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Chitosan Reduces Enteric Colonization of Campylobacter in Young Chickens, but Not on Post-Harvest Chicken Skin Samples

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Chitosan Reduces Enteric Colonization of *Campylobacter* in Young Chickens, but Not on Post-Harvest Chicken Skin Samples

Chitosan Reduces Enteric Colonization of *Campylobacter* in Young Chickens, but Not on Post-Harvest Chicken Skin Samples

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Poultry Science

By

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University of Arkansas
Bachelor of Science in Poultry Science, 2012

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ABSTRACT

Worldwide, *Campylobacter* is one of the leading causes of foodborne bacterial gastroenteritis causing an estimated 1.3 million infections in the United States alone. Consumption and/or cross-contamination of raw or undercooked poultry products have been linked as the most common source of *Campylobacter* infection, making the poultry industry a target for *Campylobacter* reduction strategies. *Campylobacter* is prevalent in most poultry flocks in the United States, with as many as 90% of flocks *Campylobacter*-positive at the time of slaughter. It is estimated that a reduction of *Campylobacter* in poultry would greatly reduce the risk of campylobacteriosis in humans. Unfortunately, there are a lack of effective intervention options to reduce *Campylobacter* in poultry. One potential strategy is the use of the natural product, chitosan, a deacetylated byproduct of crustacean shells, has been shown to reduce *E. coli* and *Salmonella*. The purpose of this study was to determine the ability of chitosan to reduce enteric *Campylobacter* colonization in pre-harvest chickens and on post-harvest chicken skin samples. In each of three trials, 100 birds were divided into 10 treatments (n=10) and were fed either 0% (controls), 0.25%, 0.5% or 1% (wt./wt.) of a low, medium or high molecular weight chitosan (300 birds total). Birds were fed treated feed for the duration of the study and were orally challenged with a four-strain mixture of wild type *C. jejuni* on day 6. On day 15, the ceca were excised and enumerated for *Campylobacter*. In all three trials, the 0.5% dose of the medium molecular weight chitosan reduced cecal *Campylobacter* counts. Because this medium molecular weight chitosan was shown to be the most effective, it was evaluated for post-harvest efficacy against *Campylobacter* on chicken skin. When a 0.5, 1 or 2% concentration was tested in three separate trials, *Campylobacter* counts were not reduced when compared to controls. These results support the use of chitosan in pre-harvest chickens but not for the reduction of

Campylobacter as a post-harvest rinse on skin for the concentrations and strategy used in this study.

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DEDICATION

This is dedicated to Mongo and Mowgli, who make me laugh on a daily basis and have taught me so much about loving life and loving others.

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CHAPTER 1
Literature Review

CHAPTER 1: Literature Review

1.1 Introduction

Campylobacter spp. is one of the leading bacterial causes of food-borne diarrheal illness in the world, causing an estimated 1.3 million infections annually in the United States alone (CDC, 2013). According to the European Food Safety Authority (EFSA, 2014), approximately nine million food related campylobacteriosis cases occur in the European Union each year. In most cases, infection with *Campylobacter* causes an acute self-limiting gastroenteritis; however *Campylobacter* infections have been associated with more severe, long-term sequelae, including Guillain-Barré syndrome (Goodyear et al., 1999; CDC, 2013b), reactive arthritis (Berden et al., 1979; Ajene et al. 2013), irritable bowel syndrome (NDDIC, 2013), and inflammatory bowel disease (CDC 2014). In many countries, including the U.S., governmental regulatory control programs have been implemented to target *Campylobacter* sources (EFSA, 2010; USDA, 2010; FSANZ, 2012). The consumption of poultry products has been identified as the most common source of *Campylobacter* infections in developed countries (Beery et al., 1988; Rosenquist et al., 2003; CDC, 2013a), including improperly cooked chicken and cross-contamination from handling raw chicken (Danis et al., 2009; Fajó-Pascual et al., 2010; CDC, 2013a). As many as 90% of U.S. broiler flocks are colonized with *Campylobacter* (Buzby et al., 1997; Stern et al., 2001); this can lead to cross-contamination of *Campylobacter* from the gut contents to the surface of the carcass in the processing plant (Hargis et al., 1995; Zhang et al., 2013) and is ultimately present on raw poultry products (Moran et al., 2009; Smole Mozina et al., 2009; Suzuki and Yamamoto, 2009). *Campylobacter jejuni* is the most common species identified in cases of food-borne campylobacteriosis (Friedman et al., 2000).

1.2 Characteristics of *Campylobacter*

1.2.1 History and discovery

Theodore Escherich first observed and described *Campylobacter* in 1886, noting a spiral-shaped and non-culturable bacteria (Escherich, 1886), and it was later isolated from aborted bovine fetuses by McFadyean and Stockman in the early 1900s (Skirrow, 2006). Over the next few decades, similar bacteria were isolated from the feces of cattle and pigs with diarrhea, which were initially classified as *Vibrio jejuni* and *Vibrio coli*, respectively (Jones et al., 1931; Doyle 1944). Later, in 1963, Sebald and Véron separated *Campylobacter* from the genus *Vibrio* and proposed a new Genus, *Campylobacter*. *Campylobacter* differs from *Vibrio* in their genomic characteristics (guanine-cytosine content for *Campylobacter* is between 29-36%, whereas G-C content for *Vibrio* is 40-50%), their characteristic non-fermentive metabolism and fastidious growth requirements (Sebald and Véron, 1963; Véron and Chatelain, 1973). The Genus *Campylobacter* is classified under the family *Campylobacteriaceae*, which also includes *Arcobacter*, *Helicobacter*, *Sulfospirillum* and *Wolinella* (Lee and Newell, 2006; Debryne et al., 2008). In 1973, additional organisms originally identified as *Vibrio*-like microaerophilic bacteria were classified under the genus *Campylobacter*, including *C. jejuni* and *C. coli* (Véron and Chatelain, 1973; Butzler, 2004).

1.2.2 Morphology

Campylobacter is derived from two Greek words, “kampulos” and “bacter”, meaning “curved” and “rod”, respectively (Sebald and Véron, 1963). The organisms in the family *Campylobacteraceae* are generally small, Gram negative, slender rods, ranging in size from 0.2-0.9 μm in width and 0.5-5 μm in length (Peterson, 1994; Debryne et al., 2008; Senok and Botta, 2009). *Campylobacters* are non-spore forming and contain single polar flagellum at one or both

ends, allowing them to be highly motile (Debryne et al., 2008; Silva et al., 2011). Multiple cells may group together, forming an “S” or “V” shape, visible under a microscope (Silva et al., 2011)

1.2.3 *In vitro* growth requirements and VBNC form

Campylobacter spp. require complex growth media for *in vitro* growth (Buck and Smith, 1986; Kelly, 2001). *C. jejuni* and *C. coli* are capable of growing at temperatures between 30 and 42°C, with 42°C being the optimum growth temperature (Nachamkin, 1995; Park, 2002; Silva et al., 2011). In addition, these organisms require microaerophilic growth conditions of a reduced oxygen atmosphere containing 5% O₂, 10% CO₂, and 85% N₂ (Park, 2002; Garénaux et al., 2008). *Campylobacter* spp. are fastidious and sensitive to fluctuating oxygen levels, freezing, salinity, moisture availability, acidic conditions (pH ≤5.0), and temperature (Altekruse et al., 1999; Park, 2002).

It has been proposed that *Campylobacter* spp. can survive in adverse conditions by converting to a viable but non-culturable (VBNC) state, in which the organism enters a physiological state that may be difficult or impossible to detect, but certain core metabolic and cellular processes still function (Portner et al., 2007). It has been suggested that these VBNC cells are still capable of infecting hosts (Saha and Sanyal, 1991), although the role of the VBNC state in microbial organisms is still unclear (Pinto et al., 2013).

