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Feed Supplementation with Natural Extracts of Cranberry and its Efficacy on Campylobacter Colonization in Poultry

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FEED SUPPLEMENTATION WITH NATURAL EXTRACTS OF CRANBERRY AND ITS EFFICACY ON *CAMPYLOBACTER* COLONIZATION IN POULTRY

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ABSTRACT

Campylobacter spp. has been identified as one of the leading causative agents of food borne diarrheal illness. Epidemiological evidence has shown that poultry is the main source for human infection. Poultry are asymptomatic carriers of *Campylobacter* within their gastrointestinal tract, with colonization levels reaching 10^6-10^8 cfu/g cecal content. Surveys of domestic poultry flocks have estimated that approximately 90% of flocks are positive for *Campylobacter* colonization. Risk assessment studies have determined that by reducing levels of *Campylobacter* colonization during rearing, incidences of human infection will be significantly reduced. Currently there are no consistently effective treatments to eliminate *Campylobacter* from poultry flocks. The use of natural plant extracts to control food borne pathogens is an area of resurgent interest due to growing consumer demand for removal of sub-theraputic administration of antibiotics in conventionally raised livestock and the increased demand for organic meat products. Extracts from American Cranberry (*Vaccinium macrocarpon*) contain proanthocyanidins which have demonstrated antimicrobial activity against other food borne pathogens including *E. coli*, *Salmonella*, and *Listeria*. However, their ability to reduce *Campylobacter* in chickens has not been reported. The objective of this study was to evaluate two different cranberry extracts, either containing a lower (1%) or higher concentration (30%) of proanthocyanidins by the manufacturer (L-PAC or H-PAC, respectively), to inhibit the growth of *Campylobacter*, *in vitro* and *in vivo*. In replicate *in vitro* trials, a 0.1 or 0.5% dose had no effect, the 1% dose produced a modest reduction and the 2 or 4% doses produced at least a 5 log reduction in *Campylobacter* counts when compared to controls 8 or 24 hours after inoculation. For the *in vivo* studies, 70 chicks were randomly assigned to one of seven treatment groups (n=10 per treatment group). Treatment groups for each trial included a positive *Campylobacter*

control (no cranberry extract) or 0.5%, 1%, or 2% of either H-PAC or L-PAC added to the feed. The same dosages were used in two replicate trials. For each trial, all birds were given feed supplemented with H-PAC or L-PAC, except for positive *Campylobacter* controls, starting at day of placement and continuing through the entire 14 day trial. At day 7 all birds were challenged with a mixture of three wild type *Campylobacter jejuni* strains by oral gavage (approximately 2.5 $x10^5$ cfu/mL). On day 14, birds were euthanized by CO_2 and cecal contents were collected for enumeration of *Campylobacter*. In both trials cecal *Campylobacter* counts were not reduced by administration of L-PAC or H-PAC in the feed. Follow up experiments are needed to increase the potency of these cranberry extracts to reduce this important food borne pathogen in chickens.

This thesis is approved for recommendation
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DEDICATION

I would like to dedicate this work to my husband Brian. Whose support and encouragement helped make this possible. Thank you and I love you. I would also like to dedicate this to my children, Emma and Brian. I hope that you will always have the love of learning, as I do.

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

One of the leading bacterial causes of food borne illness worldwide is contamination of food products by thermophilic *Campylobacter* spp. In many nations, programs have been developed to try to determine the potential disease burden caused by known food borne pathogens. In the United States, the Centers for Disease Control (CDC) estimates that yearly, *Campylobacter* spp. is responsible for approximately 850,000 food related illnesses (Scallan et al., 2011). The European Food Safety Authority (EFSA), which provides food borne illness data from its twenty seven Member States, estimates that annually *Campylobacter* spp. causes 9 million illnesses in the European Union (European Food Safety Authority, 2011b). Poultry products have been cited as the leading cause of human campylobacteriosis in developed countries (Rosenquist et al., 2003; Friedman et al., 2004; World Health Organization, 2009; European Food Safety Authority, 2010a). Source attribution and case control studies have indicated that the most frequent cause of human infection with *Campylobacter* are eating improperly cooked chicken, handling chicken and exposure to animals including poultry (Friedman et al., 2004; Danis et al., 2009; Lindmark et al., 2009; Fajó-Pascual et al., 2010). *Campylobacter jejuni* is the species most frequently identified from food borne campylobacteriosis cases (European Food Safety Authority, 2011b).

1.2 Characteristics of *Campylobacter*

1.2.1 Historical Overview

The family *Campylobacteraceae* consists of the genus *Campylobacter* and related genera including but not limited to *Helicobacter*, *Wolinella*, *Sulfurospirillum,* and *Arcobacter* (Debruyne et al., 2008). There are currently at least 17 identified species of *Campylobacter,* but

the number is expected to grow due to the continual identification of new species, aided by the advent of new molecular detection and identification tools (Lawson et al., 1998; Debruyne et al., 2008). *Campylobacter* spp. were first isolated and described in 1906 by McFayden during his investigation into increased rates of abortion in sheep and cattle due to an unknown agent (Skirrow, 2006). It was several years later, in 1919, that Smith and Taylor first classified the veterinary pathogen as *Vibrio fetus* and not until 1963 that the genus of *Campylobacter* was designated (Smith and Taylor, 1919; Sebald and Veron, 1963). Further classification of the *Vibrio* like organisms into the type species *C. fetus* along with *C. coli*, *C. jejuni*, and *C. sputorum* was proposed in 1973 by Vêron and Chatelain owing to its differing characteristics from the genus *Vibrio* (Veron and Chatelain, 1973; Butzler, 2004). *Campylobacter* spp. has risen from obscure veterinary pathogen that causes abortion and enteritis in livestock, to the leading etiological agent of food borne illness in the developed world (European Food Safety Authority, 2011b; Scallan et al., 2011). Our understanding of the nature and abundance of *Campylobacter* with regards to human illness and food safety has evolved over the last 40 years principally due to pioneering researchers that developed isolation and identification methodologies for this relatively fastidious organism (Dekeyser et al., 1972; Skirrow, 1977).

1.2.2 Morphology and *In Vitro* Culture Conditions

The family *Campylobacteraceae* is composed of small (0.2 to 0.8μm) curved or S-shaped, non-spore forming Gram negative rods with a single polar flagella at one or both ends of the cell (Debruyne et al., 2008). Its morphology is known to evolve from spiral form to coccoid depending upon age of the culture and external environmental conditions (Griffiths, 1993; Debruyne et al., 2008). Members of the genus *Campylobacter* require reduced oxygen,

microaerobic atmosphere for growth and the primary human disease causing species, *C. jejuni* and *C. coli*, are thermophiles whose optimum temperature for growth is 42^0 C (King, 1957; King, 1962).

1.2.3 Genetic Diversity

A characteristic of *Campylobacter* spp. that has been frustrating and challenging researchers alike is the extensive amount of strain variability of this organism. Research in the phenotypic characteristics of *C. jejuni* have shown it to express wide ranging rates of invasiveness into host cell lines, chicken cell lines, and toxin produced (Hu and Kopecko, 1999). Efforts have been made to better understand these variations and the use of whole genome sequencing technologies and microarray based assays are being employed to investigate the genetic diversity within the genus. The first whole genome molecular analysis of *C. jejuni* was performed by Parkhill, and from that work it was determined that *C. jejuni* has a circular chromosome of approximately 1.6Kb rich in A+T (~70% A+T) (Parkhill and Wren, 2000). Also noted upon analysis of the whole genome were the following: (1) the lack of presence of transposons; (2) traces of phage integration in the chromosome; (3) lack of other commonly utilized molecular mechanisms for gaining new genetic material such as insertion elements; and (4) few number of repeat sequences (Parkhill and Wren, 2000). Following the first whole genome sequence of *Campylobacter jejuni* strain NCTC11168, many other strains have since been sequenced and their genome information is freely available for use in further research. The availability of multiple genome sequences has enabled researchers to utilize microarray technology for multiple strain comparisons at the molecular level (Dorrell et al., 2001; Pearson et al., 2003; Parker et al., 2006; Duong and Konkel, 2009). From these multiple strain

comparisons, researchers have identified areas within the *Campylobacter* genome with the potential to generate the high degree of diversity that is notable within this genus. Areas found include hypervariable plasticity regions, of which seven have been identified so far, and they are believed to contain approximately 50% of the genes involved in generating genomic diversity (Pearson et al., 2003). Genes contained within the plasticity regions are believed to be mostly dispensable within the *Campylobacter* spp. genome, with functional activity including the modification of flagellar, outer membrane, and periplasmic proteins along with the ability to accept alternative electron sources (Pearson et al., 2003). During Parkhill's first analysis of the *Campylobacter* NCTC 11168 genome, it was noted that it contained homopolymeric tracts of nucleotides which have since been traced to hypervariable or plasticity regions (Parkhill and Wren, 2000; Pearson et al., 2003). It is suggested that these homopolymeric runs of nucleotides may work as a mechanism in phase variation to turn genes on or off leading to the large amount of strain diversity that is a hallmark of *Campylobacter*.

1.2.4 Environmental Reservoirs

Campylobacter spp. are widespread in the environment, they do however require a warm blooded host to carry out their processes of replication and are therefore common flora of wild and domestic animals and birds (Ketley, 1997). Although *Campylobacter* is currently regarded as a natural inhabitant of the avian gut microbiota, historically it has been implicated in poultry disease including vibrionic hepatitis and shown to be capable of inducing diarrhea in a chicken model (King, 1962; Smibert, 1978; Ruiz-Palacios et al., 1981; Achen et al., 1998). Environmental and non-animal sources of *Campylobacter* spp. include, but are not limited to, surface water, abattoir effluent, non-pasteurized milk, shellfish, sand and protozoans; where the

organism can persist for extended periods if given the favorable environment of protection from direct sunlight and cool moist atmosphere (Jones, 2001; Skelly and Weinstein, 2003; Axelsson-Olsson et al., 2005; European Food Safety Authority, 2010c).

