination treatments could modulate C. jejuni virulence and capacity to cause infections in humans.

6. Conclusions

We observed that GA or CH-based coating with CR produced consistent reduction of C. jejuni on chicken wingettes. The aforementioned reduction would have significant food safety implications since a 2-log10 reduction of Campylobacter from the poultry carcass could result in a 30-fold reduction in human infection (Rosenquist et al., 2003). In addition, the aforementioned treatments also reduced the expression of select virulent genes of C. jejuni. Thus, CR in combination with GA, CH coating could be used as an effective antimicrobial treatment for controlling C. jejuni and improving safety of poultry products.
7. Acknowledgement

This research was funded in part by the United States Department of Agriculture-National Institute of Food and Agriculture-Organic Agriculture Research and Extension Initiative-2017-51300-26815.
8. References


Oh, E., & Jeon, B. (2015). Synergistic anti-
Campylobacter jejuni 
activity of fluoroquinolone and 
macrolide antibiotics with phenolic compounds. Frontiers in Microbiology, 6, 1129.


## Appendix

### Tables

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

<table>
<thead>
<tr>
<th>Gene with Accession no.</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S-rRNA (NC_002163.1)</td>
<td>Forward</td>
<td>5’-ATAAGCACCAGGCTAACTCCG-3’</td>
<td>Ribosomal RNA (housekeeping gene)</td>
</tr>
<tr>
<td>(product length 78 bp)</td>
<td>Reverse</td>
<td>5’-TTACGCCCAGTGATTCCGAG-3’</td>
<td></td>
</tr>
<tr>
<td><strong>motA</strong> (NC_002163.1)</td>
<td>Forward</td>
<td>5’-AGCGGGGTATTTCAGGTGCTT-3’</td>
<td>Flagellar motor protein</td>
</tr>
<tr>
<td>(product length 75 bp)</td>
<td>Reverse</td>
<td>5’-CCCCAAGGAGCAAAAAGTGC-3’</td>
<td></td>
</tr>
<tr>
<td><strong>motB</strong> (NC_002163.1)</td>
<td>Forward</td>
<td>5’-AATGCCCAGAATGTCCAGCA-3’</td>
<td>Flagellar motor protein</td>
</tr>
<tr>
<td>(product length 51 bp)</td>
<td>Reverse</td>
<td>5’-AGTCTGCATAAGGCACAGCC-3’</td>
<td></td>
</tr>
<tr>
<td><strong>fliA</strong> (NC_002163.1)</td>
<td>Forward</td>
<td>5’-AGCTTTTCACGCCGTTACGAT-3’</td>
<td>Flagella biosynthesis RNA polymerase sigma protein</td>
</tr>
<tr>
<td>(product length 56 bp)</td>
<td>Reverse</td>
<td>5’-TCTTGCAAAAACCCCAAGAT-3’</td>
<td></td>
</tr>
<tr>
<td><strong>cetB</strong> (NC_002163.1)</td>
<td>Forward</td>
<td>5’-GCCTTTGGTGTGCTTCTGCTC-3’</td>
<td>Energy taxis protein/motility</td>
</tr>
<tr>
<td>(product length 88 bp)</td>
<td>Reverse</td>
<td>5’-TTCCGTTTCGTCGTATGC-3’</td>
<td></td>
</tr>
<tr>
<td><strong>cadF</strong> (NC_002163.1)</td>
<td>Forward</td>
<td>5’-CGCGGGATGAAAATTCGTC-3’</td>
<td>Outer-membrane fibronectin-binding protein</td>
</tr>
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<td>(product length 135 bp)</td>
<td>Reverse</td>
<td>5’-TCTTTTTTGCCCAAAAAACA-3’</td>
<td></td>
</tr>
<tr>
<td><strong>ciaB</strong> (NC_002163.1)</td>
<td>Forward</td>
<td>5’-TTCAGCTCAAGGCTGTTCCA-3’</td>
<td>Invasion antigen protein</td>
</tr>
<tr>
<td>(product length 50 bp)</td>
<td>Reverse</td>
<td>5’-GCCCGGCCTTGGAATCCATAA-3’</td>
<td></td>
</tr>
<tr>
<td><strong>jlpA</strong> (NC_002163.1)</td>
<td>Forward</td>
<td>5’-AGCACACAGGGAATCGACAG-3’</td>
<td>Surface exposed lipoprotein</td>
</tr>
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<td>(product length 66 bp)</td>
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<td>5’-TAACGCTTTGTGCGGTCTT-3</td>
<td></td>
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<tr>
<td><strong>racS</strong> (NC_002163.1)</td>
<td>Forward</td>
<td>5’-AGACAAAGGTGGCCGAAGTGTC-3’</td>
<td>Two-component sensor/histidine kinase</td>
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<tr>
<td>(product length 79 bp)</td>
<td>Reverse</td>
<td>5’-AGGCGATCTTGCTACTTCA-3’</td>
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Table 2: The efficacy of gum arabic (GA), carvacrol (CR) and their combinations on survival of *Campylobacter jejuni* on chicken wingettes