1.2.4 Environmental reservoirs of *Campylobacter*

Campylobacter spp. are most often found in warm-blooded animals, including many food animal sources, such as chickens, pigs, turkeys, lamb, cattle, dairy cows and duck (Nesbakken et al., 2003; Humphrey et al., 2007; Lin et al., 2007). Untreated water is also considered a consumption risk for humans (Schorr et al., 1994; Eberhart-Phillips et al., 1997; Friedman et al., 2004), as is raw milk, which has also been established as a route for *Campylobacter*, resulting in

human gastroenteritis (Blaser et al., 1979; Robinson et al., 1979; Porter and Reid, 1980; Potter et al., 1983). In addition to animal food sources, water and unpasteurized milk, contact with farm animals and domestic pets also increase the risk of *Campylobacter* colonization and infection (Kapperud et al., 1992; Saeed et al., 1993; Schorr et al., 1994; Studahl and Andersson; 2000).

Poultry have been established as a common *Campylobacter* reservoir. In addition to horizontal transmission from coprophagic behavior of poultry, there are many other vectors which serve as a source for *Campylobacter* in poultry (Shane, 1992). High flock concentrations, as well as cross-contamination from litter, fecal contact, farm personnel and other animals may aid in dissemination, while environmental water supplies, insects, wild birds and rodents also increase the risk of *Campylobacter* spp. colonization (Aarts et al., 1995; Line et al., 2001; Adkin et al., 2006; Horrocks et al., 2009).

1.3 Campylobacteriosis in humans

It has been established that *Campylobacter* spp. are capable of causing infections in animals since the early 1900s; however, it wasn't until about 1980 that *Campylobacter* spp. were identified as causing disease in humans (Silva et al., 2011). In recent years, it has been acknowledged that *Campylobacter* spp. is the most common bacterial agent causing enteritis in the world (Skovgaard, 2007) and consumption of poultry meat has been reported as the most common cause of campylobacteriosis in humans (Silva et al., 2011). Numerous other studies have also reported that improper handling and/or consumption of undercooked poultry or poultry products are a significant source of campylobacteriosis in humans (Beery et al., 1988; Butzler and Oosterom, 1991; Tauxe, 1992; Tauxe, 1997; Corry and Atabay, 2001; Nadeau et al., 2002; CDC, 2013a). *Campylobacter* spp. are responsible for infections in both industrialized and developing countries, and tend to cause more infections in children, immunocompromised, and

elderly persons (Tauxe 1992; Nachamkin and Skirrow, 1998; Corry and Atabay, 2001). Based on reports by the European Food Safety Authority and the European Centre for Disease Prevention and Control, campylobacteriosis is the most commonly reported zoonotic disease in the European Union; the second is salmonellosis (EFSA, 2014). In 2006, the United States reported 43,696 cases of campylobacteriosis, and it is estimated that the true value of this number should be approximately 845,024 cases, when considering under-reporting and under-diagnosing patients (Scallan et al., 2011). In addition, 80% of these cases confirmed were declared food-borne (Scallan et al., 2011). A surveillance study completed in England and Wales showed that *C. jejuni* caused at least 12 times the number of confirmed campylobacteriosis cases than *C. coli* (Friedman et al., 2000). Treatment for campylobacteriosis is often expensive and in 1994, it was estimated that the average cost to treat a *Campylobacter* spp. infection was roughly \$920 per case (CAST, 1994). As of 2010, it is estimated that treatment for campylobacteriosis is \$1,846 per case (Scharff, 2012), equating to detrimental economic costs of approximately \$1.6-1.7 billion annually (Batz et al., 2012; Hoffmann et al., 2012; Scharff, 2012).

1.3.1 Mechanisms of pathogenesis

Pathogenic mechanisms of *Campylobacter* spp. are still not fully known or understood. It has been proposed that adhesion and invasion of intestinal epithelium may play a role in producing the symptoms associated with campylobacteriosis. Potential virulence factors, including flagella-driven motility, invasive mechanisms, adherence within in the mucosa, and toxin production, have been identified and are believed to be part of *Campylobacter* spp. pathogenesis (van Vliet and Ketley, 2001; Asakura et al., 2007; Dastia et al., 2010). *C. jejuni* polar flagellum is comprised of two part system: a sensor (FlgS) and a response regulator (FlgR), that aid in the flagellum function (Dastia et al., 2010). Cytolethal distending toxin genes (cdt

genes) have been sequenced for *C. jejuni* (Pickett et al., 1996; Bang et al., 2001), as well as *C. coli* and *C. fetus* (Asakura et al., 2007; Asakura et al., 2008). These genes are responsible for preventing eukaryotic cells from entering into the mitosis phase, ultimately causing cell death (Yamasaki et al., 2006; Ge et al., 2008; Zilbauer et al., 2008).

1.3.2 Acute infection

Campylobacter is the most common cause of diarrheal illness in humans (CDC, 2013a) and common symptoms of campylobacteriosis include fever, nausea, general malaise, watery or bloody diarrhea, vomiting, and severe abdominal pain (Allos and Blaser, 1995; Rosenquist et al., 2003; Butzler, 2004). These symptoms generally last between 1 to 11 days, and the diarrhea is self-limiting in most cases (Allos and Blaser, 1995; Rosenquist et al., 2003). *Campylobacter* spp. infections can be caused by as few as 500 cells (Robinson, 1981).

1.3.3 Long-term complications

Infection with *Campylobacter* has been linked with serious post-infectious complications, such as Guillain-Barré syndrome, reactive arthritis, irritable bowel syndrome and inflammatory bowel disease.

1.3.3.1 Guillain-Barré syndrome

One potential long-term complication is Guillain-Barré syndrome, a severe, but relatively rare, syndrome that appears in roughly 1 in 1000 *Campylobacter* cases (Buzby et al., 1997; CDC, 2010b). Guillain-Barré syndrome is a neuromuscular disorder, which results in weakness of the limbs and respiratory muscles, as well as loss of reflexes (Mishu and Blaser, 1993; Mishu et al., 1993; Allos, 1997). Guillain-Barré syndrome usually starts with rapid onset of symptoms, with weakness progressing over a period of one to four weeks, while recovery generally takes many months following treatment (Ropper et al., 1991). Outcomes of Guillain-Barré vary and while

some patients are permanently paralyzed or wheelchair-bound, many recover with only minor long-term symptoms (Buzby et al., 1997). In 5-10% cases, Guillain-Barré syndrome can be fatal and death may occur due to respiratory paralysis (Kuwabara, 2004; Willison, 2005). A similar syndrome, called Miller-Fisher Syndrome, a variant of Guillain-Barré syndrome has been reported, which is characterized by symptoms such as ophthalmoplegia (paralysis of the eye muscles), areflexia (lack of neurological reflexes), and ataxia (lack of coordinated muscle movements) (Ohtsuka et al., 1988;Kuwubara, 2004)

1.3.3.2 Reactive Arthritis

Reactive arthritis (ReA), or post-infectious arthritis, is a disease defined by joint and tissue inflammation, which occurs subsequent to bacterial gastrointestinal infections, including *Campylobacter* (Wu and Schwartz, 2008; Carter, 2006; Carter and Hudson, 2009; Townes, 2010). Reportedly, 1-5% of *Campylobacter* cases may result in reactive arthritis (Pope et al., 2007), including symptoms such as joint inflammation, inflammation of eyes, skin or tendons (Pope et al., 2007; Townes, 2010). Reactive arthritis can either be self-limiting (6 months or less) or chronic (Carter, 2006). Diagnosis of reactive arthritis is subjective and currently there are no established diagnostic tests for reactive arthritis (Sieper et al., 2002; Ajene et al., 2013). Reactive arthritis is not gender-dependent, but is more common in adults than children (Carter, 2006).

1.3.3.3 Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a gastrointestinal disorder and is broadly generalized by frequent abdominal pain (more than three times per month) over repeated months (three or more) that cannot be due to any other disease (NDDIC, 2013). Common symptoms include general abdominal discomfort, more or less frequent bowel movements, abnormal stool (too loose or too hard), passing mucus and abdominal bloating (NDDIC, 2013). The link between

Campylobacter and IBS is not fully understood and the relationship between the two is continually changing (Riddle et al., 2012). According to Pimentel et al. (2008), mice have provided a model linking *C. jejuni* infections to overgrowth of bacteria in the small intestine, GI motor dysfunction and chronic inflammation.

