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The Ability of Select Probiotics to Reduce Enteric Campylobacter Colonization in Broiler Chickens

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The Ability of Select Probiotics to Reduce Enteric *Campylobacter* Colonization in Broiler Chickens.

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

By

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Bachelor of Veterinary Science & Animal Husbandry, 2010

July 2015
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ABSTRACT

Campylobacter is the leading cause of foodborne illness worldwide and is often associated with consumption and/or mishandling of contaminated poultry products. Probiotic use in poultry has been an effective strategy in reducing other enteric foodborne pathogens but has not proven consistent for *Campylobacter*. As *Campylobacter* resides and utilizes intestinal mucin for growth, isolates selected on the basis of mucin utilization might be a strategy to screen for efficacious probiotic bacterium. In this study, bacterial isolates demonstrating increased growth rates in mucin, *in vitro* (trials 1 or 2), or isolates demonstrating a reduction of *Campylobacter* counts when co-incubated with mucin, *in vitro* (trials 3 or 4) were selected for their ability to reduce *Campylobacter* colonization in four bird trials. In trials 1 or 2, ninety day-of-hatch chicks were randomly divided into 9 treatment groups (n=10 chicks/treatment) and treated individually with one of four bacterial isolates demonstrating increased growth in media containing mucin. The treatments included a positive *Campylobacter* control (no isolate) or four isolates grown in media with or without mucin prior to inoculation. In trials 3 or 4, sixty day-of-hatch chicks were divided into six treatment groups (n=10 chicks/treatment) receiving either no isolate (positive *Campylobacter* control) or dosed with five individual isolates all demonstrating the ability to reduce *Campylobacter* counts when co-incubated with mucin, *in vitro*. These isolates were grown in media containing mucin prior to inoculation. In all four trials, birds were gavaged with individual isolates at day-of-hatch and orally challenged with a four strain mixture *C. jejuni* on day 7. Ceca were collected at day 14 for *Campylobacter* enumeration. Results from these first two trials demonstrated two individual isolates, one with increased growth rates when grown in mucin or one isolate incubated without mucin, consistently reduced cecal *Campylobacter* counts (1.5 to 4 log reduction) when compared with controls. In follow-up trials with isolates selected

for their ability to directly reduce *Campylobacter* counts when co-incubated with mucin, *in vitro*, one isolate consistently reduced cecal *Campylobacter* counts by approximately 1.5 logs. These results support the potential use of mucin to preselect isolates for their ability to reduce enteric *Campylobacter* colonization.

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DEDICATION

This dissertation is dedicated to

My mother Ms. Urmila Shrestha

and

My father Mr. Arjun Kumar Shrestha

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Food-borne illness is one of the greatest problems in public health today, which is mostly due to consumption of food contaminated with bacteria, viruses, parasites and/or toxins (WHO, 2011). It has been reported that food-borne illnesses are likely to occur in developing and underdeveloped countries due to poor sanitation and poor socioeconomic conditions prevailing in those countries (WHO, 2012a). However, recent studies have documented an increasing number of food-borne diseases in developed countries (Humphery et al., 1993; CDC, 2011). Even with one of the safest food supplies in the world, it has been reported that 1 in 6 Americans get sick due to food-borne illness, resulting in 128,000 hospitalizations and 3,000 deaths each year in the United States (CDC, 2011). It has been estimated that food-borne illnesses cause economic losses worth \$77.7 billion per annum in the United States (Scharff, 2012).

Campylobacter infection in humans is one of the leading causes of food-borne illness worldwide (WHO, 2012b). In the United States alone, approximately 13.85 cases were reported per every 100,000 people in 2013 (CDC, 2014) and it is estimated to cause economic loss of \$1.7 billion annually (Hoffmann et al., 2012). Similarly in the European Union, approximately nine million human campylobacteriosis cases have been estimated with a resulting economic loss of € 2.4 billion annually (EFSA, 2011).

CHARACTERISTICS OF *CAMPYLOBACTER*

Historical overview

In 1886, Theodor Escherich first observed a unique spiral shaped bacteria in the stool samples from infants with diarrhea (Escherich, 1886), which was later identified as *Campylobacter*. During the early 1900s, McFadyean and Stockman first isolated *Campylobacter* spp. from the fetal tissues of aborted sheep (Butzler, 2004; Skirrow, 2006). Since then several

scientists isolated similar organisms from aborted bovine fetuses, dysentery in calves and sheep (Jones et al., 1931; Doyle, 1944). Initially the organisms were classified under the genus *Vibrio*, but in 1963 Sebald and Véron separated *Campylobacter* from the genus *Vibrio* and proposed a new genus, *Campylobacter* (Sebald and Véron, 1963). *Campylobacter* markedly differ from *Vibrio* due to their microaerophilic growth, non-fermentative metabolism and low DNA base composition (Sebald and Véron, 1963). In 1973, Véron and Chatelain further classified *Vibrio*-like organisms into the type species; *C. fetus* along with *C. coli*, *C. jejuni* and *C. sputorum* because of their different characteristics from genus *Vibrio* (Véron and Chatelain, 1973). The Genus *Campylobacter* was later classified under a new family *Campylobacteraceae* which also included other genera; *Helicobacter*, *Arcobacter*, *Sulfurospirillum* and *Wolinella* (Vandamme and De Ley, 1991). There are at least 17 identified species of *Campylobacter* (Lastovica, 2006; Debruyne et al., 2008); however *Campylobacter jejuni* and *Campylobacter coli* are responsible for more than 95% of the human campylobacteriosis cases (Park, 2002; Snelling et al., 2005)

Morphological characteristics

The word *Campylobacter* originated from the Greek words, “kampulos” and “bacter” meaning “curved” and “rod” respectively (Sebald and Véron, 1963). All members of the genus *Campylobacter* are gram negative non-spore forming slender spirally-curved rods, measuring 0.2-0.8 µm wide and 0.5-5 µm long (Smibert, 1978; Thomas et al., 1998; Debruyne et al., 2008). *Campylobacter* have a single polar flagellum that is approximately double the length of the cell which makes them highly motile with a characteristic cork-screw like motility (Smibert, 1978; Debruyne et al., 2008). As exceptions, some species, such as *C. gracilis*, are non-motile, and *C. showae* have multiple flagella (Debruyne et al., 2008).

In vitro growth requirements

Campylobacter spp. are fastidious organisms, needing complex growth media and microaerophilic environmental conditions for their growth (Buck and Smith, 1987; Kelly, 2001; Park, 2002; Garénaux et al., 2008). Optimal growth is observed at 42°C under microaerophilic conditions (5% oxygen, 10% carbon dioxide and 85% nitrogen; Park, 2002). Despite the high temperature requirement for their growth, *C. jejuni* displays physiological activity even at 4°C (Hazeleger et al., 1998). It has been reported that *C. jejuni* can resist environmental stressors by changing its morphology from spiral-bacilla to coccoid forms, which is characterized by loss of culturability (Kelly, 2001), however they still remain viable (Rollins and Colwell, 1986). Also, unfavorable environmental growth conditions such as changes in temperature, pH, osmolarity and loss of nutrients in the medium are responsible for transition from spiral to coccoid viable but nonculturable (VBNC) state (Rollins and Colwell, 1986; Lázaro et al., 1999; Moore, 2001). It has been found that this VBNC state possesses the ability to infect hosts (Saha et al., 1991; Cappelier et al., 1999a; Baffone et al., 2006). However, contradictory opinions have been proposed about the ability of VBNC forms to become metabolically active and produce disease upon exposure to favorable conditions (Jones et al., 1991; Beumer et al., 1992; Korsak and Popowski, 1997; Cappelier et al., 1999a; Cappelier et al., 1999b). The molecular mechanism underlying the VBNC state development and resuscitation are still unknown (Pinto et al., 2013).

Environmental reservoirs and sources of Campylobacter infection.