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>Baseline</td>
<td>7.00±0.03a</td>
<td>6.66±0.03a</td>
<td>6.51±0.03a</td>
<td>6.60±0.03a</td>
<td>6.30±0.11a</td>
</tr>
<tr>
<td></td>
<td>BPD control</td>
<td>6.11±0.07b</td>
<td>5.53±0.07b</td>
<td>5.56±0.08b</td>
<td>5.53±0.03b</td>
<td>5.55±0.08b</td>
</tr>
<tr>
<td></td>
<td>10% GA</td>
<td>5.92±0.05b</td>
<td>5.77±0.10b</td>
<td>5.78±0.09b</td>
<td>5.71±0.02b</td>
<td>5.41±0.07b</td>
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<tr>
<td></td>
<td>0.25% CR</td>
<td>4.80±0.14c</td>
<td>4.44±0.17c</td>
<td>4.46±0.12cd</td>
<td>4.60±0.16c</td>
<td>4.69±0.11c</td>
</tr>
<tr>
<td></td>
<td>0.5% CR</td>
<td>4.00±0.18de</td>
<td>4.10±0.15cd</td>
<td>3.96±0.17e</td>
<td>4.03±0.14de</td>
<td>4.20±0.06ed</td>
</tr>
<tr>
<td></td>
<td>1% CR</td>
<td>3.62±0.31c</td>
<td>3.81±0.31d</td>
<td>2.82±0.07f</td>
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<td>3.59±0.10c</td>
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<td></td>
<td>0.25% CR+10% GA</td>
<td>4.85±0.10c</td>
<td>4.70±0.19c</td>
<td>4.80±0.07c</td>
<td>4.48±0.11cd</td>
<td>4.72±0.16c</td>
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<tr>
<td></td>
<td>0.5% CR+10% GA</td>
<td>4.24±0.18d</td>
<td>4.30±0.36cd</td>
<td>4.24±0.21de</td>
<td>4.27±0.19ed</td>
<td>4.57±0.22c</td>
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<tr>
<td></td>
<td>1% CR+10% GA</td>
<td>4.14±0.21d</td>
<td>3.80±0.29d</td>
<td>4.25±0.12de</td>
<td>3.48±0.27ef</td>
<td>3.78±0.42de</td>
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<tr>
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<td>Baseline</td>
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<td>6.93±0.05a</td>
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<td>6.84±0.09a</td>
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<td>BPD control</td>
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<tr>
<td></td>
<td>10% GA</td>
<td>6.45±0.09b</td>
<td>5.93±0.05b</td>
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<td>0.5% CR</td>
<td>5.61±0.17cd</td>
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<td>1% CR</td>
<td>5.19±0.06c</td>
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<td>5.01±0.16d</td>
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<td>0.25% CR+10% GA</td>
<td>5.80±0.14c</td>
<td>5.21±0.05c</td>
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<td>5.42±0.08c</td>
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<tr>
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<td>0.5% CR+10% GA</td>
<td>5.31±0.09de</td>
<td>5.15±0.26c</td>
<td>5.12±0.19c</td>
<td>5.33±0.18c</td>
<td>5.16±0.16ed</td>
</tr>
<tr>
<td></td>
<td>1% CR+10% GA</td>
<td>5.20±0.17c</td>
<td>5.01±0.20c</td>
<td>4.50±0.27d</td>
<td>5.24±0.15c</td>
<td>5.04±0.19ed</td>
</tr>
</tbody>
</table>