1.3.3.4 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a broad category, and is used to describe chronic or recurring inflammatory responses of the gastrointestinal tract (CDC 2014). IBD encompasses multiple diseases, including ulcerative colitis and Crohn's disease (CDC 2014), chronic inflammatory diseases which are distinct from IBS. Food-borne pathogens, including *E. coli*, *M. avium*, *Salmonella* and *C. jejuni* are all suspected pathogens that may be linked to IBD (Darfeuille-Michaud et al., 1998; Boudeau et al., 1999; Sartor, 2003; Gradel et al., 2009). No medical cure currently exists and patients must manage with this chronic disease for their lifetime (CDC 2014).

1.4 *Campylobacter* in poultry

1.4.1 *Campylobacter* in retail poultry products

Research has shown that retail poultry products have much variability in regards to the presence or absence of *Campylobacter* contamination. A study by Zhao et al. (2001) found 70.7% of retail chicken meat samples to be contaminated with *Campylobacter* during a 14-month study of four major supermarket chain stores. A majority of these samples were identified as *C. jejuni*, with the remaining being *C. coli* or "other" *Campylobacter* spp. (Zhao et al., 2001). More recent studies and reports have suggested that 90-100% of raw chicken meat is contaminated (Moran et al., 2009; Smole Mozina et al., 2009; Suzuki and Yamamoto, 2009; EFSA, 2014).

Another study looked at antibacterial resistance of *Campylobacter* spp. recovered from retail meat, finding 94% of the samples to be resistant to at least one or more of the seven antimicrobials tested, which included tetracycline (82% resistant), doxycycline (77%), erythromycin (54%), nalidixic acid (41%), ciprofloxacin (35%), chloramphenicol (1%) and gentamicin (0%) (Ge et al., 2003).

1.4.2 *Campylobacter* colonization in birds

While *Campylobacter* infection in humans causes adverse reactions, *Campylobacter* spp. colonize the intestinal tract of poultry asymptotically around 2-3 weeks of age, as a commensal organism (Beery et al., 1988). Avian bodies provide an ideal growing environment for *Campylobacter* spp., including an optimum growth temperature (Luechtefeld et al., 1980; Kapperud and Rosef, 1983; Altekruse et al., 1999). *Campylobacter* is commonly isolated from the lower intestines, predominantly from the ceca, and concentrations may reach up to 10^8 colony forming units (CFU) per gram of cecal content (Beery et al., 1988; Newell et al., 2000; Cole et al., 2006). Previous studies have shown that *Campylobacter* spp. are present in poultry year-round, and present a specific concern during the warmer months (between May and October) (Willis and Murray, 1997; Nylén et al., 2002). During summer months samples tested positive for *C. jejuni* in 87-97% of those evaluated (Willis and Murray, 1997) and other studies reflect similar findings (Stern and Line, 1991; Zhao et al., 2001). It is estimated that between 40-70% of poultry flocks in the EU are *Campylobacter*-positive at time of slaughter (Denis et al., 2001; Herman et al., 2003; Reich et al., 2008), while in the United States, as many as 90% of flocks are *Campylobacter*-positive at time of slaughter (Buzby, et al. 1997; Stern et al., 2001).

1.4.3 Horizontal transmission

Horizontal transmission is the widely accepted mode of transmission of *Campylobacter* in poultry (Carrillo et al., 2005; Horrocks et al., 2009; Silva et al., 2011). Natural colonization of commercial poultry generally occurs around 2-3 weeks of age, and birds begin shedding *Campylobacter* in the feces (Mead, 2002). It has been observed that *Campylobacter* spp. spread quickly from bird to bird and can rapidly colonize an entire flock (Carrillo et al., 2005; Horrocks et al., 2009). This is aided by birds' coprophagic tendencies, in which *Campylobacter* spp. are disseminated via the fecal-oral route (Keener et al., 2004). Once internalized by the bird, a study by Cox and colleagues demonstrated rapid dissemination and long-term persistence in lymphoid organs after intra-cloacal or oral inoculation of *C. jejuni* in day-old broiler chicks (Cox et al., 2005).

1.4.4 Vertical Transmission

A more debated source of contamination is vertical transmission, in which *Campylobacter* spp. are passed from breeding flock hens to hatched eggs (Silva et al., 2011). *Campylobacter* spp. have been detected in eggs and hatchery fluff, further indicating the possibility of vertical transmission (Silva et al., 2011). Cox and colleagues (2012) discuss the high likelihood that vertical transmission as a source of *Campylobacter* contamination, citing previous research that focused on evidence of transmission from breeder hens to broiler offspring, after identifying indistinguishable *Campylobacter* isolates in both groups (Cox et al., 2002). More research is needed to determine whether vertical transmission is a likely source of *Campylobacter* contamination.

1.4.5 USDA regulations

In 2010, the United States Department of Agriculture Food Safety and Inspection Service (FSIS) initiated the implementation of *Campylobacter* monitoring and control for chickens and

turkeys (USDA, 2010). Performance standards for chickens require that no more than 10.4% (of 1 mL samples) or 46.7% (of 30 mL samples) be positive for *Campylobacter* for processing plants to pass. Performance standards for young turkeys require that no more than 3 of 56 samples be positive for *Campylobacter* (USDA, 2010). The accompanying compliance guide included pre-harvest strategies aimed at reducing *Campylobacter*, with hopes of drastically reducing *Campylobacter* cases within two years (USDA, 2010). *Campylobacter* spp. reporting actively continues through FoodNet (Foodborne Diseases Active Surveillance Network) (Scallan et al., 2011).

1.5 Pre-harvest poultry intervention strategies

The development of intervention strategies is important to the control and reduction of *Campylobacter* in poultry to reduce the human risk. A risk model developed by Rosenquist and colleagues (2003) predicted that a 2-log reduction of *Campylobacter* in poultry can reduce human infections by 30 times. Many pre-harvest strategies have been evaluated, including biosecurity, probiotics and competitive exclusion, bacteriocins, bacteriophages, vaccines and natural compounds, including medium chain fatty acids and plant extracts with varying results.

1.5.1 Biosecurity

In the last decade the necessity of biosecurity has increased, especially considering the regulatory measures implemented by the USDA. With many potential vectors for contamination, including, but not limited to, feed, water, litter, insects, air, farm personnel, and cross-contamination of feces, modified hygienic barriers have become necessary on poultry farms (Newell et al., 2011; Silva et al., 2011). A review of *Campylobacter* interventions by Newell and colleagues (Newell et al., 2011) concluded that while the determined risk levels for some factors are unknown, visitors and farm personnel pose a high risk as a transport route for *Campylobacter*

spp. Many companies and farms have implemented biosecurity hygiene barriers including hand-sanitizing and requiring boots and coveralls upon farm entrance (Silva et al., 2011). In addition, equipment, vehicles and temporary machinery may also contaminate farms, if not properly decontaminated prior to arrival (Newell et al., 2011). While many of these hygienic procedures are not strictly adhered to (Silva et al., 2011), it has been demonstrated that diligent application of basic biosecurity (i.e. boots, coveralls, foot baths) may reduce flock colonization (Kiess et al., 2007).