1.2.5 Viable But Non Culturable (VNBC)

Although *Campylobacter* spp. has particular requirements for its continual colonization within suitable hosts and may be considered a more 'fragile' organism, it also posses the capability to withstand environmental stressors during transmission from one host to another. During the transition from host to host *Campylobacter* spp. must endure unfavorable environmental conditions such as increased oxygen, temperature fluctuations and varying levels of moisture, all of which prior research has demonstrated inhibits its proliferation and survival (Park, 2002). One potential mechanism that *Campylobacter* may utilize for survival during periods of stress is the conversion to a viable but nonculturable (VNBC) state (Rollins and Colwell, 1986; Meinersmann et al., 1991). VNBC is an alteration of the bacterium from an active state of growth and metabolism to one of dormancy leading to minimal metabolism and no reproducibility, however viability can be restored under favorable conditions (Oliver, 2005; Oliver, 2010). However the role of this VNBC state and its impact as an environmental reservoir for the continual dissemination of *Campylobacter* remains to be determined (Svensson et al., 2008).

1.2.6 Mechanisms of Pathogenesis

Research on other frequent food borne bacterial pathogens that produce enteritis, such as *Salmonella enterica* and *Escherichia coli*, have shown that the production of protein toxins are intimately involved in inducing diarrhea, and therefore it could be expected that *Campylobacter*

spp. may also produce protein toxins (Pickett, 2000). The exact virulence mechanisms for the pathogenesis of *Campylobacter* spp. however still remain to be fully understood. Upon sequencing of the genomes of several strains of *C. jejuni* and performing comparisons to other enteric pathogens, it was determined that *C. jejuni* does not encode genes that are homologous to commonly associated virulence factors such as enterotoxins, adhesions, invasions or pathogenicity islands (Parkhill and Wren, 2000; Eppinger et al., 2004; Fouts et al., 2005; Hofreuter et al., 2006). Research has shown that *Campylobacter jejuni* adheres to intestinal epithelial cells leading to the translocation of the bacterium across the mucosal layer and internalization into epithelial cells (Hu and Kopecko, 2008; Watson and Galan, 2008). Some *Campylobacter* strains do posses a gene cluster that encodes a multi-subunit toxin, named cytolethal distending toxin (CDT), but its function in relation to pathogenesis is not clear (AbuOun et al., 2005; Asakura et al., 2007). Even with limited knowledge of the mechanisms of action for *Campylobacter* pathogenesis, it is generally recognized that there are five main components of *Campylobacter* pathogenicity, consisting of motility, adherence, invasion, production of toxins, intracellular survivability and secretion of specialized proteins (Larson et al., 2008).

1.3 Human Infection

With the development and introduction of sensitive and specific laboratory methods to isolate and identify *Campylobacter* spp. from human samples, a more accurate assessment of the disease burden caused by this zoonotic organism has been shown. Starting in the early 1980's, at which time it was considered an emerging food borne pathogen, and continuing to the present, *Campylobacter* spp. remains a primary agent for human enteritis in the United States (Blaser et

al., 1983; Altekruse et al., 1999; Centers for Disease Control and Prevention, 2006; Matyas et al., 2010). It is now recognized that human infection with *Campylobacter* spp. is one of the leading causes of acute bacterial gastroenteritis in developed nations throughout the world (Olson et al., 2008; European Food Safety Authority, 2011b). Human infection with *Campylobacter*, leading to campylobacteriosis, are caused by the thermophilic members of the *Campylobacter* genus; *C. jejuni, C. coli, C. lari, C. upsaliensis* and *C. helveticus*. *Campylobacter jejuni* and *C. coli* are the two species most frequently identified in association with food borne illness, epidemiologic studies have indicated that infection with *C. jejuni* is most frequent (>80%) and infection with *C. coli* occurs at a much lower level (~10%) (European Food Safety Authority, 2011a; European Food Safety Authority, 2011b). Initial studies described that ingestion of as few as 500-800cfu of *Campylobacter* can result in illness onset (Robinson, 1981; Black et al., 1988). Further investigation into the number of *Campylobacter* required to elicit illness has demonstrated that the true infective dose is more elusive, and is dependent upon other criteria such as the traits of the particular strain and the immune status of the host (Newell, 2002). General characteristics of campylobacteriosis include rapid onset of abdominal cramping and diarrhea lasting on average 7 days, initiated after an average incubation period of 3 days from time of exposure, with 30% of persons infected having general malaise and fever before diarrheal onset (Blaser, 1997; Blaser and Engberg, 2008). It has been noted that diarrhea caused by *Campylobacter* generally manifests in one of two ways; non-inflammatory diarrhea in which the stool is watery with no presence of white blood cells and red blood cells or inflammatory diarrhea in which the fecal material contains red and white blood cells (Wassenaar, 1997). Severe abdominal cramping has been known to lead to the misdiagnosis of appendicitis, however *Campylobacter* spp. is capable of extraintestinal infection of the gallbladder, pancreas, appendix and bacteremia (Blaser, 1997;

Blaser and Engberg, 2008). Antibiotic treatment is usually unwarranted but depending upon the severity of the symptoms antimicrobial therapy may be administered. *Campylobacter* spp. may be continually shed in the feces for up to three weeks, but there is documentation of its presence for up to 69 days (Kapperud et al., 1992). Infection with *Campylobacter* spp. is capable of causing late onset morbidity such as Guillain-Barré syndrome, reactive arthritis and irritable bowel syndrome.

1.3.1 Guillain-Barré Syndrome

Most frequently campylobacteriosis is an acute self-limiting illness, but a certain percentage of those afflicted will develop Guillain-Barré syndrome (GBS). It is a post-infectious neurological syndrome that manifests as weakness and tingling in the extremities and proceeds to ascending symmetrical paralysis potentially requiring medical interventions to support life (Allos, 1997). Due to the global vaccination programs that have almost completely eradicated poliomyelitis virus, GBS has become the primary cause of acute flaccid paralysis (Allos, 1997; Jacobs et al., 2008). The connection between *Campylobacter* spp. and Guillain-Barré was first suspected in the early 1980's and in the last 30 years evidence has indicated that between 30% - 40% of patients with GBS had an infection with *Campylobacter* spp. preceding the onset of GBS (Rhodes and Tattersfield, 1982; Mishu and Blaser, 1993; Allos, 1997; Jacobs et al., 2008). The onset of GBS symptoms usually occur between 10 days to 3 weeks after the diarrheal stage of *Campylobacter* infection (Blaser and Engberg, 2008). Research into the mode of action for the development of GBS has focused on the immune systems production of cross-reactive antibodies, in response to *Campylobacter* infection, that then proceed to attack the myelin sheath covering peripheral nerve tissue, leading to paralysis (Jacobs et al., 2008). During infection with

Campylobcter there exists the potential for production of autoantibodies that bind to GM1 ganglions contained within the plasma membranes of Schwann cells that make up the myelin sheath covering axons (Yuki et al., 1993; Yuki et al., 2004). These autoantibodies bind to the GM1 ganglions and elicit a recruitment of complement components and macrophage that break down the myelin sheath (Oomes et al., 1995). The duration of symptoms may last for weeks, with the greatest amount of peripheral nerve paralysis generally occurring between 2-3 weeks after onset of symptoms, after which time recovery begins (Nachamkin et al., 2000; van Koningsveld et al., 2007). Although the disease is considered self-limiting, due to the involvement of respiratory nerves, a large proportion of cases require the use of a ventilator (Jacobs et al., 2008). Recovery is slow and progresses over weeks to months with up to 20% of patients having some lasting physical disability (van Koningsveld et al., 2007; Jacobs et al., 2008).

1.3.2 Reactive Arthritis

Reactive arthritis (ReA) is a poorly defined disease characterized by sterile joint inflammation and potential inflammation of other sites including tendons, eyes and skin (Pope et al., 2007; Townes, 2010). The association between ReA and *Campylobacter* infection was first noted in the 1970's and to this day the true incidence and consequent health impacts remain to be determined (Gumpel et al., 1981; Pope et al., 2007). It has been estimated that ReA potentially arises in 1% - 5% of persons sickened by *Campylobacter*, with symptoms of ReA generally occurring on average of 14 days post-onset of *Campylobacter* associated diarrhea (Peterson, 1994; Pope et al., 2007). The course of symptoms from ReA can persist for several weeks to

months after which time persons fully recover, however chronic inflammation lasting longer periods has been documented (Blaser and Engberg, 2008; Garg et al., 2008).

1.3.3 Irritable Bowel Syndrome & Inflammatory Bowel Disease

Irritable bowel syndrome is a complication that can occur after infection with bacterial agents that cause gastroenteritis, such as *Campylobacter* spp. (Smith and Bayles, 2007; Spiller, 2007). Common causes of food borne illness, including *Campylobacter* spp. may induce irritable bowel syndrome, however the rates of persons affected by IBS following campylobacteriosis are not as yet known (Gilkin, 2005). In general, the amount of time to return to normal bowel function following an episode of IBS is lengthy, with one follow up study showing data that 5 years after onset of IBS 50% of persons still had symptoms (Spiller, 2007). *Campylobacter* infection has also been linked to the development of inflammatory bowel disease (IBD) (Gradel et al., 2009). The current theory is that the body, for unknown reasons, loses immunological tolerance to commensal organisms within the gut leading to chronic immune system stimulation within the gastrointestinal tract (Maul and Duchmann, 2008). *Campylobacter's* role in IBD is still not fully understood. A potential mechanism by which *Campylobacter* facilitates the symptoms of IBD is by its ability to translocate normal gut microflora across the intestinal epithelium leading to the inappropriate activation of T-cells (Kalischuk et al., 2009; Kalischuk et al., 2010).