Campylobacter spp. are ubiquitous and normally found in a wide range of warm-blooded animals (Humphrey et al., 2007), including food-producing animals such as pigs (Nesbakken et al., 2003), sheep (Firehammer and Myers, 1981; Stanley and Jones, 2003), beef cattle, turkeys (Zhao et al., 2001) and chickens (King, 1962; Skirrow, 1977). Apart from food-producing

animals, contact with pets can also serve as a source of human *Campylobacter* infections (Deming et al., 1987; Kapperud et al., 1992). Consumption of untreated water may be another route of human *Campylobacter* infection (Vogt et al., 1982; Palmer et al., 1983; Taylor et al., 1983; Hopkins et al., 1984). Some research articles also suggested that raw or unpasteurized milk is a source of *Campylobacter* infection resulting in human gastroenteritis (Blaser et al., 1979; Porter and Reid, 1980; Robinson and Jones, 1981). Consumption of fruits and vegetables (Evans et al., 2003) and mushrooms (Doyle and Schoeni, 1986) have been reported as minor sources of *Campylobacter* transmission to humans (Rosef and Kapperud, 1983). Among the various causes of human infections, poultry is considered the primary source of infections (King, 1962; Skirrow, 1977). Handling of *Campylobacter* contaminated chicken and consumption of undercooked chicken are the major sources for human campylobacteriosis (Hopkins and Scott, 1983; Ikram et al., 1994; Neimann et al., 2003; Friedman et al., 2004). It has also been found that cross contamination of raw chicken with other uncooked food items during food preparation in the kitchen is a major route for *Campylobacter* infections in humans (Boer and Hahne, 1990; Mylius et al., 2007; Lubber, 2009)

CAMPYLOBACTER IN HUMANS

Human Incidence

It was shown that *Campylobacter* spp. are able to cause infections in animals in the early 1900s; however it wasn't reported in humans until about 1980 (Skirrow, 1977; Silva et al., 2011). With the development of filter techniques (Dekeyser et al., 1972) and selective medium (Skirrow, 1977). *Campylobacter* has been recognized as the most common cause of food-borne diarrheal illness in humans (Allos, 2001; EFSA/ECDC, 2013; CDC, 2014). Cases of human campylobacteriosis are seen in both developed and developing countries, and are likely to occur

often in children, immune-compromised and elderly persons (Tauxe et al., 1992; Corry and Atabay, 2001). The incidence in infants (24.08 per 100,000) is higher as opposed to adults (14.54 per 100,000) in United States (CDC, 2014). *Campylobacter* infections in male to female ratio is approximately 1.2-1 (Louis et al., 2005; Olson et al., 2008). However, the reason behind such high incidence in males compared to females is not fully understood (Friedman, 2000). It has been reported that the incidence may vary with the season; peak incidences occurring from June-August in North America, Europe, UK and Canada (Nylen et al., 2002; Nelson and Harris, 2011; Lal et al., 2012).

In the United States, the CDC has reported 13.83 incidences per 100,000 people in 2013 which is greater than previous reports (CDC, 2014). This reported incidence might be lower than the actual incidence due to many underdiagnosed and underreported cases (Mead et al., 1999; Samuel et al., 2004). The European Food Safety Authority and the European Center for Disease Control and Prevention jointly estimated 220,201 *Campylobacter* cases in 2011 which is 2.2% more than in 2010 (EFSA, 2013). It is also reported as the most frequent zoonotic disease after salmonellosis in 2011 (EFSA/ECDC, 2013). In Australia, England and Wales, annual cases of 225,000 and 500,000 were reported respectively (Hall et al., 2008; Nichols et al., 2012). Other countries such as Germany, Netherlands and Finland have also reported increasing cases of campylobacteriosis (Nakari et al., 2010).

Pathogenesis of Campylobacter

The molecular mechanisms involved in the pathogenesis of *Campylobacter* are not clear (Ketley, 1997; Svensson et al., 2014). However it is believed that adhesion, colonization and invasion of host intestinal epithelium play a pivotal role in producing symptoms associated with campylobacteriosis (Ketley, 1997). *Campylobacter* possesses the fibronectin binding proteins

cadF, FlpA and periplasmic or membrane associated protein (PEB 1) that is responsible for host cell binding and colonization (Konkel et al., 1997; van Vliet and Ketley, 2001; Young et al., 2007; Konkel et al., 2010). Host cell invasion and gastroenteritis is mediated by protein secretion via the flagellar type III secretion system (Larson et al., 2008). The infection is further aided by flagellar-driven motility and *Campylobacter* invasion antigen (Dasti et al., 2010). It has been found that internalization of *C. jejuni* into host cells is triggered by the combined effects of the microfilaments and microtubules of host cells (Biswas et al., 2003). Johnson and Lior (1988) reported that *C. jejuni* produces a toxin called cytolethal distending toxin (Cdt). Cdt causes a host cell cycle arrest, preventing cells from entering the M phase, inducing host cell apoptosis (Whitehouse et al., 1998; Dasti et al., 2010). The genes encoding Cdt were sequenced for *C. jejuni* in late 1990s (Pickett et al., 1996; Bang et al., 2001) and for *C. coli* and *C. fetus* in late 2007 (Asakura et al., 2007, 2008).

Human Infections

Campylobacter infection is one of the leading causes of bacterial gastroenteritis in humans (Blaser et al., 1983; Allos, 2001; CDC, 2014). It has been suggested that children and immunocompromised people are more susceptible to *Campylobacter* infections (Allos, 2001). Thermotolerant *Campylobacter* spp. specifically *C. jejuni* and *C. coli*, together account for approximately 95% of human campylobacteriosis cases worldwide (Park, 2002; EFSA/ECDC, 2013). An infective dose as low as 500-800 live cells may be sufficient to cause illness in humans (Robinson, 1981; Black et al., 1988). The incubation period ranges from 2-5 days, but has been reported up to 10 days (Butzler, 2004). In most patients symptoms may include diarrhea, abdominal cramps, malaise, myalgia and fever (Skirrow, 1977; Butzler, 2004). Diarrhea may be loose, watery or bloody, suggestive of ulcerative colitis due to the invasive

nature of *C. jejuni* (Blaser, 1997). Extra-intestinal manifestations including meningitis (Goossens et al., 1986), osteomyelitis (Vandenberg et al., 2003) and neonatal sepsis are less frequently seen (Butzler, 2004). Campylobacteriosis is generally a self-limiting disease and the affected patients may recover without any treatment (Allos and Blaser, 1995; Rosenquist et al., 2003). Some cases of campylobacteriosis have been associated with serious post-infectious complications such as Guillain-Barré syndrome, reactive arthritis, irritable bowel syndrome and inflammatory bowel disease (Gumpel et al., 1981; Spiller and Garsed, 2009).

Guillian-Barré syndrome. Guillian-Barré syndrome (GBS) is one of the potential long-term severe complications of *Campylobacter* infection. It is a neuromuscular disease characterized by ascending paralysis that causes weakness of limbs, respiratory muscles and loss of reflexes (Allos, 1997). It has been identified that 20-40% of the GBS cases were associated with a preceding *C. jejuni* infection (Mishu and Blaser, 1993). Approximately 1 in 1000 cases of *Campylobacter* infections may develop GBS (Allos, 1997). Patients usually develop GBS 1-3 weeks after the onset of *Campylobacter* enteritis (Butzler, 2004). Approximately 20% of GBS patients requires hospitalization in the intensive care unit for respiratory ventilation (WHO 2012b).

It has been postulated that molecular mimicry between lipooligosaccharides of *C. jejuni* and host GM₁ gangliosides may cause the development of autoantibodies and play a role in the pathogenesis of GBS (Yuki et al., 1993, 2004). There are four subtypes of GBS: 1) acute motor axonal neuropathy (AMAN); 2) acute inflammatory demyelinating polyadiculoneuropathy (AIDP); 3) acute motor and sensory axonal neuropathy (AMSAN) and 4) Miller Fishers syndrome. Among these four subtypes of GBS, *Campylobacter* infections are most frequently associated with the AMSAN subtype (Kuwabara, 2004).