1.5.2 Probiotics and competitive exclusion

Probiotics are non-pathogenic organisms that can be applied singularly, but are often combined as a mixture, and colonize with the gastrointestinal tract to improve gut health (Fuller 1989; Vanbelle et al., 1990; Griggs and Jacob, 2005). In poultry research, probiotics are generally administered to day-of-hatch chicks, with the purpose of allowing beneficial probiotic bacteria to colonize the gastrointestinal tract prior to contact with pathogenic organisms in the gut (Aguiar et al, 2013). This concept is called competitive exclusion (Mead, 2002), introduced by researchers Nurmi and Rantala in 1973, while studying *Salmonella* in broilers (Nurmi and Rantala, 1973). While efficacy has been shown for *Salmonella* control, inconsistencies have been noted when aimed at *Campylobacter* (Shanker et al., 1990; Mead, 2002; Bielke et al., 2003; Bhaskaran et al., 2011; Aguiar et al. 2013). Previous research has shown mixed efficacy, but studies have reported a reduction of *C. jejuni* when birds were administered *L. acidophilus* and *Enterococcus faecium* (Morishita et al., 1997), and *B. subtilis* spp. (Aguiar et al., 2013).

1.5.3 Bacteriocins

Bacteriocins are anti-bacterial proteins or peptides, produced by bacteria, and are capable of killing or inhibiting the growth of other closely related bacteria (Cleveland et al., 2001).

Limited research is available concerning bacteriocins in relation to *Campylobacter* spp. inhibition, but it has been acknowledged that further research is warranted (Stern et al., 2005). A study by Cole et al. (2006) showed the significant reductions of cecal *Campylobacter* in turkey poultts using two orally administered bacteriocins, isolated from *P. polymyxa* and *L. salivarius*. In three separate trials, these bacteriocins reduced *Campylobacter* to non-detectible levels in comparison to the positive controls, all of which were orally challenged with approximately 10^5 - 10^6 CFU/mL *C. coli* mixture (Cole et al., 2006). Additional studies utilizing bacteriocins produced by *P. polymyxa*, *L. salivarius*, *E. durrans/faecium/hirae*, and *E. faecium* have showed similar results of non-detectible levels, which range from 2.2-6.6 log reductions in chickens (Stern et al., 2005; Svetock et al., 2005; Stern et al., 2006; Line, et al. 2008; Svetock et al., 2008). While bacteriocins present a potential effective solution, worthy of future research, bacteriocins may be limited in application (Lin, 2009). They are expensive and current regulatory issues would prevent industry-wide application. In addition, it has been proposed that *Campylobacter* spp. could develop resistance to bacteriocins and this should be determined prior to implementing bacteriocins as an intervention strategy (Hoang et al., 2011).

1.5.4 Bacteriophages

Bacteriophages are viruses capable of infecting and killing targeted bacteria (Huff et al., 2005). *Campylobacter*-specific phages have been recovered post-harvest in chilled retail poultry, proving capable of surviving further processing conditions (Atterbury et al., 2003). Two *Listeria* bacteriophage products are currently approved by the FDA and utilized in the food production industry, one for ready-to-eat foods (Bren, 2007) and another for meat and cheese products (Carlton et al., 2005). Targeted *Campylobacter* phages have been isolated from broiler and layer chicken excreta, retail poultry and other animal sources (Connerton et al., 2011). Bacteriophages

specific for *C. jejuni* have been administered to chicks, resulting in 0.5-5 log reductions of cecal *Campylobacter* (Carrillo et al., 2005). A bacteriophage cocktail containing three phages administered to chickens reduced *Campylobacter* by 2 logs, a result seen throughout the trial duration (Carvalho et al., 2010). In comparison, another study did not find reduction of *Campylobacter jejuni* or *coli* using two *Campylobacter*-targeted phages (Orquera et al., 2012). More research is needed for bacteriophage application as there are concerns about the development of phage resistance *in vitro* and *in vivo* (Lu and Koeris, 2011). Bacteria are capable of acquiring resistance to bacteriophages, making bacteriophages unlikely as an independent solution to *Campylobacter*, but potentially as a multifaceted reduction strategy (Carrillo et al., 2005).

1.5.5 Vaccination

Another route investigated to reduce or eliminate *Campylobacter* is by developing vaccines, which overall have had limited success. Previous researchers have noted the difficulty of vaccine efficacy in poultry, most likely due to the commensal interaction of *Campylobacter* in the poultry gut (Rice et al., 1997; de Zoete et al., 2007). In addition, a *Campylobacter* vaccine has been challenging to develop due to the difficulty of identifying specific antigens (Saxena et al., 2013). Baqar and colleagues (1995) tested two killed *Campylobacter* vaccines in monkeys, finding that a mucosal adjuvant played an important role in its potential efficacy, warranting future research. A research group compared two commercially available *Campylobacter* vaccines, containing *C. fetus* subsp. *fetus* and *C. jejuni* strains, in guinea pigs (Burrough et al., 2010). Results from this study suggested that an autogenous vaccine may be effective in reducing the *Campylobacter* strain used in the study. Recent vaccine attempts have had mixed success, but a commercial vaccine for poultry has still not been produced (Buckley et al., 2010;

Clavero, 2013). Vaccines are still being researched and would provide an ideal method of control if applicable at the hatchery stage prior to rearing and processing.

1.5.6 Natural compounds

In recent years, there has been consumer emphasis on the demand for safe, natural food products that are free of synthetic residues (Xu et al., 2008). Natural products, generally widely present in nature, inexpensive and many of which are considered GRAS (Generally Recognized As Safe) by USDA, have been shown to possess antimicrobial properties (Chaveerach et al., 2002; Friedman et al., 2002).

1.5.6.1 Medium chain fatty acids

Caprylic acid, along with other fatty acids, has been found to possess antimicrobial properties against an array of bacteria (Petschow et al., 1996; Sprong et al., 2001; Vasudevan et al., 2005). Caprylic acid, a medium-chain fatty acid comprised of eight carbons, is naturally found in coconut oil, bovine and breast milk (Jensen et al., 1990; Jensen, 2002; Nair et al., 2005). Studies by Solis de los Santos et al. (2008a, b) have shown that varying doses of caprylic acid are capable of reducing *C. jejuni* prophylactically and therapeutically.

1.5.6.2 Plant extracts

Plant extracts have been evaluated for antibacterial properties, especially as there is increased pressure to shift away from antibiotic use in animals (Atterbury et al., 2003; Sirsat et al., 2009). Rosemary and rosemary extract both possess antioxidant activity and also show anti-*Campylobacter* potential (Klančnik et al., 2009). A study by Murali et al. (2012) evaluated the efficacy of multiple plant extracts and teas against *C. jejuni*. Lemon extract, turmeric extract, roobios tea, mint tea and green tea were all capable of inhibiting *C. jejuni* in pre-chilled and post-chilled carcasses, as well as retail chicken meat within 36 hours of incubation (Murali et al.,

2012). *Tran*-cinnamaldehyde, eugenol, carvacrol, thymol and cranberry extract have also been evaluated and have shown mixed efficacy against *Campylobacter* spp. (Metcalf, 2008; Arsi, 2011; Woo-Ming, 2012).

1.6 Post-harvest poultry intervention strategies

Pre-harvest intervention strategies have not been effective at consistently reducing or eliminating *Campylobacter* at the farm level, therefore *Campylobacter* remains a challenge during processing and post-harvest, in which these organisms are capable of cross-contaminating other carcasses and equipment in the plant (Hargis et al., 1995; Byrd et al., 1998; Corrier et al., 1999; Zhang et al., 2013). Processing plants provide for potential increases and decreases in bacterial load. Previous studies have shown counts multiplying between 10 and 1,000 times between the farm and the plant, de-feathering or evisceration (Acuff et al., 1986; Izat et al., 1988; Stern et al., 1995). A study by Melero et al. (2012) followed two flocks, from farm to the product packaging in stores and measured *Campylobacter* prevalence at five points: on the farm, slaughter, deboning, processing and product packaging. For Farm A, *Campylobacter* was present at 89%, 70%, 100%, 69% and 59%, respectively, while for Farm B, *Campylobacter* was present at 44%, 96%, 63%, 69% and 69% during the five stages (Melero et al., 2012). *Campylobacter* spp. are generally environmentally sensitive and while chilled storage (at 4°C) doesn't appear to considerably reduce bacteria load (Simmons and Gibbs, 1979; Blankenship and Craven, 1982; Oosterom et al., 1983a; Yogasundram and Shane, 1986), freezing products (at -20°C) is capable of reducing the *Campylobacter* load by 0.5-2.5 logs (Hänninen, 1981; Oosterom et al., 1983b; Yogasundram and Shane, 1986). Once departing the processing plant, packaged poultry meat is still a significant source of *Campylobacter jejuni* (Harris et al., 1986; Altekruze et al., 1994), making post-harvest intervention a key step in *Campylobacter* reduction. Previous studies have

investigated salt solutions and antibacterial agents, including trisodium phosphate, as potential carcass sprays to reduce *Campylobacter* (Slavik et al., 1994). Natural compounds previously mentioned, including medium chain fatty acids and plant extracts, may be potentially efficacious as post-harvest interventions, applicable in poultry processing plant systems. Post-harvest applications must also take into account challenges with palatability, texture and odor alterations that may occur after application (Goode et al., 2003).