1.4 *Campylobacter* spp. in Poultry

The capability of poultry to serve as a natural reservoir for thermophilic *Campylobacter* spp. is commonly believed to be due in part to their ability to reside within the gut microbiota as a commensal and the average avian body temperature of 41.8^0C is optimum for growth of the

bacteria (Horrocks et al., 2009; Hermans et al., 2012). Infection studies performed with poultry demonstrate that *Campylobacter* primarily colonize the lower parts of the gastrointestinal tract with a preference for the cypts within the ceca (Beery et al., 1988; Meinersmann et al., 1991; Achen et al., 1998). The concentration of *Campylobacter* spp. within the ceca of chickens is generally very high, studies have shown levels to be between 10^6 – 10^8 cfu per gram of cecal contents (Evans, 1992; Stern et al., 1995; Cawthraw et al., 1996). *Campylobacter* spp. are chemoattracted to mucin, which can serve as a source of energy, and this may explain its predilection for colonizing within the mucus filled cecal crypts (Beery et al., 1988; Hugdahl et al., 1988). The ceca of the chicken is a dynamic environment, contractile movements frequently occur to mix the contents and also expel them as necessary (Clench, 1999). The exact mechanisms by which *Campylobacter* is able to maintain a presence within this constantly moving environment is not yet fully understood. Although *Campylobacter* is predominantly found within the cecal crypts several studies have shown that it is capable of extraintestinal invasion of other organs including the liver and spleen (Young et al., 1999; Meade et al., 2009). *In vivo* testing demonstrated that inoculation and subsequent colonization of the ceca of chickens with *Campylobacter* spp. does not lead to host tissue pathology or increased morbidity and mortality (Dhillon et al., 2006; Smith et al., 2008).

1.4.1 Commensalism

Due to the lack of host cell pathology and symptoms of disease *Campylobacter* is frequently termed as a commensal organism within the gut microbiota of poultry. The role of *Campylobacter* as a commensal organism within poultry is not definitive. Work by Meade and coworkers (2009) which investigated gene expression profiles of the immune response of chicks

following inoculation with C. *jejuni*, showed that *C. jejuni* is capable of eliciting a response of the chick immune system, albeit not strongly, and subsequent down regulation of several genes that produce antimicrobial peptides. Therefore, the persistent presence of *Campylobacter* and its high cell density within the ceca may be the result of *Campylobacters* ability to modulate the immune response of the chicken (Van Deun et al., 2008; Meade et al., 2009; Shaughnessy et al., 2009). The ability of *Campylobacter* to invade chicken intestinal epithelial cells is also a subject of some discord. Initial work done by Beery and coworkers (1988) demonstrated no internalization of *C. jejuni* into the intestinal mucosa, but later research by Van Deun and coworkers (2008) has demonstrated *C. jejuni's* ability to temporally invade and then exit the epithelial cells. It is proposed that *Campylobacter* may avoid cecal crypt removal by a process of invading crypt epithelial cells briefly then exiting back into the mucus layer followed by replication in the mucus (Van Deun et al., 2008). More recent studies have shown that *Campylobacter* does express adhesion proteins and that they play a role in the successful colonization of chickens, so interaction with the surface of intestinal epithelial cells potentially does occur at some point during colonization (Ziprin et al., 1999; Ashgar et al., 2007; Flanagan et al., 2009). Ultimately, the colonization of the chicken gastrointestinal tract by *Campylobacter* is dictated by many host and bacterial factors whose mechanisms require more research to be fully understood.

1.4.2 Incidence in Poultry

Surveys done to try to ascertain the percent of commercial poultry flocks positive for *Campylobacter* spp. found counts to be highly variable; with rates in EU member states between 2-100% and rates in the U.S. to upwards of 90% (Humphrey et al., 1993; Stern et al., 2001b;

European Food Safety Authority, 2010b). Studies have shown that commercial poultry typically become positive for the presence of *Campylobacter* between 2-3 weeks of age (Gregory et al., 1997; Wagenaar et al., 2008). Chickens become rapidly colonized and may shed *Campylobacter* in droppings within 24 hours of initial exposure, after which time the spread of *Campylobacter* through the flock is estimated to be approximately 2 new cases per colonized bird per day (Achen et al., 1998; Gerwe et al., 2005; Knudsen et al., 2006). The infective dose required for host colonization is strain variable, however successful colonization of poultry has been established with inoculum concentrations as low as 10 CFU/g after which time the *Campylobacter* concentration within the cecum may reach levels of 10^9 CFU/g at time of slaughter (Shanker et al., 1988; Newell et al., 2000; Chen et al., 2006; Line et al., 2008a). Contamination of the rearing environment from *Campylobacter* positive birds and the subsequent rapid transmission among the flock leads to the incidence of *Campylobacter* positive flocks estimated at between 60% -100% worldwide (Stern et al., 2001b; Herman et al., 2003; European Food Safety Authority, 2010b).

1.4.3 Horizontal Transmission

The mode of transmission of *Campylobacter* spp. to commercial poultry flocks is believed to be primarily through horizontal transmission due to the presence of bacteria in the farm environment, on and in such things as equipment, footwear, standing water and vectors including flies, mice and wild birds (Hiett et al., 2002; Herman et al., 2003; Wagenaar et al., 2008). Analysis into the impact of potential environmental sources for the horizontal transmission of *Campylobacter* have found that insect vectors, such as flies and Darkling Beetles, have a high rate of testing positive for *Campylobacter* and are therefore a potential

source for the spread of the bacterium throughout the farm (Rosef and Kapperud, 1983; Bates et al., 2004; European Food Safety Authority, 2011b). Research performed on commercial broiler flocks positive for *Campylobacter* indicate that multiple strains may circulate within the flock, which may play a part in increasing strain diversity (Jacobs-Reitsma et al., 1995; Ellerbroek et al., 2010).

1.4.4 Vertical Transmission

The role of vertical transmission of *Campylobacter* among poultry and its potential impact is an area of much debate. Research has demonstrated that the reproductive tissues of hens and their reproductive tract may be colonized with *Campylobacter* leading to contamination during egg development (Hiett et al., 2003). The presence of *Campylobacter* within semen from breeder roosters and toms has also been confirmed and serves as a potential route for transmission to the breeder hen (Cox et al., 2002; Donoghue et al., 2004; Cox et al., 2005). A potential secondary mechanism for *Campylobacter* contamination of eggs is by fecal material in contact with the shell of the egg upon lay, after which time it must penetrate the shell and remain vegetative until hatching of the chick (Sahin et al., 2003). Work done to determine the significance of *Campylobacter* contaminated eggs has shown that hens testing positive for *Campylobacter* rarely produce eggs that test positive (Doyle, 1984; Shanker et al., 1986; Sahin et al., 2003). Research into the ability of *Campylobacter* to survive on the shell and penetrate to the albumen revealed that *Campylobacter* remained on the surface for a maximum of 16 hours and was sensitive to the drying of the shell and increased oxygen levels (Shane et al., 1986). The ability of *Campylobacter* to stay viable in the albumen and airsac within the egg has been studied and results show it is unable to survive for periods longer than 8 days, potentially due to the

oxygen concentrations, increased pH levels and inhibitory substances (Sahin et al., 2003). Commercially, table eggs and hatching eggs are stored at refrigerated temperatures which may also contribute to low contamination levels (Clark and Bueschkens, 1986). A potential source for *Campylobacter* viability within the egg is by contamination of the yolk, where studies have shown it to survive for up to 14 days (Sahin et al., 2003). Though the presence of *Campylobacter* has been confirmed within the reproductive tracts of poultry, evidence for this route of transmission as having a significant impact upon colonization status in subsequent progeny flocks has not been demonstrated (Sahin et al., 2003).

1.5 Methods of Pre-harvest Intervention

Exposure to *Campylobacter* through contact with poultry and poultry products has been determined to be the leading cause of human campylobacteriosis in the United States and in Europe (Friedman et al., 2004; European Food Safety Authority, 2011b). The European Food Safety Authority has determined that approximately 20% - 30% of human campylobacteriosis is caused from inadequate handling, preparation and subsequent consumption of chicken meat, and 50%-80% of campylobacteriosis cases are related to contact with poultry and poultry products in general (European Food Safety Authority, 2010c). In Belgium during a temporary ban on consumption of poultry due to dioxin contamination, the rates of *campylobacteriosis* infection in humans dropped by approximately 40%, further establishing the link between poultry and human *Campylobacter* infection (Vellinga and van Loock, 2002). In May of 2010 the United States Department of Agriculture (USDA) announce a new monitoring program for the presence of *Campylobacter* on young chicken and young turkey carcasses (Food Safety and Inspection Service, 2011c). Initial baseline surveys performed by USDA indicated that level of

Campylobacter contamination of young chicken and turkey carcasses was 46% and 1% respectively, and from this data the new performance standards have been set at 10.4% for chicken and 0.79% for turkey (Food Safety and Inspection Service, 2011a; Food Safety and Inspection Service, 2011c; Food Safety and Inspection Service, 2011d). The USDA expects a reduction of approximately 5,000 incidents of food borne related *Campylobacter* infections according to analysis done by the USDA's Risk Assessment Division (Food Safety and Inspection Service, 2011b). Much emphasis has been placed on preharvest strategies for the elimination of *Campylobacter* in poultry. Risk assessment studies have indicated that a reduction by 3 logs of *Campylobacter* in the gastrointestinal tract of chickens would have a significant public health impact, with estimates of a potentially 90% reduction of public health risks from *Campylobacter* (European Food Safety Authority, 2011b). Strategies for eliminating *Campylobacter* colonization in poultry can be broadly grouped into three approaches, consisting of (1) increased biosecurity to limit the birds exposure to potential environmental sources (Adkin et al., 2006; Wagenaar et al., 2008), (2) utilization of naturally produced molecules from bacteria that have antibacterial activity against *Campylobacter* such as bacteriocins and bacteriophages (Carrillo et al., 2005; Stern et al., 2005; Svetoch et al., 2005; Wagenaar et al, 2005) and (3) strategy is to use methods to prevent colonization of the ceca including vaccination, probiotic treatments and administration of natural compounds (Newell and Wagenaar, 2000; Stern et al., 2001a; Zoete et al., 2007; Lin, 2009; Solis de los Santos et al., 2010; Molatova et al., 2011).