\(^1n = 5\) replicates per treatment per day per trial. Values (log\(_{10}\) cfu/sample) presented as mean±standard error of the mean. Within the same trial, within column with no common superscript differ significantly (P < 0.05).
Table 3: The efficacy of chitosan (CH), carvacrol (CR) and their combinations on survival of *Campylobacter jejuni* on chicken wingettes

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Baseline</td>
<td>6.65±0.19</td>
<td>6.31±0.21</td>
<td>6.64±0.07</td>
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<td>6.71±0.13</td>
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<tr>
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<td>BPD control</td>
<td>6.06±0.14</td>
<td>5.89±0.14</td>
<td>5.73±0.21</td>
<td>5.34±0.07</td>
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<tr>
<td></td>
<td>50 mM acetic acid</td>
<td>6.02±0.15</td>
<td>6.11±0.10</td>
<td>5.59±0.18</td>
<td>5.47±0.09</td>
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<td>2% CH</td>
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<td>0.5% CR</td>
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<td>4.68±0.14</td>
<td>4.95±0.36</td>
</tr>
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<td>1% CR</td>
<td>4.08±0.23</td>
<td>5.16±0.11</td>
<td>5.12±0.16</td>
<td>4.07±0.21</td>
<td>4.94±0.07</td>
</tr>
<tr>
<td></td>
<td>0.25% CR+2% CH</td>
<td>5.03±0.19</td>
<td>4.52±0.19</td>
<td>4.07±0.23</td>
<td>4.05±0.14</td>
<td>4.79±0.15</td>
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<td>3.14±0.48</td>
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<td>4.13±0.18</td>
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<td>4.33±0.27</td>
<td>4.54±0.20</td>
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<td>6.64±0.20</td>
<td>6.71±0.13</td>
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<td>BPD control</td>
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<td>50 mM acetic acid</td>
<td>6.38±0.11</td>
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<td>4.81±0.10</td>
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<td>4.44±0.19</td>
<td>3.85±0.24</td>
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</tr>
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</table>

1 n = 5 replicates per treatment per day per trial. Values (log10 cfu/sample) presented as mean±standard error of the mean. Within the same trial, within column with no common superscript differ significantly (P < 0.05).
Table 4: The efficacy of gum arabic (GA), carvacrol (CR) and their combinations on total aerobic counts on chicken wingettes