Reactive arthritis. Reactive arthritis (ReA) is a spondyloarthropathy and occurs subsequent to microbial gastrointestinal infections, including *Campylobacter* (Carter and Hudson, 2009; Wu and Schwartz, 2008). Symptoms of ReA may include inflammation of joints, tissues, skin and tendons (Pope et al., 2007; Townes, 2010). It has been estimated that 1-5% of *Campylobacter* cases may result in reactive arthritis (Pope et al., 2007), however estimates of up to 16% have been reported (Ajene et al., 2013). Though children are more likely to get *Campylobacter* infections, ReA is more common in adults (Carter, 2006; Pope et al., 2007). Pathophysiology of this disease is still not clear. However one hypothesis involves antibody production against pathogens having affinity to HLA-B27 and another hypothesis is impaired cellular immunity (decreased interleukin-2 production) against the inciting microorganism correlating with disease development (Wu and Schwartz, 2008).

Irritable Bowel Syndrome. Irritable bowel syndrome (IBS) is a recurring functional gastrointestinal disorder characterized by frequent abdominal pain (three or more per month) or discomfort linked with defecation or change in bowel habit and abdominal bloating (Quigley et al., 2009). Prevalence of IBS in North America and Europe ranges from 10-16% (Quigley et al., 2009). It is believed that *Campylobacter* infections as an antecedent infection account for about 10% of IBS cases (Spiller and Garsed, 2009). The exact mechanism by which *Campylobacter* causes symptoms of IBS is not completely understood, however *Campylobacter* spp. is known to produce cytotoxins and some of them are believed to be associated with development of IBS (Thornley et al., 2001).

Inflammatory Bowel Disease. Inflammatory bowel disease (IBD) is a collective term for ulcerative colitis and Crohn's disease (Papadakis and Targan, 1999). It is a chronic relapsing disease characterized by diarrhea, constipation, tenesmus, abdominal cramps, fever, pain and/or

rectal bleeding with bowel movement, (Bernstein et al., 2009). *Campylobacter jejuni* has been isolated from 10% of IBD cases (Gradel et al., 2009). *Campylobacter* promotes translocation of non-invasive bacteria by disrupting transcellular transport across the intestinal epithelium playing a role in the pathogenesis of IBD (Kalischuk et al., 2009).

Treatment

Campylobacter infections are generally self-limiting and do not require antibiotic therapy, if antimicrobial therapy is needed fluoroquinolones are the drug of choice (Allos, 2001). However, in the past few years, fluoroquinolone resistant *Campylobacter* strains are emerging (Allos, 2001). *Campylobacter* spp. may also be resistant to other antibiotics including ciprofloxacin, bacitracin, novobiocin, rifampin, trimethoprim, vancomycin and tetracycline (Taylor and Courvalin, 1988; Kuschner et al., 1995; Engberg et al., 2001). Currently, erythromycin is used most frequently to treat *Campylobacter* infection due to its low toxicity, narrow spectrum and low cost (Allos and Blaser, 1995; Allos, 2001).

CAMPYLOBACTER IN POULTRY

Epidemiology of Campylobacter in poultry

Campylobacter is ubiquitous in poultry flocks and it has been found that the percentage of broiler flocks colonized with *Campylobacter* varies from country to country (Newell and Fearnley, 2003). In the United States and Great Britain nearly 90% of flocks are colonized with *Campylobacter* (Evans and Sayers, 2000; Stern et al., 2001b), 41.1% in Germany (Atanassova and Ring, 1999) and 47.5% in Japan (Haruna et al., 2012). However, in Europe prevalence rates vary from 18 to 90%, with the northernmost countries having remarkably lower percentages than southernmost countries (Newell and Fearnley, 2003).

Many research findings have shown variability in *Campylobacter* contamination with retail poultry products. Factors such as sample collection, detection methodology, season, geographical location and production practices may contribute to variability in *Campylobacter* contamination in poultry and poultry products (Lee and Newell, 2006). An epidemiological study in Greater Washington D.C. suggested that approximately 70.7% of raw chicken meat was contaminated with *Campylobacter* (Zhao et al., 2001). Other studies have revealed as high as 90-100% of the raw chicken meat is contaminated with *Campylobacter* (Suzuki and Yamamoto, 2009).

***Campylobacter* colonization in birds**

Campylobacter is generally nonpathogenic in poultry (Beery et al., 1988; Stern et al., 1988). Environmental contamination is the primary source of infection in newly placed chicks (Shane, 1992). Chicks around the age of 2-3 weeks get colonized with *Campylobacter* in their intestinal tract as a commensal organism (Beery et al., 1988). The infectious dose for chicken has been reported to be as low as 50 organisms (Achen et al., 1998; Knudsen et al., 2006).

Campylobacter predominantly resides in the lower part of the intestine, notably in the ceca, and concentrations may reach up to 10^8 CFU per gram of cecal contents (Beery et al., 1988; Stern et al., 1988; Achen et al., 1998). A study conducted in UK reported that there is no seasonal variation regarding the prevalence of *Campylobacter* in broiler flocks (Humphery et al., 1993). Nevertheless, some studies found a summer peak in the prevalence of positive flocks (Wallace et al., 1997; Nylen et al., 2002). The mechanism of colonization in the bird's intestine is not fully elucidated. However, it is hypothesized that chemoattraction of *C. jejuni* to mucin plays a significant role in colonization; *C. jejuni* uses mucin as a substrate and colonizes in high numbers in the cecal crypts (Beery et al., 1988). Similarly, an immunological investigation on host

immune response to *Campylobacter* in chickens suggested that down regulation of certain genes in the host by *Campylobacter* plays a vital role in the persistent high level of colonization (Meade et al., 2009). In most cases, *Campylobacter* localizes in the intestines. However, systemic invasion to organs such as liver, spleen, heart and lungs has also been reported (Young et al., 1999; Meade et al., 2009).

Transmission

Horizontal transmission. Several studies have shown a wide range of hosts for *Campylobacter*, wild birds, domestic birds (Luechtefeld et al., 1980; Glünder et al., 1992), rodents (Cabrita et al., 1992) and insects (Jacobs-Reitsma et al., 1995). Horizontal transmission is the predominant mode of transmission of *Campylobacter* in poultry (Loc Carrillo et al., 2005; Silva et al., 2011). Poultry flocks naturally become colonized from the above mentioned sources and *Campylobacter* positive birds rapidly shed the organisms in the feces which act as a source for other birds (Jacobs-Reitsma et al., 1995; Achen et al., 1998; Mead, 2002), which then spreads rapidly from bird to bird making the entire flock contaminated (Loc Carrillo et al., 2005; Horrocks et al., 2009). The rapidity of the shift from un-colonized to almost 100% colonization of *Campylobacter* in a flock is aided by coprophagic behavior of chicks and via contamination of food and water sources (Montrose et al., 1985; Keener et al., 2004).

Vertical transmission. Transmission of *C. jejuni* from parent hen to chicks is controversial. Vertical transmission of any bacteria can take place by either primary (contamination of egg content in the hen's reproductive tract) or secondary (contamination of the eggshell with fecal material after lay) infection of the egg (Sahin et al., 2003a). Many researchers have found the presence of *Campylobacter* in various parts of the male and female reproductive tracts of poultry (Cox et al., 2002; Cole et al., 2004) indicating a possibility of vertical transmission of

Campylobacter to chicks. Several investigations have been conducted to verify the possibility of vertical transmission of *Campylobacter* in poultry (Doyle, 1984; Clark and Bueschkens, 1985; Shanker et al., 1986). In their earlier studies, Clark and Bueschkens (1985) inoculated fertile eggs with *C. jejuni*, and demonstrated that 11% of the resulting chicks had *Campylobacter* in their intestinal tract. However, naturally it is not easy for *Campylobacter* to get into the egg content via egg shell penetration and even if it does contaminate the egg contents, it is not likely to survive for more than 48 h stored at room temperature (Doyle, 1984; Shanker et al., 1986). In contrast to these findings, some researchers demonstrated that *Campylobacter* can remain viable inside egg yolk for up to 14 days, but <8 days inside the air sac and albumen (Clark and Bueschkens, 1986).