1.5.1 Biosecurity

Biosecurity is a strategy of preventative actions implemented to reduce the potential for transmission of infectious organisms from a reservoir to a potential host (European Food Safety Authority, 2011b). Thus, the implementation of strict biosecurity protocols on the farm may be

used to try to prevent the initial exposure of the birds to *Campylobacter*. The impact of biosecurity measures has been studied with results implicating unsatisfactory biosecurity as the greatest contributor to *Campylobacter* transmission to commercial poultry (Adkin et al., 2006). Due to horizontal transmission of *Campylobacter* to poultry as the main mechanism for flock colonization, the hygiene practices of workers on the farm play a significant role (Newell et al., 2011). Some potential difficulties regarding increased on farm biosecurity include the lack of clearly defined relative risk contributions for specific environmental factors (Carrillo et al., 2005; Wagenaar et al., 2008). However, with the vast number of potential environmental sources and the needs within commercial poultry production for interaction with the flock, implementation of very strict biosecurity measures may not be feasible (Wagenaar et al., 2008).

1.5.2 Bacteriocin

One strategy that is being investigated for the elimination of *Campylobacter* colonization in poultry is the administration of bacteriocins. Bacteriocins are small peptides of approximately 5 to 6kDa that are produced by bacteria and are toxigenic to bacteria other than the producer strain; their function is as a defense mechanism (Klaenhammer, 1993; Nes et al., 1996). Research into bacteriocins has determined that the most common genera of bacteria that produce antimicrobial peptides which may have some applicability to poultry include *Lactobacillus*, *Lactococcus* and *Bacillus*, however many other genera also produce these compounds (Svetoch and Stern, 2010). Svetoch and coworkers (2005) investigated the normal gut flora of poultry as a source for bacteria producing anti-*Campylobacter* peptides and successfully purified and characterized bacteriocins from strains of *Bacillus circulans* and *Paenibacillus polymyxa*. *In vivo* experiments with anti-*Campylobacter* bacteriocins when administered to poultry have

demonstrated efficacy to significantly reduce *Campylobacter* colonization within the gastrointestinal tract to levels below the detection limit (Stern et al., 2005; Cole et al., 2006b; Stern et al., 2006; Line et al., 2008b; Svetoch et al., 2008). Commercial usage of bacteriocins for the control of *Campylobacter* in poultry is limited by the labor and expense of production and concerns regarding development of resistance and toxicity to the host and consumer (Lin, 2009). With regards to toxicity, the risks of bacteriocins are regarded as minimal; they are produced by bacteria in commonly consumed food products including cheese and yogurt and are likely also produced by commensal bacteria contained within the human gut (Cleveland et al., 2001; Svetoch and Stern, 2010). There is concern of developing bacteriocin resistant strains of *Campylobacter* in response to their being used therapeutically. Hoang and coworkers (2011b) screened a collection of *Campylobacter jejuni* isolates from multiple sources and found that only one isolate of the 146 tested displayed some level of resistance. Limited *in vitro* testing in poultry for determining rates of resistance development after treatment with a bacteriocin (E-760) indicated that a low level resistance was present after administration of the bacteriocin, but levels of resistance never increased during the trial, indicating mechanisms for development of resistance to bacteriocins may not be highly developed (Peschel and Sahl, 2006; Hoang et al., 2011a). Another potential hurdle for implementation of bacteriocins to control *Campylobacter* in poultry is the process of obtaining regulatory approval, which potentially requires extensive studies of efficacy and toxicity along with the requisite application process.

1.5.3 Bacteriophage

Control of *Campylobacter* in the chicken gastrointestinal tract and upon retail poultry products by the introduction of anti-*Campylobacter* viruses is a potential treatment option that is

being investigated for commercial use (Connerton et al., 2008). Viruses which infect bacteria and require a bacterial host for replication and dissemination are named as bacteriophage. These infectious viruses may have a very narrow range of bacterial hosts to which they infect, some of which are single strain specific. Cytophathic effects on the host organism caused by infection of lytic bacteriophage, subsequent multiplication and mechanisms for virus spread ultimately lead to lysis of the host cell (Joerger, 2003). Bacteriophage are ubiquitous in nature and *Campylobacter* specific bacteriophage have been isolated from multiple sources, including poultry intestinal microbiota, retail poultry products, processing plant wastewater, and manure from other livestock including cattle, pigs and sheep (Grajewski et al., 1985; Salama et al., 1989; Khakhria and Lior, 1992; Atterbury et al., 2003; Carrillo et al., 2005). Limited research on the application of bacteriophage to broiler chickens for the reduction of cecal carriage of *Campylobacter* has demonstrated the capability of this strategy, rates of reduction for *Campylobacter* were between 1-5 logs (Carrillo et al., 2005; Wagenaar et al., 2005). The implementation of bacteriophage based interventions face several technical challenges. Researchers believe that significant factors influencing the efficacy of bacteriophage against *Campylobacter* include the selection of specific bacteriophage, the bacteriophage and host strain interaction, and ascertainment of optimal dosages (Carrillo et al., 2005; Wagenaar et al., 2005; Connerton et al., 2008). The potential for development of bacteriophage resistant *Campylobacter* strains is also of concern in regards to its commercial application (Connerton et al., 2008). Carrillo and coworkers (2005) reported isolation of *Campylobacter* resistant to the therapeutic bacteriophage administered during an *in vivo* trial, however it was noted that the resistant strain was not the predominant phenotype isolated and the resistant strain when re-administered to poultry reverted to a sensitive phenotype. In regards to development of resistant

strains it has been suggested that due to the environmental exposure to multiple strains of *Campylobacter* in poultry, it is feasible that during bacteriophage treatment an environmental isolate insensitive to the administered bacteriophage could become dominant instead of a resistant strain being created by selective pressure (Connerton et al., 2004).

1.5.4 Vaccination

Within commercial production of poultry the use of vaccination to prevent the infection and spread of some disease causing microorganisms is already established. Currently however there are no commercial vaccines available for the prevention of *Campylobacter* infection in poultry (Wagenaar et al., 2008; Lin, 2009). Important challenges to developing a vaccine include the nature of colonization of *Campylobacter* in poultry, our relative lack of understanding in regards to the avian immune system, the identification of conserved regions among *Campylobacter* that may serve as antigenic targets, and the short lifespan of broilers that therefore have relatively immature immune system development (Zoete et al., 2007; Lin, 2009). Zoete and coworkers (2007) have proposed that a proper *Campylobacter* candidate vaccine must include several parameters: (1) broad specificity to induce protection against multiple *Campylobacter* subtypes, due to the early exposure of birds to *Campylobacter* in the rearing environment; (2) the generation of a protective immune response must be generated quickly post vaccination and (3) the vaccination must be cost-effective and easily administered. Research on the interaction of *Campylobacter* and the avian immune system has demonstrated that *Campylobacter* specific antibodies are present upon challenge and therefore an antibody mediated mechanism due to vaccination is a possibility (Myszewski and Stern, 1990; Cawthraw et al., 1994; Widders et al., 1996; Rice et al., 1997). In a review of candidate vaccines by Zoete

and coworkers (2007) the results of various vaccination strategies were analyzed and they found that inactivated and heat killed *C.jejuni* vaccines provided little protection (approximately 1-2 log reduction in cecal counts) and recombinant vaccines in which an antigenic *Campylobacter* protein is expressed had between 2-6 log reductions in cecal colonization after homologous strain challenge. The mechanisms by which *Campylobacter* generates its extensive genotypic and phenotypic diversity contribute greatly to the difficulty of creating a vaccine with heterologous protective effects (Zoete et al., 2007; Wagenaar et al., 2008). So the challenge still remains to produce a vaccine capable of inducing a strong immune response that inhibits colonization or leads to a significant reduction of *Campylobacter* colonization within poultry, that is easily administered and inexpensive (Zoete et al., 2007)

