The Efficacy of Probiotic Bacterial Isolates in Reducing Cecal Campylobacter Colonization in Broiler Chickens

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The Efficacy of Probiotic Bacterial Isolates in Reducing Cecal *Camylobacter* Colonization in Broiler Chickens
The Efficacy of Probiotic Bacterial Isolates in Reducing Cecal *Campylobacter* Colonization in Broiler Chickens

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

by

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ABSTRACT

_Campylobacter_ is the leading cause of foodborne illness worldwide and human illnesses are often associated with consumption of poultry or poultry products. Many strategies have been tried to eliminate _Campylobacter_ from poultry with limited success. One of the strategies to reduce _Campylobacter_ colonization in poultry is by use of probiotics. We conducted 2 separate studies to evaluate the efficacy of probiotics against _Campylobacter_ in broiler chickens. For our first study, GRAS (Generally Regarded as Safe) bacteria were isolated from healthy chickens and tested their efficacy against _Campylobacter in vitro_. Twenty six isolates with _in vitro_ anti- _Campylobacter_ activity were selected and tested in broiler chickens. Only 3 out of 26 isolates tested, demonstrated a 1-2 log reduction in _Campylobacter_ colonization. To further improve the _in vivo_ efficacy of these 3 isolates, these isolates were given along with 3 different doses of a prebiotic (fructoligosaccharide/FOS or mannanoligosaccharide/MOS). Of all the treatments tested, only one isolate when combined with 0.04% MOS showed a 3 log reduction in _Campylobacter_. However, the isolates which reduced _Campylobacter in vivo_ in our initial trials failed to reduce _Campylobacter_ in subsequent trials. One possible explanation for such inconsistencies could be due to destruction of probiotic isolates in the acidic environment of stomach. Encapsulation of isolates may overcome this problem, but there is no assurance that these isolates will have efficiency in the lower intestine. In the second study, a procedure to screen the _in vivo_ efficacy of candidate isolates was developed by directly inoculating isolates in the lower intestinal tract via the cloaca. For this study GRAS bacterial isolates with enhanced motility and _in vitro_ anti- _Campylobacter_ activity were selected and tested _in vivo_ by dosing the isolates either orally or intra-cloacally. When isolates were dosed orally, only one isolate showed a 1 log reduction in _Campylobacter_, but when these isolates were administered intra-cloacally,
five of these isolates produced a 1-3 log reduction in cecal *Campylobacter* counts. These results support the strategy of evaluating the efficacy of potential probiotic isolates via cloacal inoculation prior to undergoing the effort of protecting isolates (e.g., encapsulation) for oral administration.
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DEDICATION

This dissertation is dedicated to

My mother Ms. Vara Lakshmi Arsi

and

My father Mr. Krishna Rao Arsi
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INTRODUCTION

Foodborne illnesses are a growing public health problem worldwide (WHO, 2011). Gastrointestinal symptoms are the most common clinical manifestation of foodborne illness, caused by a wide range of microorganisms including bacteria, viruses and parasites (WHO, 2013b). It has been estimated that diarrheal diseases alone are responsible for 1.7 - 2.5 million deaths globally every year and a significant proportion of these deaths are due to consumption of contaminated food and/or water (WHO, 2009). It has been indicated that foodborne diseases are more common in developing and underdeveloped countries, and are often reported to be associated with poor sanitation and poor socio-economic conditions (WHO, 2012). However, foodborne illnesses are no longer limited to developing countries. Epidemiological evidence indicates that one in three persons in developed countries are affected by foodborne illnesses each year (WHO, 2013a). In United States alone, foodborne diseases result in 47.8 million illnesses, 127,839 hospitalizations and 3,037 deaths every year (CDC, 2013a). The economic loss due to foodborne illnesses in the US was estimated to be about $77.7 billion (Scharff, 2012). Epidemiological data indicate that Campylobacter is one of the leading causes of foodborne illness in the United States after Salmonella (CDC, 2013b). Campylobacter has been reported to cause 400 million illnesses annually all over the world (Rollwagen, et al., 1993). In the United States alone, Campylobacter illness was estimated to cost $1.7 billion annually (Batz, et al., 2012; Hoffmann, et al., 2012). In Canada, Campylobacter was found to be the causative agent for 59% of waterborne or foodborne enteritis cases being reported (Hannu et al., 2002). Campylobacter has been reported to be the most common cause of foodborne illness in the European Union (EU) also, and is estimated to cause around 9 million foodborne illnesses.
resulting in economic losses of €2.4 billion a year (EFSA, 2013a). In Australia 5.4 million cases of foodborne illnesses were reported each year causing economic losses up to A$1.2 billion (AGDHA, 2006), and in New Zealand the losses were estimated to be around NZ$161.9 million, of which *Campylobacter* alone causes losses up to NZ$36 million (NZFSA, 2010).
Chapter 1