PREHARVEST CONTROL STRATEGIES OF *CAMPYLOBACTER* IN POULTRY

With increasing cases of human campylobacteriosis, development of intervention strategies are necessary to control and reduce *Campylobacter* in poultry and poultry products to minimize human infections. Reducing the bacterial concentration in poultry prior to processing would be beneficial, as cross contamination between fecal contaminated carcasses and meat may occur during processing. Risk assessment studies conducted by Rosenquist and his colleagues (2003) predicted that a 2 log reduction of the *Campylobacter* on chicken carcasses can reduce the human incidence by 30 fold. Many pre-harvest intervention strategies have been evaluated with varying results. Some of them are briefly described below which may be used as potential control measures to reduce the *Campylobacter* counts in poultry and poultry products.

Biosecurity

Biosecurity is the protection of farm animals from various types of infectious agents by using different types of measures such as use of protective clothing, cleaning and disinfecting of farm house, provision of clean water, restricting the movement of people or animals between farms, etc. (Silva et al., 2001; Vandeplass et al., 2008). Studies have demonstrated that adopting standard biosecurity methods led to an approximate 50% reduction of *Campylobacter* prevalence in broiler flocks (Gibbens et al., 2001). Similarly, a report from two Dutch broiler farms suggested that introduction of hygiene measures significantly reduced the *Campylobacter* prevalence in broiler flocks (van de Giessen et al., 1998). A review on biosecurity-based interventions by Newell and colleagues (2011) suggested that diligent application of biosecurity measures is required to reduce flock prevalence. However, complete elimination of *Campylobacter* from flocks is unlikely (Wagenaar et al., 2006; Vandeplass et al., 2008). Moreover, the costs involved with the adoption of such strict on farm biosecurity measures limits the practicality of biosecurity (Fraser et al., 2010).

Bacteriocins

Bacteriocins are small biologically active protein compounds of approximately 5- 6 kilodalton, produced by some strains of bacteria that can inhibit the growth of other closely related bacteria (Klaenhammer, 1993; Cleveland et al., 2001). Both Gram positive and Gram negative bacteria such as, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Carnobacterium*, *Enterococcus*, *Escherichia*, *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Pseudomonas* and *Clostridium* have been reported to produce bacteriocins (Svetoch and Stern, 2010). Bacteriocins application in poultry processing was initiated in 1994 with the test against *Listeria monocytogenes* using nisin (Mahadeo and Tatini, 1994). Similarly, a literature review on

bacteriocins suggest the potential use of bacteriocins for the reduction or elimination of many food-borne pathogens (Joerger, 2003). Bacteriocins produced by certain strains of *Bacillus circulans* and *Paenibacillus polymyxa* were found to be inhibitory to *Campylobacter* growth *in vitro* (Svetoch et al., 2005). Stern and colleagues (2005) reported that bacteriocins (B602) produced by *P. polymyxa* reduced cecal *C. jejuni* to undetectable levels in chickens. Subsequently, in a second study, inclusion of a microencapsulated bacteriocin (OR 7) in chicken feed for 3 days (day 7 to day 10) reduced cecal *Campylobacter* counts from 1.3 log CFU/g to undetectable levels, whereas control groups were colonized at 7-8 log CFU/g in 10-day-old broiler chickens (Stern et al., 2006). An additional study utilizing bacteriocins produced by *P. polymyxa* and *Lactobacillus salivarius* have shown cecal *Campylobacter coli* reductions to undetectable levels in turkey poults (Cole et al., 2006). Although research has shown promising results on bacteriocins against *Campylobacter* in poultry, bacteriocins can be degraded easily inside the host gut due to its proteinaceous nature (Joerger, 2003). It is expensive to adapt techniques such as microencapsulation, to prevent the enzymatic digestion of bacteriocins in the gastrointestinal tract (Joerger, 2003; Svetoch et al., 2005). In addition, implementation requires approval from the U.S. Food and Drug Administration (FDA) which would require extensive and expensive safety and efficacy studies. So far only one bacteriocin (Nisin) has GRAS (generally recognized as safe) status (Joerger, 2003). Moreover, it has been found that *Campylobacter* develops resistance against bacteriocins which further limits their use in poultry (Hoang et al., 2011a, b).

Bacteriophage

Bacteriophages are viruses capable of infecting and killing specific bacteria (Huff et al., 2005; Hagens and Loessner, 2007). Bacteriophages that infect and replicate in bacteria

subsequently killing the host cells are virulent bacteriophages, which are particularly important for reducing pathogenic bacteria (Huff et al., 2005). *Campylobacter* specific phages have been isolated from chicken excreta, retail poultry, abattoir effluent, sewage and other animal as well as human sources (Atterbury et al., 2003b; Connerton et al., 2004). Several studies were conducted to evaluate the potential application of bacteriophage to reduce cecal colonization of *Campylobacter* in broiler chickens (Loc Carrillo et al., 2005; Wagenaar et al., 2005; El-Shibiny, et al., 2009; Carvalho et al., 2010). Loc Carrillo and co-workers (2005) demonstrated phage treatment of *C. jejuni* colonized broiler chickens resulted in *Campylobacter* counts decreasing in range from 0.5 and 5 log CFU/g of cecal contents compared to the *Campylobacter* positive group. Additionally, they reported that variations in *Campylobacter* reductions were related to the administration dose of phage, type of phage used and time elapsed after administration. Although a sharp decrease in cecal *C. jejuni* is noted immediately after phage administration, *C. jejuni* re-establishes itself over time (Loc Carrillo et al., 2005; Wagenaar et al., 2005). This phenomenon supports the application of bacteriophage a few days before slaughter could be more effective for reducing *Campylobacter* counts in market age birds (Wagenaar et al., 2005). In addition, it has also been observed that bacteriophage administration in the feed is more effective than oral gavage (Carvalho et al., 2010). Introduction of *Campylobacter* specific bacteriophage on artificially *Campylobacter* contaminated chicken skin showed a promising result in reduction of recoverable *Campylobacter* cells from treated chicken skin samples (Atterbury et al., 2003a). In contrast, another study on bacteriophage application on chicken meat samples stored at 4°C did not reduce *Campylobacter* counts (Orquera et al., 2012). Bacteriophage application in food products is safe for human health (Hagens and Loessner, 2010). However, consumer acceptability, narrow host range (Janež and Loc-Carrillo, 2013) and

the possibility of resistance development (El-Shibiny et al., 2009) impedes industry wide application.

Vaccination

Vaccination could be another effort to reduce or eliminate *Campylobacter*. It has been proposed that maternal antibody against *Campylobacter* plays an important role in preventing *Campylobacter* colonization in the early stage of life in chicken (Sahin et al., 2003b). Several investigations have been conducted on the possible use of vaccination but have had limited success. A research article reported cecal *Campylobacter* reduction by approximately 2 log CFU/g of cecal contents after administration of killed *C. jejuni* whole cells and flagellin vaccine intraperitoneally at the age of 16 and 29 day of age (Widders et al., 1996). Similarly, formalin inactivated *C. jejuni* vaccine administered orally in broiler chickens reduced intestinal colonization ranging from 16 to 93% compared with a non-vaccinated control group (Rice et al., 1997). A study conducted by Wyszynska and colleagues (2004) found that oral immunization (on the day of hatch and two weeks after primary immunization) with an avirulent *Salmonella* vaccine strain carrying the *C. jejuni cjaA* gene significantly reduced cecal *Campylobacter* counts. In addition, researchers have demonstrated an increase in anti-*Campylobacter* secretory IgG with inactivated whole cell vaccine (Widders et al., 1996; Rice et al., 1997) or both IgG and IgA after recombinant *Salmonella* vaccination (Wyszynska et al., 2004). Recombinant vaccine candidates which elicit better humoral response produce better results in recent studies (Wyszynska et al., 2004; Layton et al., 2011). Vaccines against *Campylobacter* colonization in poultry are not commercially available yet. Further research in the development of effective vaccines against *C. jejuni* is warranted and should be feasible economically and practical for use in the poultry industry.