1.5.5 Probiotics and Competitive Exclusion

The supplementation of diet with beneficial microorganisms to potentially improve the health of the host, so called probiotic treatment is a strategy under investigation for control of *Campylobacter* in poultry (Fuller, 1989; Joint Food and Agriculture Organization, 2006; Wagenaar et al., 2008). The goal of the use of probiotics in poultry is to attempt to prevent colonization or alter the gut microflora so as to inhibit establishment of unwanted or detrimental bacteria by administration of helpful or beneficial bacteria (Metchnikoff and Mitchell, 1910; Wagenaar et al., 2008). The most frequently used bacterial genus for use as probiotics include *Lactobacillus, Bacillus, Lactococcus, Bifidobacterium* and *Streptococcus* (Fooks and Gibson, 2002; Lutful Kabir, 2009). One application of probiotic usage within the poultry industry is as a component of defined cultures during competitive exclusion treatments to prevent colonization of *Campylobacter* in newly hatched chicks (Cox and Pavic, 2010). The principal of competitive

exclusion (CE) is based upon work by Nurmi and Rantala (1973) who associated the direct feeding of healthy adult chicken intestinal flora to newly hatched chicks with the lack of *Salmonella infantis* colonization within the chick gastrointestinal system (Nurmi and Rantala, 1973; Rantala and Nurmi, 1973). Research into the potential mechanisms of action for the positive effects of competitive exclusion treatments have led to several proposed ideas, including competition between CE organisms and the undesirable organisms for sites to colonize within the niche environment and competition for nutrients, the production of bacteriocins and volatile fatty acids inhibitory to the target organisms and the potential modulation of host immune cell interactions with the gut microbiota (Koenen et al., 2004; Schneitz, 2005; Doyle and Erickson, 2006). The results of *in vivo* trials to evaluate the ability of probiotic strains of bacteria to eliminate *Campylobacter* colonization of the ceca have yielded inconsistent results. Work by Stern an coworkers (2001a) in which they harvested mucosally derived probiotic organisms for a CE treatment found that by administering the treatment to day of hatch chicks and artificially inoculating the chicks with *Campylobacter* 24 hours later they were able to achieve a statistically significant reduction in *Campylobacter* colonization, but it was noted that the overall effective protection afforded by this treatment was very low. Several *in vivo* trials which utilized poultry derived probiotic cultures as a competitive exclusion treatment have demonstrated the ability to reduce fecal shedding and cecal colonization but were unable to prevent to colonization of the ceca (Aho et al., 1991; Morishita et al., 1997; Newell and Wagenaar, 2000).

1.5.6 Natural Compounds

The rise in interest of natural compounds, including plant extracts, for improvement of poultry health and disease treatment has followed the general trend in the United States of increased

demand for organic poultry and increased pressure on commercial producers to eliminate use the use of arsenicals, growth promoters and antibiotics. This poses a significant challenge for producers of food animals to find alternatives for the aforementioned compounds but not increase rates of disease and maintain efficient and cost effective production practices (Tillman et al., 2011). Research into the use of naturally derived compounds for control of pathogens in poultry is limited. However preliminary data has demonstrated efficacy with administration of medium chain fatty acids, and the use of plant extracts is also being investigated.

1.5.7 Medium Chain Fatty Acids

Fatty acids are composed of long chains of carbon atoms with attached hydrogen atoms and can be classified based upon the length of the carbon chain. Fatty acids are present in animal and vegetable fats and are a component of the body's phospholipids and glycolipids. Several characteristics of medium chain fatty acids make them appealing as a natural treatment for animal health, including their generally recognized as safe (GRAS) status conferred by FDA, and toxicity studies have shown them to be non-toxic in animal diets even at high levels (Code of Federal Regulation 21CFR 184.1025, 1981; Traul et al., 2000). Medium chain fatty acids have been investigated for their potential to eliminate *Campylobacter* based upon their reported high broad spectrum antibacterial activity (Van Immerseel et al., 2004; Nair et al., 2005). Experiments by Chaveerach and coworkers. (2002, 2004) have confirmed the antimicrobial activity of organic acids when used as a component of feed and in the water system and a subsequent *in vivo* trial demonstrated that administration of organic acids through the drinking water kept the water free of *Campylobacter* and the birds had no adverse clinical or histological effects. *In vivo* trials utilizing broiler chicks demonstrated a 3-4 log reduction in cecal

Campylobacter colonization when a single medium chain organic acid, caprylic acid, was administrated as a prophylactic or theraputic treatment in feed (Solis de los Santos et al., 2008a; Solis de los Santos et al., 2008b; Solis de los Santos et al., 2009). The feeding of combinations of medium chain fatty acids has been shown to have a protective effect against colonization with *Campylobacter* by causing an increase in the required infective dose and demonstrating reduced levels of fecal shedding (van Gerwe et al., 2010; Molatova et al., 2011). To date no studies have shown the complete failure of *Campylobacter* to colonize the ceca in response to medium chain fatty acids, but its efficacy in lowering shedding could give it potential as a therapeutic administered shortly before slaughter in order to reduce the bacterial load entering the slaughter facility (Solis de los Santos et al., 2010; Molatova et al., 2011).

1.5.8 Plant Extracts

The use of compounds extracted from plants, phytogenics, by producers of food animals is an area of resurgent interest due to growing consumer demand for removal of sub-theraputic administration of antibiotics in conventionally raised livestock and the increased demand for organic meat products (Sirsat et al., 2009; Organic Trade Association, 2011). In the United States and European Union there is growing consumer demand for food products produced in a more sustainable and natural manner and the inclusion of plant extracts into food animal production aligns well with the general populations idea that ‘natural’ compounds are better and safer (Greathead, 2003).

The majority of research has focused upon supplementation of plant compounds to replace antibiotic growth promoters (AGP) and their safety and effect on the chicken growth characteristics and gut health (Windisch et al., 2008). The use of phytogenics for the control of

disease is a natural progression from research into replacements for AGP due to the knowledge regarding the bacteriostatic and bactericidal properties of numerous plant extracts (Helander et al., 1998; Smith-Palmer et al., 1998; Dorman and Deans, 2000; Guo et al., 2003). The greatest amount of research in the area of antimicrobial action of plant extracts has centered upon essential oils, however the majority of studies in which antimicrobial activity was assayed was not done so in live animals (Griggs and Jacob, 2005). The use of plant extracts in a research setting can become quite complicated due to several parameters: (1) plants may produce numerous bioactive compounds and determining which one is the source of antimicrobial activity may be cost and labor intensive (2) the concentration of bioactive compounds in plants can have significant variability due to location, growth conditions, time of harvest and method of storage (Greathead, 2003; Viskelis et al., 2009; Applegate et al., 2010).

1.6 Use of Cranberry Extract for Preharvest Control of *Campylobacter* in Poultry

The North American Cranberry (*Vaccinium macrocarpon*) is considered a plant with many positive health attributes for humans. These positive attributes originate from the phytochemicals produced as secondary metabolites which serve to protect plants from harmful environmental stressors including UV light, free radicals, plant bacterial pathogens and herbivorous predators (Dixon et al., 2005; Stevenson and Hurst, 2007). Research into the phytochemicals produced by cranberries has identified over 150 unique compounds (Pappas and Schaich, 2009). Investigation into the bioactive compounds produced by cranberries that have potential beneficial effects on health, include phenolic acids and flavonoids including anthocyanins and proanthocyanidins (Neto, 2007; Pappas and Schaich, 2009; Côté et al., 2010; Lacombe et al., 2010; Côté et al., 2011a).

1.6.1 Bioactive Compounds

Flavonoids produced by cranberries include flavonols, flavan-3-ols anthocyanins and proanthocyanidins (Sun et al., 2002; Pappas and Schaich, 2009; Côté et al., 2010). Research into anthocyanins and proanthocyanidins of cranberry have determined that they exhibit strong *in vitro* antioxidant activity as they are capable of acting as hydrogen donors (Lin et al., 2005; Caillet et al., 2011). The antioxidant activity of cranberry extracts, and of anthocyanins specifically, are affected by pH and exhibit greater antioxidant activity at lower than neutral pH (Wu et al., 2008; Caillet et al., 2011). In general, the activity of bioactive secondary metabolites is dependent upon external environmental conditions including pH, temperature and composition of the solvent (Wu et al., 2008). The health benefits of cranberry extract based upon antioxidant activity have been disputed due to research indicating that less than 5% of flavonoids are absorbed by the human body and absorbed flavonoids get rapidly metabolized and expelled from the body (Lotito and Frei, 2006; European Food Safety Authority, 2010d).

1.6.2 Antimicrobial Activity

Cranberry extract has been evaluated for antimicrobial activity and found to be inhibitory to wide range of Gram-positive and Gram-negative organisms (Puupponen-Pimiä et al., 2005a; Nohynek et al., 2006; Chi-Hua Wu et al., 2008; Lacombe et al., 2010; Côté et al., 2011a; Caillet et al., 2012). Research trials which screened different cultivars of cranberry for antimicrobial activity have indicated that overall antimicrobial efficacy can depend upon many parameters including; specific cultivar, environmental conditions during growth, methods of storing raw material and which method is utilized to isolate the bioactive compounds (Viskelis et al., 2009). Fractionation of cranberry extract and analysis of the antimicrobial activity of each fraction has

demonstrated that flavonoids, which include anthocyanins and proanthocyanidins, and phenolic acids are responsible for up to 50% of the *in vitro* antimicrobial activity (Marwan and Nagel, 1986). Anthocyanins from cranberry extract have been investigated for their potential antimicrobial activity against the food borne pathogen *E.coli* O157:H7 and results indicate that there is significant bactericidal activity at the native pH of 2 (Lacombe et al., 2010). However the use of anthocyaninis for control of bacterial pathogens can be complicated by their sensitivity to pH, oxygen levels and temperature (Lacombe et al., 2010).

Cranberries contain a large amount of phenolic acid compounds, which are considered as one of the sources of the health promoting qualities of fruits and vegetables (Sun et al., 2002). It has been suggested that the phenolic content of cranberry extract is responsible for the greatest amount of demonstrated antimicrobial activity (Wu et al., 2008). Prior research has indicated that phenolics from berries were bactericidal to select human pathogens, including *Salmonella* spp., *Escherichia coli*, and *Staphylococcus* spp. (Puupponen-Pimiä et al., 2001; Nohynek et al., 2006; Lacombe et al., 2010). In an assay screening for potential antibacterial activity by various berry phenolic extracts, Nohynek and coworkers (2006) found that cranberry phenolic compounds had inhibitory activity against *B. cereus, C. perfringens, S. epidermidis* and *Candida albicans* but found no inhibition against *C. jejuni*, *H. pylori* and *S. aureus*.