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LITERATURE REVIEW

1.1 HISTORY

_Campylobacter_ was first described by Theodor Escherich in 1886. Escherich (1886) observed spiral shaped non-culturable bacteria from the colon of children who died of cholera infantum. In 1906, two British veterinarians, McFadyean and Stockman isolated spiral shaped organisms from aborting ewes during their investigation of epizootic abortions in animals in the United Kingdom (Véron and Chatelain, 1973). Later Smith (1919) isolated similar organisms from aborted bovine fetuses. Smith along with Taylor (1919) speculated the organisms reported by McFadyean’s group and Smith to be the same and proposed the name _Vibrio fetus_ for the microaerophilic organism responsible for abortions in cattle and sheep. Jones and colleagues (1931) demonstrated curved vibrio-like bacteria from the jejunum of calves with winter dysentery and proposed the name _Vibrio jejuni_. A few years later, Doyle (1944) isolated similar organisms from the intestine of swine with dysentery and named the organism _Vibrio coli_. King first studied the strains of _Vibrio_ causing diarrhea in humans and reported an unusually high optimal growth temperature requirement of some of the strains (King, 1957, 1962). She referred to these strains as related _Vibrios_, which were later renamed as _C. jejuni_ and _C. coli_ (Véron and Chatelain, 1973). Dekeyser and his colleagues successfully isolated _Campylobacter_ from stool samples (Dekeyser, et al., 1972). Sebald and Véron separated _Campylobacters_ from _Vibrio_ and proposed the new genus _Campylobacter_ with _Campylobacter fetus_ as the type species (Sebald and Véron, 1963). Véron and Chatelain studied the previously reported microaerophilic _Vibrios_ and reclassified _V. fetus, V. coli, V. jejuni, V. sputorum sub spssputorum_ and _V. sputorum sub sps_
prior to 1977, many scientists attempted to isolate Campylobacter using filtration techniques, but the development of selective media for Campylobacter (Skirrow, 1977) marked the beginning of the “era of Campylobacter research”.

1.2 GENERAL CHARACTERISTICS

1.2.1 Genus Campylobacter

The word Campylobacter is derived from a Greek word “kampulos” meaning curved and “bacter” meaning rod (Sebald and Véron, 1963). The genus Campylobacter was classified under the family Campylobacteraceae and other members of the family include Arcobacter, Sulfurospirillum and Bacteroides ureolyticus (Debruyne, et al., 2008). Currently there are 17 recognized species under the genus Campylobacter and C. jejuni has been recognized as the predominant species responsible for majority of human enterocolitis infection (Butzler, et al., 1973; Skirrow, 1977; Phillips, 1995; Debruyne, et al., 2008). Approximately 5-10% of human infections may be caused by C. coli and rarely other species such as C. lari and C. upsaliensis can cause similar disease in some people (EFSA, 2013b).