1.7 Chitosan

1.7.1 Overview of chitosan

Chitosan is a natural biopolyaminosaccharide produced by deacetylation of chitin, more commonly recognized as crustacean shell waste from crab, lobster, shrimp and other species (No and Meyers, 1995; Qin et al., 2006). Chitosan is produced commercially, deacetylated at different degrees (usually 40-98%) (Ravi Kumar, 2000) and varies in molecular weight (No and Meyers, 1995; Genta et al., 1998). According to No et al. (2007), chitosan is the “second most abundant natural biopolymer”, following cellulose, making it available in large quantities and, ideally, inexpensive (Rabea et al., 2003; Qin et al., 2006), as well as biodegradable, non-toxic and biocompatible (Rinaudo, 2007). Unmodified chitosan is insoluble in water (Rabea et al., 2003; Qin et al., 2006) and tends to coagulate or come out of solution completely at higher pH levels (pH > 6.5) (Rabea et al., 2003). Chitosan is soluble in organic acids, including acetic acid (Rabea et al., 2003), and increases in viscosity as concentration increases. Chitosan isn't a novel natural product as it has been previously applied in commercial industry practices, including biomedical, food production, pharmaceutical, and chemical sectors (Muzzarelli, 1977; Knorr, 1984; Chung and Chen, 2008). Because of the chelating properties of chitosan, it has been applied in waste water treatment (No and Meyers, 2000). Its anti-inflammatory, hemostatic,

antitumor, antioxidant, antimicrobial and immunostimulatory properties (Park et al., 2011) make it an ideal potential applicant for further biomedical research.

1.7.2 Mechanism of action

The antimicrobial mode of action of chitosan (as well as chitin and other derivatives) is largely unknown (Rabea et al., 2003; Raafat et al., 2008). A study by Rabea and colleagues (2003) observed chitosan primarily acting on the outer membrane of bacteria, concluding that chitosan is capable altering cell permeability (Rabea et al., 2003). Previous research has indicated that chitosan is a chelating agent (Muzzarelli et al., 1980; No and Meyers, 2000) and may be capable of binding trace metals, therefore inhibiting toxin production or microbial growth (Cuero et al., 1991), as well as fats, cholesterol and proteins (No and Meyers, 2000).

Chitosan is not soluble in water, but is soluble in many acids, including acetic, formic, lactic, ascorbic, tartaric and propionic acids (Piskin et al., 1986; No et al., 2002). The acidic medium may be key for chitosan to have an antimicrobial effect (Rabea et al., 2003; Qin et al., 2006).

1.7.3 Chitosan research

Chitosan possesses promising antibacterial potential, as a natural product with a broad killing spectrum and low toxicity to mammalian cells (Franklin and Snow, 1981; Takemono, 1989; Liu et al., 2001). Chitosan has demonstrated efficacy against other Gram- positive and negative organisms, as well as fungi, including *E. coli*, *Salmonella*, *Pseudomonas* and *Campylobacter* (El Ghaouth et al., 1992b; Laflamme et al., 2000; Ganan et al., 2009; Friedman and Juneja, 2010). Ganan and colleagues (2009) analyzed the efficacy of three different molecular weight chitosans (120, 400 and 643 kDa) individually on 3 strains of *C. jejuni* and 1 strain of *C. coli*. The *C. coli* strain was reduced to undetectable levels (1.48 CFU/mL) compared

to the control (median log of 6.3 CFU/mL) by all three chitosans at a concentration of 0.05% (Ganan et al., 2009). The three *C. jejuni* strains were reduced to undetectable levels compared to the controls (median log 8.23-9.09 CFU/mL) by the 120 and 400 kDa chitosans, and significantly reduced by the 643 kDa chitosan at a concentration of 0.05% (Ganan et al., 2009). Another group studied the efficacy of chitosan against *S. aureus*, finding a concentration of 1-1.5% necessary to completely inactivate this bacterium (Wang et al., 1992). *E. coli* inhibition was possible with concentrations of 0.5-1% chitosan, and could be completely inactivated with concentrations greater than 1% (Wang et al., 1992). Chitosan has displayed anti-fungal properties in corn and peanut studies, in which chitosan inhibited *Aspergillus flavus* growth (Cuero et al., 1991a,b; Cuero et al. 1992), and also in *in vitro* studies of *F. acuminatum* and *Cylindrocladium floridanum*, which were completely inhibited (Laflamme et al., 2000).

Rabea et al. (2003) has suggested that chitosan within the molecular weight range of 10,000-100,000 kDa is ideal for restraining bacterial growth. Other research suggests chitosan within the molecular weight range of 200,000-300,000 kDa has shown higher efficacy against Gram-negative and Gram-positive organisms (Kim et al., 2007). While no specific range has been declared more efficacious, it has been suggested that lower molecular weight chitosans are more inhibitory against microorganisms than higher molecular weight chitosans (Kim et al., 2006; Ganan et al., 2009).

1.7.4 Application in poultry

Many studies have been performed analyzing the inclusion of chitosan in poultry feed and the potential performance effects, with consistent results among the different studies. Studies have evaluated inclusion levels of chitosan in the poultry diet, indicating that a higher level of chitosan (3%) results in negative performance effects, including reduced live weight and feed

intake (Razdan and Pettersson, 1994; Razdan et al., 1997). Another evaluated lower levels of chitosan between 0.02 and 0.5% and determined that concentrations between 0.05 and 0.1% improved broiler performance in comparison to untreated birds (Shi et al., 2005). A study in which broiler treatment groups were fed chitosan salt, which constituted 3% of the poultry diet, resulted in slightly decreased weight gain of 2-2.5% compared to the control group (Balicka-Ramisz et al., 2007). However, the results of this study showed birds treated with chitosan prior to exposure to *S. gallinarum* were highly resistant to *S. gallinarum* infection (Balicka-Ramisz et al., 2007), which may be considered as a potential trade-off in the poultry industry. Another study has evaluated the fat deposition and lipase effects of chitosan in broilers, concluding that dietary chitosan reduces excessive abdominal deposition, without sacrificing feed intake, body weight gain or feed efficiency (Kobayashi et al., 2002). A similar study evaluating dietary chitosan found body weight gain and feed intake were increased in birds fed chitosan compared to birds fed no chitosan, but that there were no differences in feed efficiency or breast meat or drumstick weight (Khambualai et al., 2009). Researchers' consensus appears to conclude that lower inclusion levels of chitosan are not inhibitory to bird growth or performance.

Chitosan presents potential for application in a processing plant as it has previously been applied in the food industry as an antimicrobial against a range of food-borne microorganisms (No et al., 2002; Sagoo et al., 2002; Beverly et al., 2008). Chitosan has been applied as a spray product on fresh fruit, including tomatoes and strawberries, capable of extending shelf life of fresh foods (El Ghaouth et al., 1992a; El Ghaouth et al., 1992c). Menconi and colleagues (2013) evaluated the efficacy of 0.5% chitosan against *Salmonella* Typhimurium on dipped chicken skin. Chitosan effectively reduced *Salmonella* after 24 hours in one study, and then reduced

Salmonella to undetectable levels after 1 and 24 hours, and 3, 6, 9 and 12 days in a secondary study (Menconi et al., 2013).