1.6.3 Mechanism of Action

The mechanisms of antimicrobial action due to cranberry extract are not well understood and more investigation is required in this area. However, the current research has indicated some potential mechanisms including inhibiting the uptake of necessary substrates, destabilization of bacterial membranes, and retarding bacterial growth by interruption of metabolism (Vattem et

al., 2004; Puupponen-Pimiä et al., 2005c; Nohynek et al., 2006). Another potential mechanism of action for the antimicrobial activity of cranberry extract includes the ability of flavonoids, including proanthocyanidins and phenolic acids, to sequester free iron and thus prevent its availability for pathogenic bacteria (Dixon et al., 2005; Guo et al., 2007). Bactericidal and bacteriostatic characteristics of cranberry have been attritubuted to the ability of some components of cranberry to prevent the adherence of bacteria to epithelial cells, which prevents colonization (Sobota, 1984; Zafriri et al., 1989; Howell et al., 1998). Research into the prevention of urinary tract infections and the connection between consumption of cranberry products has led researchers to focus in on proanthocyanidins specifically, and results have indicated that they block the specific P fimbriae of *Escherichia coli* from attachment to uroepithelial cells (Howell et al., 1998; Foo et al., 2000; Howell et al., 2005). The anti-adherence properties of cranberry extract have also been investigated in regards to gastritis caused by *H. pylori* and dental caries caused by adherence of Gram-negative anaerobes to the gingival surface (Burger et al., 2000, 2002; Weiss et al., 2002). The inhibition of *H. pylori* to attach to gastric epithelial cells was originally reported to be due to a high molecular weight component of cranberry and further work has suggested proanthocyanidins are responsible for this activity (Burger et al., 2000; Burger et al., 2002).

1.6.4 Factors Influencing Bioactive Compounds of Cranberry

As noted, the phytochemicals of cranberries can be influenced by many environmental factors both pre-processing and during juice extraction (Wu et al., 2008; Viskelis et al., 2009). It is during the multiple steps of juice extraction, including freezing, crushing heating and evaporation that the bioactivity of cranberries compounds can be significantly affected (Côté et

al., 2011b). Work by Caillet and coworkers (2011) demonstrated that the solvent utilized in the extraction process affected the antioxidant activity of cranberry phenolic compounds, whereby aqueous solvents increased antioxidant properties when compared to extraction using organic solvents. The antimicrobial properties of cranberry juice and the resultant mash left over from processing are also influenced by juice extraction processes due in great part to flavonoids (anthocyanidins and phenolics) being readily oxidized and subsequently degraded (Côté et al., 2011b). Côte and coworkers (2011b) analyzed samples from each step of the juice making process for antimicrobial activity and concluded that in general the juicing process lowered the antibacterial potential of extracted flavonoids.

Cranberry extracts have been studied within the poultry industry as a potential replacement to antibiotic growth promoters and found to have no adverse effect on the performance characteristics of broilers (Leusink et al., 2010). The anti-adhesive properties of cranberry extract against *E.coli* and *H.pylori* make it a candidate for evaluation of these characteristics against *Campylobacter jejuni* colonization in poultry. The objective of our study was to evaluate the efficacy of two cranberry extracts, differing in proanthocyanidin concentration, of selected doses to prevent the colonization of the ceca of broiler chickens with *Campylobacter jejuni*.

Chapter 2

Feed Supplementation with Natural Extracts of Cranberry and its Efficacy on *Campylobacter* Colonization in Poultry

Feed Supplementation with Natural Extracts of Cranberry and its Efficacy on *Campylobacter* Colonization in Poultry

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ABSTRACT

Campylobacter spp. has been identified as one of the leading causative agents of food borne diarrheal illness. Epidemiological evidence has shown that poultry is the main source for human infection. Poultry are asymptomatic carriers of *Campylobacter* within their gastrointestinal tract, with colonization levels reaching 10^6-10^8 cfu/g cecal content. Surveys of domestic poultry flocks have estimated that approximately 90% of flocks are positive for *Campylobacter* colonization. Risk assessment studies have determined that by reducing levels of *Campylobacter* colonization within the gastrointestinal tract, incidences of human infection will be significantly reduced. Currently there are no consistently effective treatments to eliminate *Campylobacter* from poultry flocks. The use of natural plant extracts to control food borne pathogens is an area of resurgent interest due to growing consumer demand for removal of sub-theraputic administration of antibiotics in conventionally raised livestock and the increased demand for organic meat products. Extracts from American Cranberry (*Vaccinium macrocarpon*) contain proanthocyanidins which have demonstrated antimicrobial activity against other food borne pathogens including *E. coli*, *Salmonella*, and *Listeria*. However, their ability to reduce *Campylobacter* in chickens has not been reported. The objective of this study was to evaluate the ability of two different cranberry extracts, either containing a lower (1%, L-PAC) or high

concentration (30%, H-PAC) of proanthocyanidins, to inhibit the growth of *Campylobacter*, *in vitro* and *in vivo*. In replicate *in vitro* trials, treatments with 0.1 or 0.5% had no effect, the 1% treatment of L-PAC or H-PAC produced variable reductions of between 1 – 5 logs, and the 2 or 4% treatments produced at least a 5 log reduction in *Campylobacter* counts when compared to controls 8 or 24 hours after inoculation for both the L-PAC or H-PAC. For the *in vivo* studies, 70 chicks were randomly assigned to one of seven treatment groups (n=10 per treatment group). Treatment groups for each trial included a positive *Campylobacter* control (no cranberry extract) or 0.5%, 1%, or 2% of either H-PAC or L-PAC added to the feed. The same dosages were used in two replicate trials. For each trial, all birds were given feed supplemented with H-PAC or L-PAC, except for positive *Campylobacter* controls, starting at day of placement and continuing through the entire 14 day trial. At day 7 all birds were challenged with a mixture of three wild type *Campylobacter jejuni* strains by oral gavage (approximately 2.5 $x10^5$ cfu/mL). On day 14, birds were euthanized by CO_2 and cecal contents were collected for enumeration of *Campylobacter*. In both trials cecal *Campylobacter* counts were not reduced by administration of L-PAC or H-PAC in the feed. Follow up experiments are needed to increase the potency of these cranberry extracts to reduce this important food borne pathogen in chickens.

INTRODUCTION

One of the leading bacterial causes of food borne illness worldwide is contamination of food products with *Campylobacter* spp. (World Health Organization, 2001; European Food Safety Authority, 2011a; Scallan et al., 2011). Estimates of food borne illness attributed to *Campylobacter*, based on data collected from the Centers for Disease Control (CDC), put the rate at approximately 850,000 cases per year (Scallan et al., 2011). The numbers of estimated illness, and the true incidence are most likely much higher due to the fact that most *Campylobacter*

caused illnesses are sporadic in nature, large outbreaks are infrequent and microbiological methods for detection are variable (European Food Safety Authority, 2010a). In rare cases, infection with *Campylobacter* has been associated with late onset morbidity including Guillain-Barré syndrome, reactive arthritis and irritable bowel syndrome (Pope et al., 2007; Smith and Bayles, 2007; Spiller, 2007; Jacobs et al., 2008).

Exposure and subsequent infection with *C. jejuni*, by way of contaminated poultry products, is one of the predominant causes of food borne related illness in the United States (Altekruse et al., 1999; Rosenquist et al., 2003; Friedman et al., 2004). Epidemiological studies have indicated that the most frequent routes for human infection with *Campylobacter* are eating improperly cooked chicken, handling chicken and exposure to animals including poultry (Friedman et al., 2004; Danis et al., 2009; Lindmark et al., 2009; Fajó-Pascual et al., 2010). *Campylobacter* is able to colonize the chicken as a commensal, after which they become asymptomatic carriers (Beery et al., 1988; Dhillon et al., 2006). *Campylobacter* preferentially colonize the crypts within the ceca, where they are able to reach levels as high as 10^6-10^8 cfu/gram of cecal material (Beery et al., 1988; Meinersmann et al., 1991; Evans, 1992; Stern et al., 1995; Cawthraw et al., 1996; Achen et al., 1998). The prevalence of *Campylobacter* spp. within poultry flocks in the United States is reported to be as high as 90% (Stern et al., 2001b). The Food Safety and Inspection Service has estimated the percent of chicken carcasses positive for *Campylobacter* in the processing plant to be approximately 46%, which can be attributed to the feathers, the skin and the gastrointestinal tract having a high *Campylobacter* load which cannot be completely eliminated during processing (Jacobs-Reitsma et al., 1995; Wagenaar et al., 2008). High levels of *Campylobacter* on and in the bird at the time of slaughter have a significant impact on carcass contamination; so a reduction of *Campylobacter* in poultry pre-

harvest should lead to a reduction in human campylobacteriosis cases from contaminated poultry products (Rosenquist et al., 2003; Reich et al., 2008; European Food Safety Authority, 2011b).

Multiple strategies have been tried to reduce *Campylobacter* colonization in poultry which include: (1) increased biosecurity to limit the birds exposure to potential environmental sources (Adkin et al., 2006; Wagenaar et al., 2008); (2) utilization of naturally produced molecules from bacteria that have antibacterial activity against *Campylobacter* such as bacteriocins and bacteriophage (Carrillo et al., 2005; Stern et al., 2005; Svetoch et al., 2005; Wagenaar et al., 2005); (3) and the use of methods to prevent colonization of the ceca including vaccination, probiotic treatments and administration of natural compounds (Newell and Wagenaar, 2000; Stern et al., 2001a; Zoete et al., 2007; Lin, 2009; Solis de los Santos, 2010; Hoang et al., 2011b; Molatova et al., 2011). Unfortunately, most of these strategies have met with limited success and additional treatments need to be developed to reduce this food borne pathogen in poultry.