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>5.34±0.18&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.71±0.31&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.60±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.97±0.23&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>8.67±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BPD control</td>
<td>5.22±0.21&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.30±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.05±0.24&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.20±0.13&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>8.02±0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10% GA</td>
<td>5.69±0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.87±0.25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.22±0.23&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.48±0.19&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.98±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.25% CR</td>
<td>5.17±0.22&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.37±0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.98±0.25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.55±0.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.44±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>0.5% CR</td>
<td>4.66±0.22&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.37±0.17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.55±0.32&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.65±0.11&lt;sup&gt;de&lt;/sup&gt;</td>
<td>7.56±0.18&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1% CR</td>
<td>4.30±0.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.00±0.49&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.04±0.56&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>7.33±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.14±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>0.25% CR+10% GA</td>
<td>4.76±0.32&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.12±0.32&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.91±0.32&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>7.51±0.22&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>8.53±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.5% CR+10% GA</td>
<td>4.06±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.65±0.36&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.60±0.16&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.74±0.29&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.98±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1% CR+10% GA</td>
<td>4.65±0.33&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.34±0.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.91±0.27&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>8.12±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.60±0.19&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>4.24±0.04&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.32±0.16&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>7.38±0.09&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.09±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.70±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BPD control</td>
<td>4.22±0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.11±0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.74±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.99±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.57±0.11&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10% GA</td>
<td>4.83±0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.14±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.67±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.84±0.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>8.86±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.25% CR</td>
<td>3.72±0.28&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>5.57±0.22&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>6.90±0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.30±0.11&lt;sup&gt;de&lt;/sup&gt;</td>
<td>8.26±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>0.5% CR</td>
<td>3.79±0.12&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>5.16±0.17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.32±0.10&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.23±0.06&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8.37±0.07&lt;sup&gt;bc&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>1% CR</td>
<td>3.50±0.12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.56±0.18&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>6.80±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.88±0.14&lt;sup&gt;f&lt;/sup&gt;</td>
<td>8.17±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.25% CR+10% GA</td>
<td>5.54±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.73±0.23&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.65±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.68±0.08&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>8.08±0.09&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.5% CR+10% GA</td>
<td>6.18±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.30±0.14&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>7.16±0.21&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.54±0.09&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>8.33±0.10&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1% CR+10% GA</td>
<td>5.98±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.80±0.20&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.03±0.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.33±0.05&lt;sup&gt;de&lt;/sup&gt;</td>
<td>8.36±0.07&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>n = 5 replicates per treatment per day per trial. Values (log<sub>10</sub> cfu/sample) presented as mean±standard error of the mean. Within the same trial, within column with no common superscript differ significantly (P < 0.05).
<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Baseline</td>
<td>7.06±0.12a</td>
<td>9.68±0.05a</td>
<td>8.16±0.05a</td>
<td>9.18±0.02a</td>
<td>9.08±0.15ab</td>
<td></td>
</tr>
<tr>
<td>1 BPD control</td>
<td>6.96±0.15a</td>
<td>9.56±0.03ab</td>
<td>8.18±0.07a</td>
<td>9.21±0.13a</td>
<td>9.27±0.67a</td>
<td></td>
</tr>
<tr>
<td>1 50 mM acetic acid</td>
<td>6.62±0.18b</td>
<td>9.15±0.12bc</td>
<td>8.00±0.11a</td>
<td>9.09±0.08a</td>
<td>9.28±0.16a</td>
<td></td>
</tr>
<tr>
<td>1 2% CH</td>
<td>6.15±0.11de</td>
<td>8.74±0.19cd</td>
<td>7.55±0.19ab</td>
<td>8.32±0.06cd</td>
<td>8.59±0.25cd</td>
<td></td>
</tr>
<tr>
<td>1 0.25% CR</td>
<td>6.40±0.07bcd</td>
<td>8.55±0.06d</td>
<td>8.00±0.07a</td>
<td>8.92±0.16ab</td>
<td>8.83±0.08bc</td>
<td></td>
</tr>
<tr>
<td>1 0.5% CR</td>
<td>6.01±0.09c</td>
<td>8.34±0.03de</td>
<td>8.11±0.20a</td>
<td>8.85±0.17ab</td>
<td>8.20±0.05c</td>
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</tr>
<tr>
<td>1 1% CR</td>
<td>6.29±0.14bcde</td>
<td>8.64±0.17d</td>
<td>7.91±0.49ab</td>
<td>8.6±0.10bc</td>
<td>8.31±0.06de</td>
<td></td>
</tr>
<tr>
<td>1 0.25% CR+2% CH</td>
<td>6.58±0.10bc</td>
<td>7.93±0.20ef</td>
<td>7.5±0.44ab</td>
<td>8.56±0.09bc</td>
<td>8.32±0.31de</td>
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</tr>
<tr>
<td>1 0.5% CR+2% CH</td>
<td>6.27±0.11cde</td>
<td>7.69±0.14f</td>
<td>7.02±0.05b</td>
<td>7.96±0.21d</td>
<td>8.57±0.04cd</td>
<td></td>
</tr>
<tr>
<td>1 1% CR+2% CH</td>
<td>6.07±0.08de</td>
<td>7.04±0.38g</td>
<td>7.59±0.33ab</td>
<td>8.08±0.19d</td>
<td>8.19±0.16e</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Baseline</td>
<td>4.5±0.15a</td>
<td>5.73±0.23a</td>
<td>6.79±0.24a</td>
<td>7.32±0.08ab</td>
<td>7.42±0.04ab</td>
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</tr>
<tr>
<td>2 BPD control</td>
<td>4.06±0.23ab</td>
<td>5.72±0.20a</td>
<td>6.02±0.16ab</td>
<td>7.45±0.17a</td>
<td>7.44±0.10a</td>
<td></td>
</tr>
<tr>
<td>2 50 mM acetic acid</td>
<td>3.78±0.15bc</td>
<td>5.16±0.22b</td>
<td>5.77±0.18b</td>
<td>7.0±0.27bc</td>
<td>7.04±0.09cd</td>
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</tr>
<tr>
<td>2 2% CH</td>
<td>3.41±0.14c</td>
<td>3.84±0.21de</td>
<td>4.92±0.26ed</td>
<td>6.56±0.16c</td>
<td>6.68±0.23d</td>
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</tr>
<tr>
<td>2 0.25% CR</td>
<td>4.36±0.14a</td>
<td>4.70±0.23bc</td>
<td>5.43±0.10bc</td>
<td>6.89±0.11bc</td>
<td>7.50±0.05a</td>
<td></td>
</tr>
<tr>
<td>2 0.5% CR</td>
<td>3.79±0.17bc</td>
<td>4.35±0.23cd</td>
<td>5.91±0.13b</td>
<td>6.80±0.08c</td>
<td>7.45±0.11a</td>
<td></td>
</tr>
<tr>
<td>2 1% CR</td>
<td>3.5±0.18bc</td>
<td>3.75±0.16e</td>
<td>4.31±0.61de</td>
<td>6.79±0.15e</td>
<td>7.43±0.13ab</td>
<td></td>
</tr>
<tr>
<td>2 0.25% CR+2% CH</td>
<td>3.6±0.26bc</td>
<td>4.18±0.08cde</td>
<td>4.31±0.32de</td>
<td>6.79±0.07c</td>
<td>7.22±0.12abc</td>
<td></td>
</tr>
<tr>
<td>2 0.5% CR+2% CH</td>
<td>3.64±0.17cde</td>
<td>4.13±0.05cde</td>
<td>4.24±0.07de</td>
<td>6.73±0.18c</td>
<td>7.33±0.06abc</td>
<td></td>
</tr>
<tr>
<td>2 1% CR+2% CH</td>
<td>3.31±0.30c</td>
<td>4.06±0.14de</td>
<td>4.05±0.22c</td>
<td>6.58±0.16c</td>
<td>7.07±0.20bc</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) n = 5 replicates per treatment per day per trial. Values (log\(_{10}\) cfu/sample) presented as mean±standard error of the mean. Within the same trial, within column with no common superscript differ significantly (P < 0.05).
**Table 6a**: The effect of gum arabic (GA), chitosan (CH), carvacrol (CR) and their combinations on lightness chicken wingettes