Chitosan may also have an application in poultry packaging. A study by Petrou and colleagues (2012) found chitosan combined with oregano oil added during packaging reduced mesophilic total plate counts, lactic acid bacteria, *Brochothrix thermosphacta*, *Enterobacteriaceae*, *Pseudomonas* spp., and yeast molds during the 21-day storage period. A chitosan-thyme combination was evaluated for efficacy against the same challenges mentioned in the previous study on packaged chicken kebabs, and chitosan-thyme significantly reduced total plate counts, lactic acid bacteria, *Bronchotrrix thermosphacta*, *Enterobacteriaceae*, *Pseudomonas* spp., and yeast molds in comparison the controls after 12 days of storage (Giatrakou et al., 2010). A chitosan-arginine solution was tested on *E. coli*-inoculated chicken juice at varying doses (between 100-500 $\mu\text{g mL}^{-1}$) to demonstrate potential application in poultry packaging (Lahmer et al., 2012). Results showed significant reduction of *E. coli* by treatments 200 $\mu\text{g mL}^{-1}$ and greater between 3-72 hours incubation (Lahmer et al., 2012).

Reductions of other bacteria *in vivo* and post-harvest, combined with previous research indicating that lower levels of chitosan would not inhibit growth performance in broilers, make chitosan an ideal potential intervention strategy for *Campylobacter* research. Previously performed research indicates potential for chitosan as a pre-harvest intervention strategy in poultry feed, as well as a post-harvest strategy applied in the processing plant or poultry product packaging.

CHAPTER 2
Chitosan Reduces Enteric Colonization of *Campylobacter* in Young Chickens, but Not on Post-Harvest Chicken Skin Samples

CHAPTER 2: Chitosan Reduces Enteric Colonization of *Campylobacter* in Young Chickens, but Not on Post-Harvest Chicken Skin Samples

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ABSTRACT

Worldwide, *Campylobacter* is one of the leading causes of foodborne bacterial gastroenteritis causing an estimated 1.3 million infections in the United States alone. Consumption and/or cross-contamination of raw or undercooked poultry products have been linked as the most common source of *Campylobacter* infection, making the poultry industry a target for *Campylobacter* reduction strategies. *Campylobacter* is prevalent in most poultry flocks in the United States, with as many as 90% of flocks *Campylobacter*-positive at the time of slaughter. It is estimated that a reduction of *Campylobacter* in poultry would greatly reduce the risk of campylobacteriosis in humans. Unfortunately, there are a lack of effective intervention options to reduce *Campylobacter* in poultry. One potential strategy is the use of the natural product, chitosan, a deacetylated byproduct of crustacean shells, has been shown to reduce *E. coli* and *Salmonella*. The purpose of this study was to determine the ability of chitosan to reduce enteric *Campylobacter* colonization in pre-harvest chickens and on post-harvest chicken skin samples. In each of three trials, 100 birds were divided into 10 treatments (n=10) and were fed either 0% (controls), 0.25%, 0.5% or 1% (wt./wt.) of a low, medium or high molecular weight chitosan (300 birds total). Birds were fed treated feed for the duration of the study and were orally challenged with a four-strain mixture of wild type *C. jejuni* on day 6. On day 15, the ceca were excised and enumerated for *Campylobacter*. In all three trials, the 0.5% dose of the medium

molecular weight chitosan reduced cecal *Campylobacter* counts. Because this medium molecular weight chitosan was shown to be the most effective, it was evaluated for post-harvest efficacy against *Campylobacter* on chicken skin. When a 0.5, 1 or 2% concentration was tested in three separate trials, *Campylobacter* counts were not reduced when compared to controls. These results support the use of chitosan in pre-harvest chickens but not for the reduction of *Campylobacter* as a post-harvest rinse on skin for the concentrations used in this study.

Keywords: *Campylobacter jejuni*, chitosan, broiler, pre-harvest, post-harvest

INTRODUCTION

Worldwide, *Campylobacter* is one of the most frequently reported food-borne pathogens and causes an estimated 1.3 million infections in the United States annually (CDC 2013a). Seasonal variation of campylobacteriosis occurs in some developed countries, generally peaking in the summer and fall (Nylen et al., 1992; Willis and Murray, 1997; Altekruse et al., 1999). However, this is not the case in many developing countries where *Campylobacter* infections consistently appear year-round (Taylor 1992; Oberhelman and Taylor, 2000). While the majority of *Campylobacter* cases result in acute gastroenteritis, infection has also been associated with more severe diseases, including Guillain-Barré syndrome (Goodyear et al., 1999; CDC, 2013b), reactive arthritis (ReA) (Berden et al., 1979; Ajene et al, 2013), irritable bowel syndrome (IBS) (NDDIC, 2013), and inflammatory bowel disease (IBD) (CDC, 2014). Epidemiological evidence supports the most common source for *Campylobacter* infections in humans is due to consumption of poultry products (Beery et al., 1988; Tauxe, 1997; CDC, 2013a). This is typically due to the consumption of improperly cooked chicken or cross-contamination from handling raw chicken (Danis et al., 2009; Fajó-Pascual et al. 2010; CDC, 2013a). *Campylobacter* colonization in poultry is common; for example, as many as 90% of U.S. broiler flocks are

contaminated with this food-borne pathogen (Buzby et al. 1997; Stern et al., 2001). In addition, because pre-harvest intervention strategies have not yet been successful in reducing *Campylobacter*, this bacterium is present and cross-contaminates the processing plant, between carcasses and equipment (Hargis et al., 1995; Zhang et al., 2013). Therefore, a reduction or elimination of *Campylobacter* in pre- and post-harvest poultry is a research priority to reduce the burden of this pathogen in humans.

Many pre-harvest strategies have been evaluated in an attempt to reduce *Campylobacter* in poultry, including biosecurity, probiotics, competitive exclusion, bacteriocins, bacteriophages, vaccines, and natural compounds, often with limited success (Mead, 2002; Cole et al., 2006; Santos et al., 2008a,b; Newell et al., 2011; Murali et al., 2012; Orquera et al., 2012; Saxena et al., 2013). Recently, the natural product chitosan has shown potential to reduce colonization of another food-borne pathogen, *Salmonella* Typhimurium, in pre-harvest poultry (Menconi, et al. 2013) and may have application against *Campylobacter*. Chitosan has also shown efficacy against other Gram-negative species, including *Escherichia coli* and *Pseudomonas fluorescens*, (No, et al., 2002; Rabea, et al. 2003). Chitosan, a natural byproduct, derived from the deacetylation of chitin, which is produced from crab and shrimp shell waste (No and Meyers, 1995; No, et al. 2007). Chitosan is a potential natural food preservative, with broad antimicrobial benefits (No, et al. 2002; Sagoo, et al. 2002; Beverlyya, et al. 2008). Although the exact mode of action of chitosan is unknown, researchers have previously determined that chitosan is capable of interacting with the outer cell membrane, altering its permeability (Helander, et al., 2001; Rabea, et al. 2003). To our knowledge, the ability of chitosan to reduce *Campylobacter* colonization in poultry has not been evaluated. The purpose of this study was to determine the efficacy of chitosan on *Campylobacter* colonization in broiler chicks and as a post-harvest intervention

applied on chicken skin. Young chickens were used in this study because previous results from our laboratory demonstrated that young birds can be used as a reliable model to study *Campylobacter* colonization in market age birds (Solis de los Santos, et al. 2008a; Solis de los Santos, et al. 2009).

MATERIALS AND METHODS

Chitosan Materials

Chitosan of molecular weight 50-190 kDa and 190-310 kDa was obtained from Sigma-Aldrich (St. Louis, MO), and 400-600 kDa chitosan was purchased from Spectrum Chemicals (New Brunswick, NJ).