Natural compounds

Medium Chain Fatty Acids. Medium chain fatty acids (MCFAs) such as caproic, caprylic, capric, lauric, etc., possess antimicrobial activity against various microorganisms making them a viable alternative to antibiotics (Bergsson et al., 1998; Decuypere and Dierick, 2003). One extensively studied MCFA is caprylic acid (eight-carbon saturated fatty acid), also known as octanoic acid which is naturally present in coconut oil, palm-kernel oils, bovine and breast milk (Jensen et al., 1990; Sprong et al., 2001; Jensen, 2002). Caprylic acid is classified as a generally regarded as safe (GRAS) compound by Food and Drug Administration (21 CFR184.1025, FDA, 2014). Cecal *Campylobacter* counts were reduced by 3-4 log CFU/g by therapeutic or prophylactic supplementation of various concentrations of caprylic acid in feed (Solis de los Santos et al., 2008a, b, 2009, 2010). Similarly, a study conducted by Molatová and co-workers (2011) concluded that feed supplementation with an encapsulated or non-encapsulated mixture of capric and caprylic acid (1:1) reduces cecal *Campylobacter* colonization consistently for 4 days post-inoculation of *Campylobacter*, with better results obtained from the encapsulated mixture. In contrast, Hermans and co-workers (2010) observed no significant efficacy of caprylic acid against cecal *Campylobacter* colonization despite marked *in-vitro* anti-*Campylobacter* activity. A study by Metcalf and co-workers (2011) reported water administration of the soluble form of caprylic acid produced an inconsistent reduction in cecal *Campylobacter* counts, *in vivo*. It has been hypothesized that the water soluble form of caprylic acid does not consistently reduce *Campylobacter* counts due to the protective action of intestinal mucus, making *Campylobacter* less susceptible to MCFAs. (Van Deun et al., 2008; Hermans et al., 2010). Mixed results obtained from various studies on potential use caprylic acid and its soluble form to reduce cecal *Campylobacter* suggests the need for further studies in this area.

Plant extracts. In the last few decades consumer awareness and preference towards organic food products in addition to increased pressure to find alternative to antibiotic use in animals has led researchers to evaluate the antimicrobial properties of plant extracts (Atterbury et al., 2003b; Sirsat et al., 2009). The phytochemicals from various medicinal plants possess antimicrobial properties (Cowan, 1999). Use of medicinal plants by humans has a long history and it has been observed that other primates repeatedly consume certain plants which have medicinal properties (Glander, 1994; Baker, 1996; Halberstein, 2005). Friedman and colleagues (2002) evaluated the antimicrobial activity of various plant essential oils against several food-borne pathogens including *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes* and *Campylobacter jejuni*, *in vitro*. Similarly, a study conducted by Johny and co-workers (2010) on the effect of trans-cinnamaldehyde, eugenol, carvacrol and thymol against *C. jejuni* in cecal contents demonstrated significant reductions of *C. jejuni*, *in vitro*. However, these compounds did not produce consistent results in bird studies conducted by various researchers (Metcalf, 2008; Hermans et al., 2011; Arsi et al., 2014). Similarly, Woo-Ming (2012) reported efficacy of cranberry extracts against *C. jejuni*, *in vitro*, but not *in vivo*. It has been suggested that the failure of plant extracts to work in these *in vivo* trials may be due the compounds being absorbed in the upper digestive tract and unable to reach the target site (ceca) in adequate concentrations to reduce *C. jejuni* counts (Woo-Ming, 2012). More research is needed to determine the most effective plant extracts and the appropriate administration strategy to reduce cecal *Campylobacter* counts in poultry production.

Probiotics

‘Probiotic’ means ‘for life’ in Greek and has been described many ways by multiple scientists over time (Fuller, 1992). Almost a century ago, Metchnikoff (1907) first described the

beneficial effect of consuming fermented milk on human health. Lilley and Stillwell (1965) first used the term ‘probiotic’ to describe the secretory substances produced by one microorganism that promotes the growth of other microorganisms. Later on, “probiotic” has been redefined as “microbial growth stimulating tissue extract” (Sperti, 1971) or “microorganisms and substances that contributes to intestinal microbial balance” (Parker, 1974). The terminology has been well defined over the last few decades. The extensively used definition of probiotic as given by Fuller is “live microorganisms which when administered in adequate amounts can confer beneficial effects on host health” (Fuller, 1989). Salminen and colleagues (1998) redefined probiotics as “a live microbial food ingredient which is beneficial to health”.

More than four decades ago, Nurmi and Rantala (1973) demonstrated that administration of probiotics (undefined mixture of bacteria from adult birds) at an early age can prevent the colonization of *Salmonella* Infantis in chickens. The precise mechanism by which probiotics produce beneficial effects is not clearly elucidated. However, some researchers predict that probiotics provide beneficial effects by producing bacteriocins (Meghrouis et al., 1990), reducing pH due to production of metabolites such as organic acids (Sanders, 1993), by competing for substrates or attachment sites (Fooks and Gibson, 2002) or by increasing macrophage mediated phagocytic activity (Hatcher and Lambrecht, 1993). Initially, this concept was used to control *Salmonella* infection in poultry (Nurmi and Rantala, 1973; Impey et al., 1982; Nurmi et al., 1992; Blankenship et al., 1993; Stavric and D'aoust, 1993; Hume et al., 1998). Lately, this concept is being used to reduce the prevalence of various enteric pathogens such as *E. coli* (Soerjadi et al., 1981; Hakkinen and Schneitz, 1996), *Clostridium perfringens* (La Ragione and Woodward, 2003) and *C. jejuni* (Soerjadi-Liem et al., 1984; Stern et al., 2001a). Even though probiotic strains reduced *Campylobacter in vitro*, most of them failed to demonstrate similar

efficacy against *Campylobacter in vivo* (Santini et al., 2010; Robyn et al., 2012). One possible reason for such variability in *in vivo* studies may be due to failure of probiotics to survive the acidic pH of the upper gastrointestinal tract (Ding and Shah, 2009). Recent studies from our laboratory demonstrated that protecting the probiotic isolates from stomach acids by making them available in the lower intestinal tract via intercloacal transfer significantly reduced *C. jejuni* colonization in broiler chickens (Arsi et al., 2015).

Several investigations on probiotics against *Campylobacter jejuni* colonization emphasized the need to develop effective probiotics through better screening methods and/or by using effective methods of probiotic administration. It has been found that mucin (mucus glycoprotein) acts as a chemoattractant to *C. jejuni* and provides a source of carbon and energy for the growth of *Campylobacter* (Berry et al., 1988; Hugdahl et al., 1988). Research findings also suggest the affinity of *Campylobacter spp.* towards mucin as an essential factor for both colonization and infection (Slomiany et al., 1987; Sylvester et al., 1996). We hypothesized that probiotic bacteria with affinity towards mucin may competitively inhibit *Campylobacter* at the preferred sites of colonization. Thus, selecting bacterial isolates with affinity to utilize and grow in the presence of mucin could be an effective strategy to reduce *Campylobacter jejuni* in poultry.

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Chapter 2

The Ability of Select Probiotics to Reduce Enteric *Campylobacter* Colonization in Broiler Chickens.

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The Ability of Select Probiotics to Reduce Enteric *Campylobacter* Colonization in Broiler Chickens.

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ABSTRACT

Campylobacter is the leading cause of foodborne illness worldwide and is often associated with consumption and/or mishandling of contaminated poultry products. Probiotic use in poultry has been an effective strategy in reducing other enteric foodborne pathogens but not consistently for *Campylobacter*. As *Campylobacter* resides and utilizes intestinal mucin for growth, isolates selected on the basis of mucin utilization might be a strategy to screen for efficacious probiotic bacterium. In this study, bacterial isolates demonstrating increased growth rates in mucin, *in vitro* (trials 1 or 2), or isolates demonstrating a reduction of *Campylobacter* counts when co-incubated with mucin, *in vitro* (trials 3 or 4) were selected for their ability to reduce *Campylobacter* colonization in four bird trials. In trials 1 or 2, ninety day-of-hatch chicks were randomly divided into 9 treatment groups (n=10 chicks/treatment) and treated individually with one of four bacterial isolates (*Bacillus* sp.) demonstrating increased growth in media containing mucin. The treatments included a positive *Campylobacter* control (no isolate) or four isolates grown in media with or without mucin prior to inoculation. In trials 3 or 4, sixty day-of-hatch chicks were divided into six treatment groups (n=10 chicks/treatment) receiving either no isolate (positive *Campylobacter* control) or dosed with five individual isolates all demonstrating

the ability to reduce *Campylobacter* counts when co-incubated with mucin, *in vitro*. These isolates were grown in media containing mucin prior to inoculation. In all four trials, birds were gavaged with individual isolates at day-of-hatch and orally challenged with a four strain mixture *C. jejuni* on day 7. Ceca were collected at day 14 for *Campylobacter* enumeration. Results from these first two trials demonstrated two individual isolates, one with increased growth rates when grown in mucin or one isolate incubated without mucin, consistently reduced cecal *Campylobacter* counts (1.5 to 4 log reduction) when compared with controls. In follow-up trials with isolates selected for their ability to directly reduce *Campylobacter* counts when co-incubated with mucin, *in vitro*, one isolate consistently reduced cecal *Campylobacter* counts by approximately 1.5 logs. These results support the potential use of mucin to preselect isolates for their ability to reduce enteric *Campylobacter* colonization.