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>72.20±0.31</td>
<td>72.76±0.45</td>
<td>71.68±0.38</td>
</tr>
<tr>
<td>BPD control</td>
<td>73.16±0.33</td>
<td>73.60±0.22</td>
<td>72.96±0.22</td>
</tr>
<tr>
<td>10% GA</td>
<td>71.64±0.59</td>
<td>71.89±0.72</td>
<td>72.17±1.26</td>
</tr>
<tr>
<td>50 mM acetic acid</td>
<td>72.68±0.44</td>
<td>72.18±0.68</td>
<td>71.25±0.83</td>
</tr>
<tr>
<td>2% CH</td>
<td>72.91±0.43</td>
<td>73.05±0.74</td>
<td>72.48±1.22</td>
</tr>
<tr>
<td>0.25% CR</td>
<td>72.61±0.55</td>
<td>73.43±0.57</td>
<td>72.25±0.81</td>
</tr>
<tr>
<td>0.5% CR</td>
<td>73.10±0.30</td>
<td>72.65±0.55</td>
<td>72.07±0.61</td>
</tr>
<tr>
<td>1% CR</td>
<td>72.93±0.28</td>
<td>73.36±0.32</td>
<td>72.62±0.32</td>
</tr>
<tr>
<td>0.25% CR + 10% GA</td>
<td>71.73±1.35</td>
<td>71.89±0.60</td>
<td>71.53±0.44</td>
</tr>
<tr>
<td>0.5% CR + 10% GA</td>
<td>72.24±0.52</td>
<td>72.05±0.60</td>
<td>71.46±0.43</td>
</tr>
<tr>
<td>1% CR + 10% GA</td>
<td>71.53±0.74</td>
<td>72.03±0.45</td>
<td>71.19±0.59</td>
</tr>
<tr>
<td>0.25% CR + 2% CH</td>
<td>73.11±0.92</td>
<td>73.22±1.00</td>
<td>72.70±1.02</td>
</tr>
<tr>
<td>0.5% CR + 2% CH</td>
<td>72.50±0.71</td>
<td>73.03±0.71</td>
<td>72.30±0.56</td>
</tr>
<tr>
<td>1% CR + 2% CH</td>
<td>73.24±0.88</td>
<td>73.43±0.49</td>
<td>72.55±0.74</td>
</tr>
</tbody>
</table>