***In vitro* susceptibility of *C. jejuni* to chitosan**

Antimicrobial activity of each molecular weight chitosan, low (50-190 kDa), medium (190-310 kDa) and high (400-600 kDa), in a 0.5% (wt./vol.) solution was determined by inoculating each solution with a four-strain mixture of wild-type *Campylobacter jejuni*. Preparation of the *Campylobacter* inoculum was done as described previously by Farnell and others (2005). In brief, working stock cultures of the four wild-type strains of *C. jejuni* were obtained by individually inoculating each strain into fresh *Campylobacter* Enrichment Broth (CEB) from frozen glycerol stock and successively sub-culturing twice at 42°C for 48 hours under microaerophilic conditions. Strain mixtures were then combined centrifuged at 3000 x g for 10 minutes and the cell pellet re-suspended in 10 mL Butterfield's Phosphate Diluent (BPD). A 1% stock solution (wt./vol.) of each molecular weight of chitosan was prepared in 50 mM acetic acid as described by Ganan and others (2009). For the experiment, the stock concentration of each of the chitosan solutions and the acetic acid control was diluted 1:1 with an inoculum containing 10⁸ CFU/mL of *C. jejuni*, resulting in a final concentration of 0.5% for each chitosan.

Sample time points included 0, 2, 4 and 8 hours post-inoculation. At each time point an aliquot from the treatments and control was taken and 1:10 serial dilutions were direct plated on Campy Line Agar (Line, 2001). The plates were incubated for 48 hours at 42°C in a microaerophilic atmosphere. Direct enumeration of *Campylobacter* colonies was converted to CFU/mL for each treatment. Each susceptibility assay was repeated in duplicate.

***In vivo* susceptibility of *C. jejuni* to chitosan**

Day of hatch Cobb broiler chicks (Siloam Springs, AR) from a local commercial hatchery were utilized for the animal experiments. In each of three replicate trials, 100 chicks per trial were randomly divided into 10 treatments, which consisted of three concentrations (0.25%, 0.5%, or 1% wt./wt.) of each molecular weight chitosan, which was added to the feed and a positive control (0% chitosan). Birds were placed in floor pens and provided feed and water *ad libitum*; treated feed was provided throughout the entire trial.

The *Campylobacter* challenge was prepared with the same method as mentioned previously for *in vitro* susceptibility. Birds were challenged by oral gavage with 0.25 mL of a four strain mixture of wild-type *Campylobacter jejuni* on day 6, at a concentration of 10^7 - 10^8 CFU/mL. On day 15, birds were euthanized and the ceca were excised for *Campylobacter* enumeration. Cecal contents were serially diluted 10-fold with BPD and plated on CLA for direct enumeration. Plates were incubated at 42°C under microaerophilic conditions for 48 hours and enumerated for *Campylobacter* colonies as previously described by our laboratory (Aguiar et al., 2013).

Post-harvest susceptibility of *C. jejuni* to chitosan

For each experiment, skin from commercially available chicken thighs was cut into 2.0 g pieces (± 0.05 g). A total of 40 skin samples were divided into 4 treatments (n=10/treatment),

which consisted of three concentrations (0.5%, 1%, or 2% wt./vol.) of medium molecular weight chitosan made soluble in 50 mM acetic acid and a positive control (50 mM acetic acid). The *Campylobacter* challenge was prepared as mentioned previously. Each piece of skin was inoculated individually with 50 μ L of the 4 strain *Campylobacter jejuni* mixture (approximately 10^8 CFU/mL). The *Campylobacter* was allowed to adhere to the skin for 30 minutes at room temperature prior to application of the treatments. Per each treatment group, skin samples were simultaneously dipped into each treatment for 30 seconds, removed and allowed to air dry for 2 minutes. Samples were then transferred individually to conical tubes, serially diluted, plated on Campy Line Agar (Line, 2001) and incubated for 48 hours at 42°C in a microaerophilic atmosphere as described above. Direct enumeration of *Campylobacter* colonies was converted to CFU/mL of treatment. The trial was repeated in triplicate.

Statistical analysis

Cecal and skin *Campylobacter jejuni* counts were logarithmically transformed before analysis to achieve homogeneity of variance (Byrd et al., 2003). Analysis of the data was done using the PROC GLM procedure of SAS (SAS Institute, 2002). Treatment means were partitioned by LSMEANS analysis (SAS Institute, 2002) and probability of $P < 0.05$ was required for statistical significance.

RESULTS

Chitosan *in vitro*

Campylobacter counts were reduced by approximately 1 log at 2 and 4 hour when co-incubated with 0.5% for all three molecular weights of chitosan when compared with controls (Table 1). At 8 hours, all three chitosan preparations produced a 4½ to 5 log reduction in counts when compared with controls.

Chitosan *in vivo*

In trial 1, *Campylobacter* counts were reduced in six of the chitosan treatments: 0.25% and 0.5% LMW, 0.25% and 0.5% MMW, 0.25% and 1% HMW, in comparison to the positive control (Table 2). Trial 2 showed significant reduction of *Campylobacter* by four of the chitosan treatments: 0.5% LMW, 1% LMW, 0.25% MMW, and 0.5% MMW (Table 2). Results from Trial 3 showed significant reduction of *Campylobacter* by one of the chitosan treatments: 0.5% MMW (Table 2).

Chitosan post-harvest

Campylobacter counts were not reduced by 0.5%, 1% or 2% chitosan treatments applied to the skin for any of the three studies (Table 3). In trials 1 and 2, there were no differences between the four treatments; however, in the trial 3, there were higher *Campylobacter* counts from the skin treated with 2% medium molecular weight chitosan, in comparison to the positive control, 0.5% or 1% medium molecular weight.

DISCUSSION

Preliminary *in vitro* results utilizing a 0.5% dose demonstrate that the three molecular weight chitosan treatments reduce *Campylobacter* counts in comparison to the untreated controls (Table 1). To evaluate the ability of chitosan to reduce enteric *Campylobacter* colonization in chickens, the 0.5% concentration of all three molecular weight chitosans, plus a lower (0.25%) and higher dose (1%) was also evaluated. In the first trial, cecal *Campylobacter* counts were reduced in 6 out of 8 of the treatments (Table 2). When conducted in a second trial, 4 of the 8 treatments were effective whereas in a third replicate trial, the 0.5% MMW reduced enteric *Campylobacter* counts when compared with controls (Table 2). Although there is variability

among replicate trials, the 0.5% MMW chitosan dose consistently reduced *Campylobacter* in all three trials.

The significance of replicating results demonstrating a significant reduction in enteric *Campylobacter* counts in pre-harvest poultry cannot be underestimated. Previous research conducted by our laboratory (Solis de los Santos et al., 2008; Metcalf et al., 2011) and others (Hakkinen and Schneitz, 1999; Hilmarsson et al., 2006; Robyn et al., 2013) have highlighted the variability among trials when evaluating pre-harvest treatments against enteric *Campylobacter*. Because of this inherent variability associated with *Campylobacter* colonization studies, results from a single preharvest study may not fully evaluate the consistency, or ruggedness of a *Campylobacter* intervention strategy (Carvalho et al., 2010; Molatová et al., 2010; Van Deun et al., 2010; Neal-McKinney et al., 2012).

Although the 0.5% dose of the MMW chitosan was effective in consistently reducing *Campylobacter* counts in pre-harvest chickens (Table 2), it was not effective when applied to processed skin samples (Table 3). In fact, *Campylobacter* counts were actually higher in one of the trials utilizing the 2% dose. The inability of chitosan to be effective on chicken skin may be due to its viscosity in solution, especially at higher concentrations. Chitosan appears to coat chicken skin possibility preventing the removal of loosely adhering *Campylobacter* cells from the skin surface. Therefore, it may not be a suitable post-harvest treatment in poultry processing plants.