Key words: *Campylobacter*; Probiotic; Chicken, mucin

INTRODUCTION

Campylobacter infections are one of the leading causes of bacterial gastroenteritis in humans worldwide (WHO, 2011; CDC, 2013). In the United States alone, 1.3 million cases of human *Campylobacter* infections have been reported annually (CDC, 2013). More than 17 *Campylobacter* spp. has been identified (Lastovica, 2006; Debruyne et al., 2008), of which, *Campylobacter jejuni* alone is responsible for approximately 95-99% of cases of human campylobacteriosis (Friedman, 2000; Park, 2002; Snelling et al., 2005). Most of the *Campylobacter* enteritis cases are self-limiting (Allos and Blaser, 1995; Coker et al., 2002; Rosenquist et al., 2003), however, some severe post-infectious sequelae such as, Guillain-Barré syndrome and reactive arthritis have been reported (Rhodes and Tattersfield, 1982; Butzler, 2004; Pope et al., 2007; Ajene et al., 2013). Various sources of *Campylobacter* have been identified, among them poultry is regarded as the principal source of infection for humans (King, 1962; Skirrow, 1977; Rosenquist et al., 2003; CDC, 2013). It has been reported that more than 90% of the US poultry flocks are contaminated with *Campylobacter jejuni* (Stern et al., 2001b), which potentially present a serious threat for humans (Friedman et al., 2004; Mylius et al., 2007). Hence, reduction or elimination of *Campylobacter* in poultry flocks would significantly reduce the human incidence of campylobacteriosis (Rosenquist et al., 2003). Several preharvest intervention strategies such as biosecurity, bacteriocins, bacteriophages, plant extracts, vaccine, medium chain fatty acids, and probiotics have been evaluated aiming to reduce *Campylobacter* prevalence in poultry flocks (Widders et al., 1996; Gibbens et al., 2001; Loc Carrillo et al., 2005; Stern et al., 2006; Solis de los Santos et al., 2008a,b; Metcalf et al., 2011 Arsi et al., 2015a,b). Unfortunately, none of them are successful in completely eliminating *Campylobacter* from poultry (Hermans et al., 2011). Application of probiotic bacteria is one strategy that may

potentially inhibit/reduce *Campylobacter* colonization in poultry. Probiotics are “live microorganisms which when administered in adequate amounts can confer beneficial effects on host health” (Fuller, 1989). Probiotics effectively reduced food-borne pathogens such as, *Salmonella*, *E. coli*, *Listeria*, *Clostridium*, etc., (Soerjadi et al., 1981; Impey et al., 1982; Hakkinen and Schneitz, 1996; Hume et al., 1998a,b). However, administration of probiotics can produce inconsistent reductions in *Campylobacter* colonization in broiler chickens (Stern et al., 2001a ; Robyn et al., 2013; Arsi et al., 2015a). Such inconsistent results against *Campylobacter* colonization suggested the need of better screening methods of probiotic bacteria. It has been observed that supplementation of porcine intestinal mucin in broth media induces the cell surface proteins in *Lactobacillus reuteri* strains and improve the mucus-binding properties *in vitro* (Jonsson et al., 2001). Since *Campylobacter* colonizes in intestinal mucus and uses mucin as a source of carbon and energy (Lee et al., 1986; Beery et al., 1988; Hugdahl et al., 1988), selection of probiotic isolates which utilize intestinal mucin could be an effective approach to competitively inhibit the enteric colonization of *Campylobacter*.

The objective of this research was to screen probiotic isolates that can eliminate/reduce cecal *Campylobacter* counts in poultry. In this study we used selected bacterial isolates that are generally regarded as safe (GRAS) and possess efficacy against *Campylobacter*, *in vitro*. These isolates were further screened for their ability to utilize mucin or inhibit *Campylobacter* in the presence of mucin. Isolates which demonstrated increased growth or anti-*Campylobacter* activity *in vitro*, in the presence of mucin, were selected and tested *in vivo*.

MATERIALS AND METHODS

Probiotic isolates

In this study, we used selected GRAS bacterial isolates (*Bacillus* and *Lactobacillus* spp.) with efficacy against *Campylobacter*, *in vitro*, using a soft agar overlay technique. The selected bacteria were isolated and identified from the cecal contents of healthy birds during earlier studies from our laboratory (Arsi et al., 2015a,b).

In vitro studies

Screening of mucin utilizing probiotic bacteria. Sixty-eight isolates were screened for increased growth in the presence of mucin. The procedure involved growing selected bacterial isolates separately in Tryptic Soy Broth (TSB, BBL® Becton Dickinson and Company, MD) and in TSB supplemented with 3% porcine gastric mucin (Sigma-aldrich, St. Louis, MO). The isolates were incubated aerobically at 37°C for 24 h. The cultures were then serially diluted with Butterfield's Buffered Phosphate Diluent (BPD, Difco™ Becton Dickinson and company, Sparks, MD) and plated on Tryptic Soy Agar (TSA, Difco™ Becton Dickinson and company, MD) for enumeration of each bacterial isolate. The four isolates which demonstrated greatest increase in counts in the presence of mucin were selected and evaluated *in vivo*.

Screening for bacteria with the ability to reduce Campylobacter counts when co-incubated with mucin. Each bacterial isolate was co-cultured with four-strain mixture of wild type *C. jejuni* in 5 mL of TSB (no mucin) and 5 mL of TSB containing 3% porcine gastric mucin separately. The tubes were incubated microaerophilically at 42°C for 24 h. Each co-culture was then serially diluted in BPD and plated on Campy Line Agar (Line, 2001) for enumeration. The *Campylobacter* colonies were enumerated and each isolate was evaluated for its efficacy to reduce *Campylobacter* when co-cultured in the presence or absence of mucin in the growth

media. The five isolates which demonstrated the greatest reduction of *Campylobacter* counts, *in vitro*, in the presence of mucin compared to non mucin media, were selected and further evaluated *in vivo*.

In vivo studies

Experimental animals and housing. For all *in vivo* trials, day of hatch broiler male chicks were procured from a local commercial hatchery. Chicks were weighed at the beginning and at the end of each trial. Birds were raised in floor pens with pine shavings, with *ad libitum* access to feed and water throughout the 14-day trial period.

Experimental design. A total of 4 bird trials were conducted at the poultry farm facility of University of Arkansas. Four probiotic isolates which had shown higher growth in the presence of mucin in the broth media were selected for *in vivo* studies. Two replicate trials were conducted (trials 1 and 2) and in each trial, a total of 90 male chicks were randomly divided into 9 treatment groups (n=10 chicks/treatment). The treatment groups include a *Campylobacter* control (*Campylobacter*, no isolate) and 8 treatment groups each receiving a separate bacterial isolate grown in the presence or absence of mucin prior to oral administration.

For trials 3 and 4, we selected isolates that reduced *Campylobacter in vitro*, in the presence of mucin instead of selection for increased growth in the presence of mucin. Five isolates which inhibited *Campylobacter in vitro*, in the presence of mucin in the broth media were selected and tested in replicate trials 3 and 4. In each trial, 60 male chicks were randomly divided into 6 treatment groups (n=10 chicks/ treatment) and treatment groups include control (*Campylobacter*, no isolate) and 5 treatment groups each receiving a separate isolate grown in the presence of mucin prior to oral.