1.2.2 Morphological and growth characteristics of the genus Campylobacter

Members of the genus Campylobacter are Gram negative curved rods measuring 0.5-5 μm long and 0.2-0.8 μm wide (Smibert, 1978; Debruyne, et al., 2008). The organisms are motile by means of a single polar flagellum at one or both ends (fig 1, Pead, 1979) and a show a characteristic corkscrew motility (Smibert, 1978). Some exceptions have been reported, such as multiple flagella in C. showae and no flagella in species like C. gracilis (Percival, et al., 2004).
Optimal growth was observed at 42°C under microaerophilic conditions, with an atmosphere containing 5% oxygen, 10% carbon dioxide, 85% nitrogen (King, 1957, 1962). *Campylobacters* are known for their fastidious growth requirements and are believed to be sensitive to environmental stressors, including changes in pH, temperature and exposure to high oxygen concentration (Butzler and Oosterom, 1991; Park, 2002). Contrary to the fastidious nature of the organism in the laboratory, *Campylobacters* can survive in water, as well as a wide range of food stuffs including meat, milk and other dairy products (Ziprin, 2004). Some researchers have proposed that *Campylobacter* can resist environmental stressors and survive as a viable but non-culturable form (VBNC, Cappelier, et al., 1999; Höller, et al., 1998; Rollins and Colwell, 1986; Tholozan, et al., 1999). Rollins and Colwell (1986) first described the ability of *Campylobacter* to transform into a coccoid viable, but non-culturable state when exposed to environmental stress, such as fluctuations in temperature, pH, high oxygen concentration and starvation. However contradictory opinions were expressed about the ability of VBNC forms to become metabolically active and produce disease on exposure to favorable conditions (Cappelier, et al., 1999; Park, 2002; Thomas, et al., 2002; Ziprin, et al., 2003; Ziprin and Harvey, 2004; Baffone, et al., 2006).

### 1.3 *CAMPYLOBACTER INFECTIONS IN HUMANS*

#### 1.3.1 Incidence and epidemiology of *Campylobacter*

During the 1970’s *Campylobacter* emerged as a frequent cause of diarrheal illness in Europe and North America (Skirrow, 1982). *Campylobacter* infections are the most frequently occurring foodborne gastroenteritis in the world (WHO, 2011). It has been estimated that
Campylobacter infections are 2-7 times more frequent than Salmonella or Shigella infections (Allos, 2001). It is often underreported even in developed countries like the United States. A recent report shows that only one case out of every 38 C. jejuni infections has been reported (CDC, 2013b). As per the CDC estimates, approximately 14.3 cases are reported per 100,000 persons annually in the United States (CDC, 2013b). Reported incidences are much higher during warmer months, especially during summer and fall (Allos, 2001). Based on epidemiological data, the average incidence of Campylobacter in the European Union is estimated to be 50.28/100,000 population (EFSA, 2013b). Surveillance data representing the incidence of campylobacteriosis per 100,000 persons from various countries has been shown in Table 1. The number of cases reported from developing countries are often the number of laboratory confirmed cases (Coker, et al., 2002). The true incidence in such countries could be many fold more than what is being reported (Coker, et al., 2002). A very high incidence of 40,000-60,000 cases per 100,000 population has been reported in children below 5 years in developing countries (Olson, et al., 2008). Such a high prevalence of Campylobacter in developing countries pose increased risk to people travelling to such countries (Skirrow, 1990).