Feed application of chitosan is a viable application for reducing *Campylobacter* colonization in chickens, however, water application is also a possible option. Unfortunately, chitosan is insoluble in water within the normal pH range (Chandumpai et al., 2004; Qin et al., 2004). This problem can be resolved by mildly acidifying the water, as accomplished in our *in*

vitro and post-harvest skin trials. It is possible this will enhance the efficacy of this compound as proposed by Qin and co-workers (2006). Acidifying water lines is already being performed in some poultry operations and is reported to reduce another foodborne pathogen, *Salmonella* (Byrd et al., 2003), and may also aid in the reduction of *Campylobacter* in the water lines and during feed withdrawal prior to processing, without altering the gut epithelium (Byrd et al., 2001; Chaveerach et al., 2004). Thus, acidifying water in poultry houses could have a number of positive effects on bird health and reduce the potential zoonotic transfer of pathogens to humans. This possibility is currently under investigation.

The use of pre-harvest intervention strategies to reduce *Campylobacter* colonization (e.g., chitosan) can be part of a multifaceted approach to reduce the incidence of this foodborne pathogen. It has been proposed that a 2-log reduction in *Campylobacter* on the chicken carcass could reduce the risk of human campylobacteriosis by up to 30-fold (Rosenquist, et al. 2003). Perceivably “small” reductions of *Campylobacter* in chickens could result in large reductions of campylobacteriosis incidences in humans. Olsen and colleagues (2008) compiled data relevant to the consistent rise of campylobacteriosis incidences from the 1980s through 2006 in many countries, including Denmark, England, Wales, Norway, Sweden, New Zealand, and Australia, many of which are currently monitored by the ECDC. In the 2000s, New Zealand focused on poultry as the primary source of *Campylobacter* and applying required regulatory implementations, along with the assistance of voluntary interventions, New Zealand saw a 54% decline in campylobacteriosis incidences in 2008 compared to the 2002-2006 years (Sears, et al. 2011). This decline was associated with only a relatively small reduction of approximately ½-log *Campylobacter* counts on chicken carcasses (French, 2010). New Zealand’s well-

documented reduction of campylobacteriosis cases sets precedence for global reduction of *Campylobacter* by focusing intervention strategies on the poultry industry.

In conclusion, enteric *Campylobacter* counts were consistently reduced for the 0.5% MMW chitosan in three replicate trials but this treatment was not effective on post-harvest skin samples. The use of this chitosan in pre-harvest poultry may be incorporated into a multifaceted strategy to reduce *Campylobacter* counts in chickens.

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Table 1: The effect of different molecular weight chitosans on *in vitro* growth of *Campylobacter jejuni*^{1,2,3}

Campylobacter* counts, *in vitro

Treatment	Hours			
	0	2	4	8
Positive controls	6.35 x 10 ⁷	8.15x10 ⁷	5.45x10 ⁷	3.5x10 ⁷
Low Molecular Weight	3.42x10 ⁷	6.8x10 ⁶	1.24x10 ⁶	3.0x10 ²
Medium Molecular Weight	8.55x10 ⁷	2.55x10 ⁶	1.82x10 ⁶	5.5x10 ²
High Molecular Weight	7.45x10 ⁷	2.59x10 ⁶	2.00x10 ⁶	6.5x10 ²

¹0.5% concentration of: low molecular weight chitosan is 50-190 kDa; medium molecular weight chitosan is 190-310 kDa; or high molecular weight chitosan is 400-600 kDa, in 50 mM acetic acid

²*Campylobacter* inoculum was added to each chitosan treatment and then sampled at 0, 2, 4, and 8 hours; samples were plated and enumerated after 48 hour incubation

³Values represent average campylobacteriosis counts of two separate replicate trials

Table 2: The effect of different concentrations and molecular weight chitosans on cecal *Campylobacter jejuni* counts (means±SEM) in 15-day old broiler chicks during three separate trials^{1, 2, 3, 4}

<i>Campylobacter</i> counts, <i>in vivo</i>				
	Chitosan dose	Trial 1	Trial 2	Trial 3
Positive controls	0%	8.77±.17 ^a	7.05±.69 ^a	8.36±.24 ^a
Low Molecular Weight	.25%	7.06±.58 ^{cde}	7.1±.29 ^{ab}	8.59±.20 ^a
	.5%	7.68±.27 ^{bcd}	3.96±1.02 ^c	7.88±.38 ^{ab}
	1%	7.96±.15 ^{abc}	ND ^d	7.76±.40 ^{ab}
Medium Molecular Weight	.25%	6.76±.34 ^{de}	4.83±1.08 ^{bc}	8.47±.21 ^a
	.5%	7.4±.38 ^{bcd}	3.25±.94 ^c	7.28±.70 ^b
	1%	8.03±.14 ^{abc}	7.45±.34 ^a	8.57±.17 ^a
High Molecular Weight	.25%	7.45±.19 ^{bcd}	7.49±.31 ^a	8.16±.29 ^{ab}
	.5%	8.43±.18 ^{ab}	7.8±.35 ^a	8.34±.26 ^a
	1%	6.3±.74 ^e	7.31±.30 ^a	8.51±.19 ^a

¹Low molecular weight chitosan is 50-190 kDa; medium molecular weight chitosan is 190-310 kDa; high molecular weight chitosan is 400-600 kDa

²ND= non-detectible

³Day-of-hatch birds were fed chick starter treatments of 0.25%, 0.5% or 1% of either low molecular weight, medium molecular weight or high molecular weight chitosan, respectively, for the entire 15-day study; birds were inoculated with *Campylobacter jejuni* mixture on day 6 and cecal contents were collected on Day 15 for campylobacteriosis enumeration

⁴Means within columns with no common superscript differ significantly (p<.05)

Table 3: The effect of different concentrations of medium molecular weight chitosan on skin *Campylobacter jejuni* counts (means±SEM) *in vitro* ^{1, 2, 3}

***Campylobacter* counts on skin**

Treatment	Trial 1	Trial 2	Trial 3
Positive controls (0% chitosan)	3.78±.44 ^a	4.94±.68 ^a	2.75±.41 ^b
0.5% Medium Molecular Weight	3.93±.44 ^a	3.79±.53 ^a	2.40±.48 ^b
1% Medium Molecular Weight	3.78±.37 ^a	4.93±.22 ^a	2.20±.42 ^b
2% Medium Molecular Weight	4.27±.13 ^a	5.16±.19 ^a	3.97±.15 ^a

¹Medium molecular weight chitosan is 190-310 kDa

²*Campylobacter* inoculum was added to each skin sample, allowed to adhere for 30 minutes, and then skin samples were dipped into the respective solutions; samples were plated and enumerated after 48 hour incubation

³Means within columns with no common superscript differ significantly (p<.05)

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February 9, 2012

MEMORANDUM

TO: Dr. Dan Donoghue

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 06021

Protocol Title: "Reducing Food Borne Pathogens in Poultry"

Approved Project Period: Start Date: February 14, 2012
Expiration Date: February 13, 2015

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol 06021, "Reducing Food Borne Pathogens in Poultry". You may continue your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

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



MEMORANDUM

TO: Annie Donoghue
Jonathan Moyle

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: May 11, 2011

SUBJECT: **IACUC PROTOCOL APPROVAL**
Expiration date : **May 8, 2014**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol #11037-**"USING NATURAL COMPOUNDS TO REDUCE CAMPYLOBACTER IN PRE-HARVEST CHICKENS"**. You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing **prior** to initiating the changes. If the study period is expected to extend beyond **05-08-2014**, you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian



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for Poultry Science



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Attn: University of Arkansas Graduate School

March 26, 2014

Dear Sir or Madam,

I attest that Hanna Arambel was the primary author of the manuscript cited below and completed at least 51% of the work for the paper:

H. Arambel, A.M. Donoghue, K. Arsi, A. Woo-Ming, P.J. Blore, K. Venkitanarayanan and D.J. Donoghue. Chitosan reduces enteric colonization of *Campylobacter* in young chickens, but not on post-harvest chicken skin samples.

Sincerely,

Dan J. Donoghue
Professor
Department of Poultry Science
POSC O-114
University of Arkansas
Fayetteville, AR 72701
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E-mail: ddonogh@uark.edu