Bacterial dosing in chicks. In each trial, at day of hatch, chicks from all the treatment groups except *Campylobacter* control were orally gavaged individually with 0.25 mL of specific probiotic isolate containing approximately 10^6 - 10^8 CFU/mL as previously described (Arsi et al., 2015a). On day 7, all the chicks were orally gavaged with a cocktail of 4 strains of wild type *Campylobacter* containing approximately 10^8 CFU/mL organisms as previously described (Farnell et al., 2005). On day 14, bird's ceca were aseptically collected 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 h and *Campylobacter* colonies were enumerated and expressed as CFU/g.

Statistical analysis

To achieve homogeneity of variance, cecal *Campylobacter jejuni* counts were logarithmically transformed (Log CFU/mL) before analysis of data (Byrd et al., 2003). Data were analyzed by using the PROC GLM procedure of SAS (SAS, 2011). Treatment means were partitioned by least square means (LSMEANS) analysis and a probability of $P < 0.05$ was required for statistical significance.

RESULTS

A total of 68 GRAS isolates were tested *in vitro* in this study and the four isolates (called isolate 1, 2, 3 and 4) which showed a greatest increase in counts when growth in mucin-supplemented media compared with the unsupplemented media (data not shown) were selected for the *in vivo* studies in trials 1 and 2 (Table 1). In addition, the five isolates demonstrating the largest reduction in *Campylobacter* counts when incubated with mucin (data not shown) were selected for the *in vivo* studies in trials 3 and 4 (Table 2).

In trial 1, isolate 1 or 4 grown without mucin prior to inoculation reduced cecal *Campylobacter* counts (approximately 2-3 logs CFU/g) whereas isolates 2, 3 or 4 incubated with mucin prior to inoculation reduced *Campylobacter* counts (approximately 2-3 logs CFU/g; Table 1) when compared with the controls. In trial 2, isolates 1, 2 or 3 grown without mucin reduced *Campylobacter* counts by approximately 1.5 to 4 logs CFU/g in the ceca whereas only isolate 4 incubated with mucin reduced *Campylobacter* counts (Table 1) compared to controls. When compared across trials, isolate 1 grown without mucin or isolate 4 incubated with mucin consistently reduced *Campylobacter* counts in two separate trials (Table 1). When isolates were selected based on their ability to reduce *Campylobacter* counts when co-incubated with mucin *in vitro*, isolates 5, 7 or 8 or isolates 5 or 6 reduced *Campylobacter* counts in chicks when compared with controls for trials 3 or 4, respectively (Table 2). None of these isolates adversely affected body weight gains at 14 days of age when compared with controls (Tables 3 and 4).

DISCUSSION

Campylobacter is a flagellated, highly motile, microaerophilic bacterium able to colonize heavily in cecal crypt mucus (Beery et al., 1988; Hugdahl et al., 1988). One theory of why probiotics are ineffective against enteric *Campylobacter* colonization is because *Campylobacters* are sequestered in the intestinal mucus laden cyrpts and the probiotic bacteria are not able to penetrate and inhibit their colonization in these locations (Aguiar et al., 2013). In an effort to overcome this potential issue, four bacterial isolates demonstrating the ability to inhibit *Campylobacter* growth and which grew better in mucin, *in vitro*, were evaluated against *Campylobacter* colonization in chickens. These isolates were also grown in mucin media prior to inoculation to determine if this would enhance efficacy, possibly due to changes in gene expression associated with mucin co-incubation (Naughton et al., 2014). In the first bird trial,

two out of four isolates grown without mucin prior to inoculation reduced cecal *Campylobacter* counts (approximately 2-3 logs CFU/g) whereas three out of four of these isolates incubated with mucin prior to inoculation reduced *Campylobacter* counts (approximately 2-3 logs CFU/g; Table 1). In trial 2, many of these isolates also reduced *Campylobacter* counts by approximately 1.5 to 4 logs CFU/g in the ceca. When compared across trials, two isolates consistently reduced *Campylobacter* counts in two separate trials (Table 1). Isolate 4 was more efficacious when grown in mucin prior to inoculation with an approximate 1.5 to 2.5 log reduction in *Campylobacter* counts whereas isolate 1 produced a greater reduction when not incubated with mucin prior to inoculation with an approximate 2-4 log reduction in *Campylobacter* counts. None of these isolates adversely affected body weight gains at 14 days of age when compared with controls (Tables 3 and 4).

In an effort to select isolates with even greater efficacy, follow-up trials were conducted selecting isolates with the ability to directly reduce *Campylobacter* counts when co-incubated with mucin, *in vitro*. The five most efficacious isolates, *in vitro*, were evaluated in two separate bird trials. In these trials, one isolate consistently reduced cecal *Campylobacter* counts in two separate trials (Table 2) by approximately 1.5 CFU/g in the ceca. Results from these trials support the preselection of probiotic isolates with the ability for increased growth rates in the presence of mucin or the ability of isolates to inhibit *Campylobacter* counts when co-incubated with *Campylobacter*, *in vitro*. It is unclear if incubating these preselected isolates in the presence mucin prior to inoculation enhances their efficacy against *Campylobacter* in poultry.

Although cecal *Campylobacter* counts were consistently reduced by three isolates in the current study, these isolates were not able to eliminate *Campylobacter* colonization in chickens. It is unknown why these isolates are effective in liquid culture but do not eliminate

Campylobacter colonization in chickens. Although the precise mechanism by which the probiotic bacteria produce beneficial effects in host is not fully established, it has been proposed that probiotic bacteria exert beneficial effect by producing bacteriocins (Meghrouh et al., 1990), organic acids (Sanders, 1993), by competing for substrates or attachment sites (Fooks and Gibson, 2002), or by increasing macrophage mediated phagocytosis (Hatcher and Lambrecht, 1993). Other mechanisms by which they produce beneficial effects include production of volatile fatty acids and antimicrobial substances (Fuller, 1991).

Probiotics also have to survive in proventriculus or gizzard at low pH condition (2.5-3.5), in bile salt in the small intestine during their transit (Ding and Shah, 2009) and be able to colonize in the ceca (Santini et al., 2010). Probiotics bacteria should be present at more than 10^7 CFU/g or mL of products to elicit beneficial effects such as stimulation of the immune system and enteric bacterial enzyme activities (Ouweland and Salminen, 1998). Ding and Shah (2009) suggested that most of the probiotic bacteria, even acid tolerant *Lactobacillus* spp. are susceptible to low pH (pH 2) and show poor viability during their gastrointestinal passage. Previous research conducted in our laboratory (Arsi et al., 2015b) demonstrates that probiotic isolates can maintain their efficacy when administered directly into the lower intestinal tract, bypassing these condition in the upper tract. The gastrointestinal tract also contains a large, dynamic and complex microflora (Zhu et al., 2002), which makes the gut an extremely competitive environment. The interaction between the various types of bacteria in gut lumen is complex (Berg, 1996) and these interactions may also inhibit or reduce the efficacy of probiotic isolates within the GI tract. Thus, the preselected bacterial isolates administered in the current study may not have been able to completely eliminate *Campylobacter* colonization in chickens possibly due to a reduction in the number of isolates reaching or penetrating the cecal crypts

containing *Campylobacter*. Even though these isolates did not eliminate *Campylobacter* colonization, they did reduce *Campylobacter* counts by 1.5 to 4 logs. Risk assessment studies conducted by Rosenquist and his colleagues (2003) predicted that a 2 log reduction of the *Campylobacter* on chicken carcasses can reduce the human incidence by 30 times. Therefore bacterial isolates demonstrating the reduction in counts produced in the current study could significantly reduce the incidence of this disease in humans.

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Table 1: The effect of selected bacterial isolates on *cecal Campylobacter* counts (log CFU/g of cecal contents) in 14-day old broiler chicks (Mean \pm SEM)¹.