1 n = 5 replicates per treatment per day. Values (mean±standard error of the mean). No significant different within the same column or within the same row (P > 0.05).
Table 6b: The effect of gum arabic (GA), chitosan (CH), carvacrol (CR) and their combinations on redness of chicken wingettes

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>2.98±0.42bc</td>
<td>3.71±0.14ab</td>
<td>3.80±0.23a</td>
</tr>
<tr>
<td>BPD control</td>
<td>2.98±0.44a</td>
<td>2.56±0.27a</td>
<td>2.99±0.35a</td>
</tr>
<tr>
<td>10% GA</td>
<td>4.04±0.42a</td>
<td>3.68±0.40a</td>
<td>4.03±0.40a</td>
</tr>
<tr>
<td>50 mM acetic acid</td>
<td>3.81±0.44a</td>
<td>3.31±0.29a</td>
<td>3.86±0.34a</td>
</tr>
<tr>
<td>2% CH</td>
<td>3.57±0.26a</td>
<td>3.14±0.31a</td>
<td>3.63±0.66a</td>
</tr>
<tr>
<td>0.25% CR</td>
<td>3.48±0.74a</td>
<td>2.67±0.37a</td>
<td>3.90±0.22a</td>
</tr>
<tr>
<td>0.5% CR</td>
<td>3.33±0.66a</td>
<td>3.35±0.75a</td>
<td>4.27±0.28a</td>
</tr>
<tr>
<td>1% CR</td>
<td>3.79±0.16a</td>
<td>3.01±0.14b</td>
<td>3.71±0.24a</td>
</tr>
<tr>
<td>0.25% CR + 10% GA</td>
<td>4.43±0.68a</td>
<td>2.93±0.29b</td>
<td>3.90±0.60ab</td>
</tr>
<tr>
<td>0.5% CR + 10% GA</td>
<td>4.38±0.30a</td>
<td>3.54±0.35a</td>
<td>3.85±0.37a</td>
</tr>
<tr>
<td>1% CR + 10% GA</td>
<td>4.50±0.82a</td>
<td>2.90±0.66a</td>
<td>3.76±0.77a</td>
</tr>
<tr>
<td>0.25% CR + 2% CH</td>
<td>4.44±0.78a</td>
<td>3.13±0.64a</td>
<td>3.31±0.43a</td>
</tr>
<tr>
<td>0.5% CR + 2% CH</td>
<td>4.42±0.66a</td>
<td>3.24±0.64a</td>
<td>3.78±0.51a</td>
</tr>
<tr>
<td>1% CR + 2% CH</td>
<td>3.79±0.22a</td>
<td>3.18±0.29a</td>
<td>4.02±0.54a</td>
</tr>
</tbody>
</table>

1 n = 5 replicates per treatment per day. Values (mean±standard error of the mean). No significant different within the same column (P > 0.05). Means with no common letter differ significantly (P < 0.05) within the same row.
Table 6c: The effect of gum arabic (GA), chitosan (CH), carvacrol (CR) and their combinations on yellowness of chicken wingettes