1.3.2 Sources of infection

King first reported the possible role of handling and consumption of poultry as a major source of Campylobacter infections in humans (King, 1957, 1962). This was further supported by many case control studies conducted in different parts of the world (Skirrow, 1982; Kapperud, et al., 2003; Stafford, et al., 2008; Danis, et al., 2009). Other sources of infection for humans include unpasteurized milk (Blaser, et al., 1979b; Robinson, et al., 1979; Robinson and Jones, 1981), water (Skirrow, 1982), fresh vegetables and fruits (Evans, et al., 2003; Danis, et al., 2009;
Verhoeff-Bakkenes, et al., 2011). Contact with domestic animals and pets has also been demonstrated as a risk factor for human infections (Kapperud, et al., 2003; Stafford, et al., 2008; Danis, et al., 2009). Poor hygiene and improper handling of food in domestic kitchens may cross contaminate fresh foods and thus may also act as sources for human infections (Danis, et al., 2009). Even though *Campylobacter* is prevalent in other food animals, retail pork and other red meats are less frequently contaminated with *Campylobacter* (Butzler and Oosterom, 1991; Evans, et al., 2003; Kapperud, et al., 2003; Zhao, et al., 2001; Danis, et al., 2009). The high prevalence of *Campylobacter* in retail poultry can be attributed to high levels in ceca and the carcass handling in conventional processing plants (Butzler and Oosterom, 1991).

1.3.3 Symptoms and sequaele

*Campylobacter* is one of the most common causes of foodborne enteritis in both developing and developed countries. Many species of *Campylobacter* can cause illness in humans, but the majority of human infections (80-90%) were caused by *C. jejuni* (Butzler, et al., 1973; Skirrow, 1977; Phillips, 1995; Debruyne, et al., 2008). *C. jejuni* predominantly causes a self-limiting diarrheal illness in humans (Coker, et al., 2002). A dose as low as 500 organisms can infect, colonize and produce disease symptoms in humans (Robinson, et al., 1979; Robinson and Jones, 1981). Usually, the incubation period is between 2-11 days (Skirrow, 1977).

Symptoms may range from mild watery diarrhea to severe mucoid bloody diarrhea (Wassenaar and Blaser, 1999). Other symptoms associated with campylobacteriosis are abdominal pain, severe malaise, fever, headache, anorexia, nausea, vomiting and myalgia (Blaser, et al., 1979a). The affected patients may sometimes require supportive treatment, and in most cases affected patients recover without any antibiotic therapy (Peterson, 1994). However antibiotic therapy is
recommended in cases of extra-intestinal infections and in immunocompromised people (Coker, et al., 2002). It has been reported that *Campylobacter* may sometimes result in post infectious complications such as, Guillian Barré syndrome and Reactive arthritis (Peterson, 1994; Moore, et al., 2005).

1.3.3.1 Guillain-Barré syndrome

Guillain, Barré and Strohl first described this neurological syndrome in two French soldiers exhibiting neurological signs (Guillain, et al., 1916). Guillain-Barré syndrome (GBS) is an autoimmune disorder, affecting the peripheral neurons, causing flaccid paralysis (Allos, 1997; Nachamkin, et al., 1998; Willison, 2005). It has been described as a self-limiting disorder with a recovery period of several weeks to months (Nachamkin, et al, 1998). However, 20% of the affected people may develop respiratory paralysis and may require mechanical ventilation (Nachamkin, et al, 1998; Kuwabara, 2004). It has been estimated that the annual global incidence of Guillain-Barré syndrome is 1-4 cases/100,000 population (Hughes, et al., 1999; Kuwabara, 2004). Case control studies have demonstrated that 1/1000 *C. jejuni* cases may develop post infectious complications like Guillain-Barré syndrome (Mishu, et al., 1993). A preceding *Campylobacter* infection has been identified as one of the most frequent cause of Guillain-Barré syndrome (Kaldor and Speed, 1984; Rees, et al., 1995; Koga, et al., 1998). Apart from *Campylobacter*, preceding infections with other agents such as *Mycoplasma pneumonia*, Cytomegalo virus, Epstein-barr virus, Varicella-zoster virus and vaccinations against swine influenza and rabies may predispose to Guillain-Barré syndrome (Nachamkin, 2002; Hughes and Cornblath, 2005).
Several clinical forms of Guillain-Barré syndrome have been recognized such as Acute Inflammatory Demyelinating Polyradiculoneuropathy (AIDP), Acute Motor-Axonal Neuropathy (AMAN), Acute Motor-Sensory Axonal Neuropathy (AMSAN) and Miller Fisher syndrome (Hughes, et al., 1999). *C. jejuni* infections are more commonly associated with the AMAN form of Guillain-Barré syndrome (Kuwabara, 2004; Komagamine and Yuki, 2006). It has been proposed that the molecular mimicry between lipooligosaccharides of *C. jejuni* and host gangliosides may result in production of auto antibodies and thus, play a role in pathogenesis of Guillain-Barré syndrome (Yuki, et al., 2004; Komagamine and Yuki, 2006). Researchers demonstrated that some strains of *C. jejuni* such as penner serotype HS: 19 are frequently associated with Guillain-Barré syndrome (Nachamkin, et al., 2002; Takahashi, et al., 2005). Also, the incidence was higher in males compared to females (Takahashi, et al., 2005).