Renewed interest has been placed on plant extracts for the control of pathogens in food animals due in part to restrictions by the Food and Drug Administration (FDA) on antibiotic usage in livestock production (Food and Drug Administration, 2/24/12). This challenges food animal producers to find alternatives to antibiotics and still maintain animal health and welfare (Tillman et al., 2011). Alternatives to antibiotics may come from phytogenics, or plant derived compounds, some of which have the benefit of being designated as generally recognized as safe (GRAS) by FDA. Compounds with GRAS designation as deemed safe to be used in foods and require no lengthy approval process, which allows them to adopted for use quickly (Code of Federal Regulation 21CFR 184.1025, 1981).

Plant extracts from the American Cranberry (*Vaccinium macrocarpon*) are GRAS and have many bioactive compounds, some of which demonstrate antimicrobial activity (Hong and Wrolstad, 1986; Marwan and Nagel, 1986; Puupponen-Pimiä et al., 2005b; Wu et al., 2008; Côté et al., 2010; Côté et al., 2011a). The bioactive compounds of cranberries are linked to secondary metabolites called flavonoids, which include anthocyanins, proanthocyanidins and phenolic acids (Marwan and Nagel, 1986; Pappas and Schaich, 2009; Côté et al., 2010). Research on flavonoids has found activity including: potent antioxidant action (Lin et al., 2005; Caillet et al., 2011); iron chelation (Dixon et al., 2005; Guo et al., 2007), and inhibitory activity against bacterial adhesion to epithelial cells (Sobota, 1984; Zafriri et al., 1989; Howell et al., 1998). Cranberry extract has documented *in vitro* antibacterial activity to the food borne pathogens *E.coli* O157:H7, *Salmonella Typhimuium*, and *Listeria monocytogenes* (Wu et al., 2008; Caillet et al., 2012). To our knowledge, cranberry extracts have not been evaluated for their ability to reduce *Campylobacter* in poultry.

For this study two commercially available GRAS designated cranberry extracts were tested. These extracts are standardized to contain a lower (1%) or higher concentration (30%) of proanthocyanidins by the manufacturer (L-PAC or H-PAC, respectively). The aim of this research was to determine if cranberry extracts, L-PAC and H-PAC, are inhibitory to *Campylobacter jejuni, in vitro* and if efficacious, examine their potential for use in young chickens to prevent *Campylobacter* colonization.

MATERIALS AND METHODS

Cranberry Plant Extracts

Two extracts of the North American Cranberry (Vaccinium macrocarpon) were used for both the *in vitro* and *in vivo* trials; (1) cranberry concentrate powder standardized to contain 1% proanthocyanidins (L-PAC) and (2) dried cranberry extract powder standardized to contain 30% proanthocyanidins (H-PAC) obtained from Decas Botanical Synergies (Carver, MA).

In vitro Antimicrobial Activity of Cranberry Extracts

Assessment of *in vitro* antimicrobial activity was determined using a mixture of three wild type *Campylobacter jejuni* strains previously isolated and identified from poultry. *Campylobacter* was prepared as previously described (Farnell et al., 2005). Briefly, a 10 µL loop of frozen glycerol stock of each *C. jejuni* strain was inoculated into 5 mL of *Campylobacter* Enrichment Broth (CEB) and incubated at 42^0C for 48 hours under microaerophilic conditions. After 48 hours, 10 µL of each of the primary broth cultures were inoculated individually into 5 mL of fresh CEB and incubated at 42^0C for 24 hours under microaerophilic conditions. Following incubation, broth cultures were pooled in a 25 mL centrifuge tube. A wet mount preparation was done to observe the mixed strain broth culture for viability and motility. The broth culture was centrifuged for 10 minutes at 3,500 x g, and the supernatant was discarded. The remaining cell pellet was resuspended with an equal amount of Butterfield's Phosphate Diluent (BPD). For each assay, duplicate samples were tested which included eleven treatments, a positive control (no cranberry extract) and five concentrations (4%, 2%, 1%, 0.5% or 0.1%) of each cranberry extract. Each assay was replicated two times. To prepare the various concentrations of cranberry extract, 900 µL of fresh CEB was aliquoted into sterile test tubes and appropriate weights of L-PAC and H-PAC were added to the CEB and resuspended by vortexing. A 100 µL aliquot of the three strain *C. jejuni* culture was inoculated into each

cranberry extract tube and a positive control tube (no cranberry extract). Broth cultures were then incubated at 42^0C under microaerophilic conditions and samples were taken at 8 hours or 24 hours post-inoculation. A 100 µL sample of each of the *C. jejuni* plus cranberry extracts was serially diluted (1:10) in Butterfield's Phosphate Diluent (BPD) and direct plated onto Campy-Line Agar (CLA) (Line, 2001) then incubated at 42^0C under microaerophilic conditions for 48 hours. Presumptive *Campylobacter* colonies were directly enumerated and converted to cfu/mL of broth culture. Bacterial colonies were confirmed by latex agglutination test (PANBIO Inc., Columbia, MD) and by API Campy (Biomeriuex Durham, NC).

Animal Studies

Day of hatch broiler chicks were obtained from a commercial hatchery. Birds were weighed individually, placed in floor pens and provided free access to feed and water during the entire duration of the trials. For each trial, 70 chicks were randomly assigned to one of seven treatment groups (n=10 per treatment group). Treatment groups for each trial included a positive *Campylobacter* control (no cranberry extract) or 0.5%, 1%, or 2% of either H-PAC or L-PAC added to the feed. The same dosages were used for Trial 2. For each trial, all birds were given feed supplemented with H-PAC and L-PAC, except for positive *Campylobacter* control, starting at day of placement and continuing through the entire 14 day trial. At day 7 all birds were challenged with a mixture of three wild type *Campylobacter jejuni* strains by oral gavage. Each bird received 0.25 mL of an approximately 10^6 cfu/mL *Campylobacter jejuni* mixture. On day 14, birds were euthanized by CO_2 and cecal contents were collected for enumeration of *Campylobacter*. Feed consumption was determined by subtracting the amount of remaining feed at day 14 from the amounts at day 1.

Campylobacter Challenge Preparation

The *Campylobacter* challenge was prepared as previously described by Farnell and coworkers (Farnell et al., 2005). Briefly, glycerol stock of the wild type *Campylobacter jejuni* strains, originally isolated from poultry, were inoculated individually into 5 mL of CEB. Broth tubes were incubated at 42^0C for 48 hours under microaerophilic conditions. For the second passage, 10 µL loops from each strain were inoculated into 5 mL fresh CEB and incubated for 24 hours at 42^0C under microaerophilic conditions. Following incubation, broth strains were pooled in a 25 mL centrifuge tube. A wet mount preparation was done to observe the mixed broth culture for viability and motility. The broth culture was centrifuged for 10 minutes at 3,500 x g, and the supernatant was discarded. The remaining cell pellet was resuspended with an equal amount of BPD. For quantitation of challenge dose a 100 µL aliquot was removed and serially diluted. Each dilution was plated onto CLA then incubated at 42^0C for 48 hours under microaerophilic conditions. After the 48 hour incubation *Campylobacter* colonies were enumerated to determine the challenge dose.

Campylobacter Enumeration from Ceca

Campylobacter counts in the ceca were determined as described previously in our laboratory by Cole and coworkers (Cole et al., 2006a). On day 14 cecal contents were collected and serially diluted (1:10) with BPD and inoculated onto CLA plates. The CLA plates were incubated for 48 hours at 42^0C under microaerophilic conditions. Presumptive *Campylobacter* colonies were directly enumerated and converted to cfu/mL of cecal contents. Bacterial colonies were confirmed by latex agglutination test (PANBIO Inc., Columbia, MD) and by API Campy (Biomeriuex Durham, NC).

Statistical Analysis

Data were analyzed using PROC GLM procedure of SAS (SAS Institute, 2002). The *Campylobacter jejuni* counts from the ceca were logarithmically transformed (log cfu/mL) before analysis to achieve homogeneity of variance (Byrd et al., 2003). Treatment means were partitioned by LSMEANS analysis (SAS Institute, 2002) and probability of $P < 0.05$ was required for statistical significance.

RESULTS

In Vitro Antibacterial Activity of Cranberry Extracts

There was no consistent reduction (>1 log) in *Campylobacter* counts for the 0.1 or 0.5% treatments at 8 or 24 hours post-inoculation for either the L-PAC (Table 1) or H-PAC (Table 2) treatments when compared with the controls in either trial. For the 1% treatment, there was at least a one log reduction in *Campylobacter* counts for both compounds at 8 or 24 hours post dosing for both trials. For the 2 or 4% doses, there was a greater reduction in counts for both time points and trials when compared with control for the L-PAC treatment (Table 1). For H-PAC, the 2 or 4% doses eliminated detectable *Campylobacter* in both trials (Table 2).