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>10.19±0.63\textsuperscript{a}</td>
<td>9.30±0.42\textsuperscript{a}</td>
<td>9.05±0.83\textsuperscript{a}</td>
</tr>
<tr>
<td>BPD control</td>
<td>9.62±0.57\textsuperscript{a}</td>
<td>8.10±0.24\textsuperscript{a}</td>
<td>8.30±0.51\textsuperscript{a}</td>
</tr>
<tr>
<td>10% GA</td>
<td>9.54±0.33\textsuperscript{a}</td>
<td>7.96±0.50\textsuperscript{a}</td>
<td>8.8±0.60\textsuperscript{a}</td>
</tr>
<tr>
<td>50 mM acetic acid</td>
<td>9.65±0.49\textsuperscript{a}</td>
<td>8.61±0.33\textsuperscript{a}</td>
<td>8.33±0.74\textsuperscript{a}</td>
</tr>
<tr>
<td>2% CH</td>
<td>10.32±0.73\textsuperscript{a}</td>
<td>8.66±0.96\textsuperscript{a}</td>
<td>8.8±0.94\textsuperscript{a}</td>
</tr>
<tr>
<td>0.25% CR</td>
<td>10.01±1.70\textsuperscript{a}</td>
<td>8.10±1.50\textsuperscript{a}</td>
<td>9.18±1.04\textsuperscript{a}</td>
</tr>
<tr>
<td>0.5% CR</td>
<td>10.40±1.25\textsuperscript{a}</td>
<td>9.60±0.96\textsuperscript{a}</td>
<td>9.51±1.09\textsuperscript{a}</td>
</tr>
<tr>
<td>1% CR</td>
<td>11.27±0.82\textsuperscript{a}</td>
<td>9.3±1.00\textsuperscript{a}</td>
<td>9.08±1.11\textsuperscript{a}</td>
</tr>
<tr>
<td>0.25% CR + 10% GA</td>
<td>11.97±1.36\textsuperscript{a}</td>
<td>9.73±0.59\textsuperscript{a}</td>
<td>9.59±1.37\textsuperscript{a}</td>
</tr>
<tr>
<td>0.5% CR + 10% GA</td>
<td>10.78±0.79\textsuperscript{a}</td>
<td>8.03±0.94\textsuperscript{a}</td>
<td>8.41±1.27\textsuperscript{a}</td>
</tr>
<tr>
<td>1% CR + 10% GA</td>
<td>10.98±0.37\textsuperscript{a}</td>
<td>8.66±1.09\textsuperscript{b}</td>
<td>8.97±0.68\textsuperscript{b}</td>
</tr>
<tr>
<td>0.25% CR + 2% CH</td>
<td>10.72±0.69\textsuperscript{a}</td>
<td>8.6±1.00\textsuperscript{a}</td>
<td>9.23±0.71\textsuperscript{a}</td>
</tr>
<tr>
<td>0.5% CR + 2% CH</td>
<td>11.82±0.74\textsuperscript{a}</td>
<td>9.62±0.56\textsuperscript{b}</td>
<td>9.98±0.32\textsuperscript{b}</td>
</tr>
<tr>
<td>1% CR + 2% CH</td>
<td>11.55±0.66\textsuperscript{a}</td>
<td>8.56±0.71\textsuperscript{b}</td>
<td>9.06±0.55\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{1} n = 5 replicates per treatment per day. Values (mean±standard error of the mean). No significant different within the same column (P > 0.05). Means with no common letter differ significantly (P < 0.05) within the same row.
Figure 1. The effect of 0.002% carvacrol (CR), 0.0125% chitosan (CH) and 0.002% CR + 0.0125% CH on the expression of selected virulent genes of *C. jejuni* S-8. 16S-rRNA served as endogenous control. Results are averages of two independent experiments, each containing duplicate samples (mean and SEM). *Indicates significantly down or up-regulated genes (P < 0.05).
IBC Protocol Approval

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.
**PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:**