Treatments	Log CFU/g (Mean \pm SE)	
	Trial 1	Trial 2
<i>Campylobacter</i> positive control	7.95 \pm 0.23 ^a	9.19 \pm 0.15 ^a
Isolate 1	5.61 \pm 0.93 ^{bcd}	4.98 \pm 0.81 ^d
Isolate 2	7.11 \pm 0.33 ^{ab}	6.94 \pm 0.54 ^c
Isolate 3	5.79 \pm 0.95 ^{abcd}	7.78 \pm 0.40 ^{bc}
Isolate 4	4.55 \pm 1.16 ^{cd}	8.56 \pm 0.14 ^{ab}
Isolate 1 incubated with mucin ²	6.80 \pm 0.85 ^{abc}	8.37 \pm 0.23 ^{ab}
Isolate 2 incubated with mucin ²	5.62 \pm 0.95 ^{bcd}	8.36 \pm 0.27 ^{ab}
Isolate 3 incubated with mucin ²	4.47 \pm 1.08 ^d	9.11 \pm 0.20 ^{ab}
Isolate 4 incubated with mucin ²	5.28 \pm 0.43 ^{bcd}	7.89 \pm 0.24 ^{bc}

^{a,b,c,d} Means within columns with no common superscript differ significantly (P < 0.05).

¹Chicks were orally challenged on day 7 with 0.25 mL of approximately 1 x 10⁸ CFU/mL of a 4 strain mixture of wild type *Campylobacter jejuni* (n=10/treatment group) in each trial.

²These isolates were incubated with mucin prior to oral challenge in chicks

All *Campylobacter* data were log₁₀ transformed for statistical analysis.

Table 2: The effect of selected bacterial isolates on cecal *Campylobacter* counts (log CFU/g of cecal contents) in 14-day old broiler chicks (Mean \pm SEM)¹.

Treatments	Log CFU/g (Mean \pm SE)	
	Trial 3	Trial 4
<i>Campylobacter</i> positive control	8.16 \pm 0.24 ^a	7.91 \pm 0.16 ^a
Isolate 5 co-incubated with mucin ²	6.93 \pm 0.42 ^c	6.38 \pm 0.32 ^c
Isolate 6 co-incubated with mucin ²	8.06 \pm 0.16 ^a	6.79 \pm 0.22 ^{bc}
Isolate 7 co-incubated with mucin ²	7.27 \pm 0.26 ^{bc}	7.89 \pm 0.15 ^a
Isolate 8 co-incubated with mucin ²	7.24 \pm 0.15 ^{bc}	7.4 \pm 0.22 ^{ab}
Isolate 9 co-incubated with mucin ²	7.70 \pm 0.15 ^{ab}	7.31 \pm 0.26 ^{ab}

^{a,b,c} Means within columns with no common superscript differ significantly (P < 0.05).

¹Chicks were orally challenged on day 7 with 0.25 mL of approximately 1 x 10⁸ CFU/mL of a 4 strain mixture of wild type *Campylobacter jejuni* (n=10/treatment group) in each trial

²These isolates were co-incubated with mucin in the *in vitro* test and incubated with mucin prior to oral challenge in chicks

All *Campylobacter* data were log₁₀ transformed for statistical analysis.

Table 3: The effect of selected bacterial isolates on body weight gain (grams) in 14-day old broiler chicks (Mean \pm SEM)¹.

Treatments	Body weight gain (g)	
	Trial 1	Trial 2
<i>Campylobacter</i> positive control	349.5 \pm 27.96 ^{abc}	199.4 \pm 14.55 ^d
Isolate 1	403.0 \pm 14.44 ^a	264.4 \pm 23.97 ^{bcd}
Isolate 2	349.5 \pm 30.53 ^{abc}	279.0 \pm 25.07 ^{bc}
Isolate 3	333.4 \pm 22.22 ^{abc}	283.6 \pm 24.89 ^{bc}
Isolate 4	346.3 \pm 26.27 ^{abc}	313.2 \pm 21.37 ^{ab}
Isolate 1 incubated with mucin ²	326.5 \pm 38.59 ^{bc}	240.8 \pm 22.76 ^{cd}
Isolate 2 incubated with mucin ²	376.8 \pm 24.75 ^{ab}	253.4 \pm 24.78 ^{ghcd}
Isolate 3 incubated with mucin ²	352.5 \pm 24.80 ^{abc}	196.6 \pm 31.11 ^d
Isolate 4 incubated with mucin ²	285.5 \pm 30.23 ^c	360.4 \pm 17.35 ^a

^{a,b,c,d} Means within columns with no common superscript differ significantly (P < 0.05).

¹A total of 90 birds were randomly divided into 9 treatment groups with 10 birds in each treatment per trial. Chicks were weighed at the beginning and at the end of the trial period (day 14). The average body weight gain in grams is shown in the table.

²These isolates were incubated with mucin prior to oral challenge in chicks

Table 4: The effect of selected bacterial isolates on body weight gain (grams) in 14-day old broiler chicks (Mean \pm SEM) during Trial 3 and 4.

Treatments	Body weight gain (g)	
	Trial 3	Trial 4
<i>Campylobacter</i> positive control	319.6 \pm 21.40 ^b	358.4 \pm 11.50 ^a
Isolate 5 co-incubated with mucin ²	369.9 \pm 10.52 ^a	361.1 \pm 14.26 ^a
Isolate 6 co-incubated with mucin ²	383.2 \pm 11.14 ^a	332.6 \pm 16.88 ^a
Isolate 7 co-incubated with mucin ²	369.7 \pm 13.08 ^a	347.2 \pm 13.76 ^a
Isolate 8 co-incubated with mucin ²	364.1 \pm 15.40 ^a	370.2 \pm 08.02 ^a
Isolate 9 co-incubated with mucin ²	358.1 \pm 15.90 ^a	359.4 \pm 15.29 ^a

^{a,b} Means within columns with no common superscript differ significantly (P < 0.05).

¹A total of 60 birds were randomly divided into 6 treatment groups with 10 birds in each treatment per trial. Chicks were weighed at the beginning and at the end of the trial period (day 14). The average body weight gain in grams is shown in the table.

²These isolates were co-incubated with mucin in the *in vitro* test and incubated with mucin prior to oral challenge in chicks



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: Dan Donoghue

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

DATE: 3/13/14

SUBJECT: IACUC APPROVAL
Expiration date: 3/12/17

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol 14030: "Testing the efficacy of probiotic cultures against *Campylobacter* colonization in chickens."

In granting its approval, the IACUC has approved only the protocol provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond 3/12/17 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 involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

CONCLUSION

In the later few decades, *Campylobacter* spp. has been identified as a leading cause of foodborne illness in the United States, and epidemiological evidence indicates that consumption and/or mishandling of contaminated poultry products is often associated with *Campylobacter* infection in humans. Probiotic use in poultry has been an effective strategy in reducing other enteric foodborne pathogens but not consistently for *Campylobacter*. As *Campylobacter* resides and utilizes intestinal mucin for growth, isolates selected on the basis of mucin utilization might be a strategy to screen for efficacious probiotic bacterium. In this study, bacterial isolates demonstrating increased growth rates or anti-*Campylobacter* property in the presence of mucin in broth were tested in a total of four bird trials. In both trials 1 and 2, ninety day-of-hatch chicks were randomly divided into 9 treatment groups (n=10/treatment) and treated individually with one of four bacterial isolates (*Bacillus* sp.) grown in media with or without mucin prior to inoculation or a *Campylobacter* positive control (no probiotics). In trials 3 and 4, sixty day-of-hatch chicks were divided into 6 treatment groups (n=10/treatment) and were dosed with five individual isolates (*Lactobacillus* sp.) all grown in mucin prior to inoculation or a *Campylobacter* positive control (no probiotic). All birds were gavaged with individual isolates at day-of-hatch and orally challenged with a four strain mixture *C. jejuni* on day 7. Ceca were collected at day 14 for *Campylobacter* enumeration. *Campylobacter* counts were logarithmically transformed (log₁₀ CFU/g) and treatment means were partitioned by LSMEANS analysis (P < 0.05). Results from these trials demonstrated three individual isolates grown in mucin prior to inoculation consistently reduced cecal *Campylobacter* counts (1.5-4 log reduction). These results support the potential use of preselection and growth of isolates in mucin in evaluating bacterial

isolates with the ability to reduce enteric *Campylobacter* colonization. Further research in probiotics is warranted to reduce/eliminate *cecal Campylobacter* from chicken.