Cecal *Campylobacter* Counts, Body Weights and Feed Consumption

Cecal *Campylobacter* counts in 14 day old broiler chicks were not reduced by administration of L-PAC or H-PAC in Trial 1 or Trial 2 when compared with the *Campylobacter* positive control (Table 3). Body weights were not affected feeding any dose of L-PAC in either trial when compared with controls (Table 3). Body weights were, however, reduced in Trial 1 at the highest concentration and for the 1% and 2% H-PAC treatments in Trial 2 when compared

with controls (Table 4). Although feed consumption was only determined for each pen at the end of the trials, the only consistent change for both trials was an increase in feed consumption in the 0.5% H-PAC treated birds versus the controls

DISCUSSION

In this study, we evaluated the potential for various doses of two different cranberry extracts, L-PAC and H-PAC, to inhibit the growth of *Campylobacter jejuni* in broth culture and, if efficacious, to test these compounds for their ability to reduce or eliminate *Campylobacter* colonization in young broiler chickens. The *in vitro* antimicrobial susceptibility assays demonstrated that L-PAC and H-PAC at the lowest concentrations had little effect on *Campylobacter* growth. However the higher concentrations of L-PAC was able to reduce *Campylobacter* by greater than 5 logs at 8 hours and to undetectable levels 24 hours after treatment. For the H-PAC treatment, at both 8 and 24 hours, *Campylobacter* was reduced to undetectable levels. These results indicated an apparent dose response relationship between the increasing concentrations of cranberry extracts and increasing efficacy against *Campylobacter in vitro*. Furthermore, it appears that H-PAC is more effective against *Campylobacter* than L-PAC in these *in vitro* trials. H-PAC has a higher concentration of proanthocyanidins than L-PAC which may explain its increased antibacterial activity against *Campylobacter*. Experiments with proanthocyanidins have shown them to have antioxidant and iron scavenging properties (Dixon et al., 2005; Lin et al., 2005; Guo et al., 2007; Caillet et al., 2011) and may play a role in the *in vitro* anti-*Campylobacter* activity observed in our trials. Although these compounds were effective *in vitro*, they were not able to consistently reduce cecal *Campylobacter* counts in young broiler chickens.

It is possible that the inability of these cranberry extracts to reduce *Campylobacter* in young birds is because it was absorbed prior to reaching the ceca in the lower intestine and did not reach the ceca in concentrations high enough to reduce *Campylobacter* counts. Although increasing the dose in the feed may be an option, it appears, at least for the higher doses of H-PAC, this extract adversely affected the birds as demonstrated by a reduction in body weights.

It is also known that pH and temperature affect the antioxidant activity of cranberry extracts (Wu et al., 2008; Côté et al., 2011b), which may not be optimal in birds. Transition through the digestive system of the bird would subject these extracts to drastic changes in pH and the elevated body temperature of the chicken may also be a contributing factor (Thornton, 1962). Another possible reason that these cranberry extracts were not efficacious *in vivo* may be due to the niche *Campylobacter* occupies in the intestine. Even if cranberry extracts reached the ceca at high concentrations it is possible that penetration into the crypts within the ceca may not occur. *Campylobacter* is able to sequester itself deep within the mucous filled crypts of the ceca, due to its chemoattraction to mucin, where it remains protected (Beery et al., 1988; Hugdahl et al., 1988; Mead, 2002; Cole et al., 2006a). Previous research from our laboratory observed that even when antibiotic treatment eliminated *Campylobacter* within other sites along the gastrointestinal tract, the crypts remained colonized (Farnell et al., 2005).

The ability of *Campylobacter* to remain protected within the mucous in the crypts may also prevent exposure to the anti-adhesive capabilities of cranberry extracts (Foo et al., 2000; Howell et al., 2005). Cranberry proanthocyanidins are able to prevent actin filaments of host cells from rearrangement, which is a mechanism used by some pathogenic bacteria for host cell invasion (Burger et al., 2000; Burger et al., 2002; Harmidy et al., 2011). *In vitro* studies to

assess host cell invasion by *Campylobacter jejuni* have determined that it utilizes adhesions and secreted proteins to alter the actin cytoskeleton leading to membrane 'ruffling' then invasion (Eucker and Konkel, 2012). The significance of this mechanism is supported by *in vivo* studies demonstrating that modified strains are unable to produce adhesion proteins are not capable of colonizing chickens (Biswas et al., 2003; Flanagan et al., 2009). Therefore, treatments which reduce the mucous crypt concentrations may expose *Campylobacter* to the anti-pathogenic properties of cranberry extracts. Research with bismuth compounds has demonstrated reductions in mucous viscosity and partial efficacy against *Campylobacter* colonization of the ceca (Wagstaff et al., 1988; Farnell et al., 2006). Follow up experiments are planned to evaluate if co-administration of bismuth and cranberry extracts can further reduce cecal *Campylobacter* colonization.

Cranberry extracts contain many flavinoid compounds including anthocyanins and proanthocyanidins (Sun et al., 2002; Pappas and Schaich, 2009; Côté et al., 2010). Research into the addition of flavonoids to improve poultry nutrition have shown that gut microbiota plays an essential role in the bioavailability of flavonoids, which require deglycosylation in order to be absorbed in the gut (Iqbal and Zhu, 2009). Iqbal and coworkers (2009) were able to isolate and identify three *Lactobacillus* strains from chicken cecal contents that significantly improved the bioavailability of flavonoids. This presents the opportunity for further study of cranberry extracts plus *Lactobacillus* strains as a potential pre-harvest intervention for *Campylobacter* colonization of poultry.

The two cranberry extracts tested in this study were able to inhibit *Campylobacter in vitro* but not when tested in young chickens. Follow up experiments are needed to increase the

potency of cranberry proanthocyanidins, such as combining them with lactic acid bacteria strains or bismuth compounds, which may reduce this important food borne pathogen in chickens.

Table 1. The effect of different concentrations of cranberry extract L-PAC on *in vitro* growth of *Campylobacter jejuni* in Trials 1 and 2[1, 2].

Cranberry extract L-PAC

		8 hours				24 hours	
		Trial 1	Trial 2			Trial 1	Trial 2
Control	0%	$4.8x10^8$	$5.7x10^7$	**Control**	0%	$2.5x10^8$	$5.8x10^7$
	0.1%	$4.3x10^7$	$1.1x10^8$		0.1%	$2.1x10^8$	$1.8x10^8$
	0.5%	$3.0x10^8$	$3.8x10^7$		0.5%	$1.1x10^8$	$1.0x10^5$
L-PAC	1%	$3.2x10^7$	$5.4x10^5$	**L-PAC**	1%	$1.7x10^7$	ND
	2%	$3.2x10^3$	ND		2%	ND	ND
	4%	$1.0x10^3$	ND		4%	ND	ND

[1] L-PAC was inoculated with a three strain mixture of wild type *Campylobacter jejuni* and incubated at 42^0C under microaerophilic conditions for 8 hours or 24 hours.

[2] ND = not detectable; detection limits of assay are $1.0x10^2$.

Table 2. The effect of different concentrations of cranberry extract H-PAC on *in vitro* growth of *Campylobacter jejuni* in Trials 1 and 2[1, 2].

Cranberry extract H-PAC

		8 hours				24 hours	
		Trial 1	Trial 2			Trial 1	Trial 2
Control	0%	$4.8x10^8$	$5.7x10^7$	**Control**	0%	$2.5x10^8$	$5.8x10^7$
	0.1%	$3.9x10^7$	$3.0x10^7$		0.1%	$8.8x10^7$	$1.3x10^7$
	0.5%	$5.8x10^6$	$8.1x10^6$		0.5%	$2.6x10^7$	$1.3x10^7$
L-PAC	1%	$3.7x10^4$	$3.2x10^5$	**L-PAC**	1%	$3.2x10^2$	ND
	2%	ND	ND		2%	ND	ND
	4%	ND	ND		4%	ND	ND

[1] L-PAC was inoculated with a three strain mixture of wild type *Campylobacter jejuni* and incubated at 42^0C under microaerophilic conditions for 8 hours or 24 hours.

[2] ND = not detectable; detection limits of assay are $1.0x10^2$

Table 3. The effect of different treatments of cranberry concentrate L-PAC and H-PAC on cecal *Campylobacter* counts (log cfu/mL of cecal contents) in 14 day old broiler chicks (Means±SEM) during Trials 1 and 2[1].

		Trial 1	Trial 2
Control	0%	7.01±0.23[a]	5.97±1.05[ab]
	0.5%	7.37±0.67 [a]	6.55±0.76[ab]
L-PAC	1%	5.64±0.81[a]	6.43±0.74[ab]
	2%	7.00±0.31 [a]	5.7±0.54[b]
	0.5%	5.97±0.66 [a]	7.25±0.23[ab]
H-PAC	1%	7.05±0.20 [a]	7.69±0.26[a]
	2%	6.90±0.22 [a]	7.10±0.33[ab]

[a,b]Means within columns with no common superscript differ significantly ($P<0.05$).

[1]Chicks were given feed supplement with H-PAC or L-PAC from day of hatch to the end of the 14 day study. At day 7 birds were challenged by oral gavage (0.25mL) with approximately 10^6 cfu/mL of three strains of *Campylobacter jejuni* in both trials. All *Campylobacter* data were $\log_{10}$ transformed for statistical analysis.

Table 4. The effect of different treatments of cranberry extracts L-PAC and H-PAC on body weight gain (Means±SEM) and feed consumption in 14 day old broiler chicks during Trials 1 and 2[1].

		Trial 1				Trial 2	
		Body Wt (g)	Feed Consumption (g)			Body Wt (g)	Feed Consumption (g)
Control	0%	344±14[a]	548	**Control**	0%	443±41[a]	732
	0.5%	340±12[a]	496		0.5%	403±13 [abc]	739
L-PAC	1%	314±9.7[ab]	537	**L-PAC**	1%	409±19 [ab]	873
	2%	327±7.9[ab]	539		2%	403±15 [abc]	815
	0.5%	326±17[ab]	564		0.5%	383±21 [abc]	781
H-PAC	1%	323±13[ab]	547	**H-PAC**	1%	346±19[bc]	903
	2%	290±20[b]	542		2%	339±27[c]	781

[1]Chicks were given feed supplement with H-PAC or L-PAC from day of hatch to the end of the 14 day study.

[a,b]Means within columns with no common superscript differ significantly ($P<0.05$).

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