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

<table>
<thead>
<tr>
<th>Name: (first and last) - POSITION (Title, academic degrees, certifications, and material field of expertise)</th>
<th>QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Example:</strong> Bob Biohazard - Associate Professor, PhD Microbiology</td>
<td>14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.</td>
</tr>
<tr>
<td>Dan J. Donoghue, Ph.D. Professor, Food Safety</td>
<td>over 20 years experience as PI running research laboratory working with BL-2 pathogens.</td>
</tr>
<tr>
<td>Ann M. Donoghue, Ph.D. Research Leader, Food safety</td>
<td>over 20 years experience as PI running research laboratory working with BL-2 pathogens.</td>
</tr>
<tr>
<td>Komala Arsi, Ph. D. Post-doctoral fellow</td>
<td>Post-doctoral fellow trained under Dr. Donoghue's supervision</td>
</tr>
<tr>
<td>Abhinav Upadhyay, Ph.D. Post-doctoral fellow</td>
<td>Post-doctoral fellow trained under Dr. Donoghue's supervision</td>
</tr>
<tr>
<td>Indu Upadhyaya, Ph. D. Post-doctoral fellow</td>
<td>Post-doctoral fellow trained under Dr. Donoghue's supervision</td>
</tr>
<tr>
<td>Basanta Raj Wagle, Ph.D, Program Associate</td>
<td>Program Associate under Dr. Donoghue's supervision</td>
</tr>
<tr>
<td>Sandip Shrestha</td>
<td>Graduate assistant trained under Dr. Donoghue's supervision</td>
</tr>
</tbody>
</table>

Additional Personnel Information (if needed):
Conclusions

*Campylobacter jejuni* is one of the leading causes of bacterial gastroenteritis worldwide and its infection in humans is strongly associated with the consumption and/or mishandling of raw contaminated poultry products. Thus, interventions aiming to reduce *C. jejuni* counts on poultry products could greatly reduce the incidence of human campylobacteriosis. With increasing consumer demand for natural and minimally processed foods, the use of generally recognized as safe (GRAS) status antimicrobials is gaining attention for improving safety of poultry products.

In this project we evaluated the antimicrobial efficacy of a GRAS compound, carvacrol (CR; derived from oregano oil), as an antimicrobial wash treatment to reduce *C. jejuni* on chicken skin (Study 1). Three delivery systems of CR: suspension, emulsion and nanoemulsion were used. Skin samples were inoculated with a cocktail of four wild strains of *C. jejuni* (~8 log\textsubscript{10} cfu/sample), and were randomly divided to CR (0.25, 0.5, 1 or 2%) suspension, emulsion or nanoemulsion. Samples were washed in the respective treatment solutions for 1 min, followed by drip drying for 2 min, and sampled at 0, 8 or 24 h post-storage at 4°C. *C. jejuni* counts were reduced up to 4 log\textsubscript{10} cfu/sample by 2% dose of CR suspension at 0 h (P < 0.05). Carvacrol emulsion or nanoemulsion did not show any additional reduction in *C. jejuni* counts when compared to suspension.

In the second study, the efficacy of gum arabic (GA) or chitosan (CH) coatings fortified with CR to reduce *C. jejuni* on chicken wingettes was investigated as an additional intervention to increase the antimicrobial activity of CR. Inoculated chicken wingettes (~7.5 log\textsubscript{10} cfu of *C. jejuni*/sample) were randomly assigned to baseline, control (0%), CR (0.25, 0.5 or 1%), GA (10%), CH (2%) or their combinations. After 1 min of coating, wingettes were air dried (1 h) and sampled at days 0, 1, 3, 5, and 7. Results showed that all three doses of CR, CH or GA-based coating fortified with CR reduced *C. jejuni* from day 0 through 7 by up to 3.0 log\textsubscript{10} cfu/sample (P < 0.05).
Moreover, the antimicrobial efficacy of GA was improved by CR and the coatings reduced \textit{C. jejuni} by \(\sim 1\) to \(2\ \log_{10}\text{ cfu/sample}\) at day 7. In addition, CH-CR coatings reduced total aerobic counts on majority of storage time when compared with non-coated samples. No significant difference in the color of chicken wingettes was observed between treatments. Exposure of this pathogen to sublethal concentrations of CR, CH or combination significantly modulated select genes encoding for energy taxis (\textit{cetB}), motility (\textit{motA}), binding (\textit{cadF}) and attachment (\textit{jlpA}).

In conclusion, carvacrol as an antimicrobial wash on chicken skin and the coating treatments on chicken wingettes was effective in reducing \textit{C. jejuni}. Since a 2-\(\log_{10}\) reduction of \textit{C. jejuni} from poultry carcass translates into more than 30-fold reduction in the risk of human \textit{Campylobacter} infections, the aforementioned treatments represent a safe, effective and natural approach that could improve poultry product safety.