*C. jejuni* may also cause Miller Fisher syndrome, a variant of Guillain-Barré syndrome, which is characterized by ataxia, loss of tendon reflexes (areflexia), ophthalmoplegia and presence of anti GQ_{1b} antibodies in serum (Hughes, et al., 1999).

### 1.3.3.2 Reactive arthritis

Up to 16% of *Campylobacter* infections may result in post infectious sequelae like Reactive arthritis in humans (Hannu, et al., 2002; Pope, et al., 2007). Reactive arthritis, also referred as post infectious arthritis or Reiter’s syndrome, is characterized by inflammation of joints and tissues (Townes, 2010). Preceding enteric or urogenital infections caused by pathogens such as *Campylobacter, Salmonella, Shigella, Yersinia* and *Chlamydia* may serve as
risk factors for Reactive arthritis (Townes, 2010). Incidence was higher in adults than in children and also in the presence of Human leukocyte antigen, HLA-B27 (Pope, et al., 2007).

1.3.4 Factors affecting *Campylobacter* infections in humans

Factors such as age, sex, seasonality and immune status may influence the incidence of campylobacteriosis in humans.

i. **Age:** *Campylobacter* infections are more common in children (below 5 years) and in young adults (Butzler, 2004; Friedman and Neimann, 2000). The high incidence in young adults (15-44 years age), is commonly seen in developed countries, and could be due to food habits and frequent travel associated with this age group (Olson, et al., 2008). However, in developing countries, the incidence is much higher in children below 1 year and adults are relatively resistant to *Campylobacter* infections (Allos, 2001; Lee and Newell, 2006).

ii. **Sex:** For reasons unknown, males were 1.2-1.5 times more susceptible than females (Koehler, et al., 2006; Olson, et al., 2008).

iii. **Season:** Sporadic outbreaks of campylobacteriosis were common during warmer months, mainly during summer and early fall (Skirrow, 1987; Skirrow, 1990; Olson, et al., 2008; Senok and Botta, 2009; Jore, et al., 2010). Seasonal influence on *Campylobacter* infections is more pronounced in temperate regions compared to developing countries in tropics (Allos, 2001; Nylen, et al., 2002).

iv. **Immune status:** Immunocompromised and HIV patients are 40-100 times more susceptible to *Campylobacter* infections (Wassenaar and Blaser, 1999; Butzler, 2004).
1.3.5 Mechanism of pathogenesis in humans

The mechanism by which Campylobacter causes disease symptoms may involve adhesion, colonization and invasion of intestinal epithelium. As explained by Hugdahl and group (1988), flagellar motility and chemo attraction of Campylobacter towards mucin components (L-fucose and L-serine) may guide the organisms towards the favorable niche for colonization. Adhesion of C. jejuni to the intestinal epithelium is mediated by factors such as flagellar adhesins, outer membrane proteins, fimbriae-like structures and lipooligosaccharide (Wassenaar and Blaser, 1999). Subsequent colonization and invasion are influenced by the virulence factors that are expressed by C. jejuni (van Vliet and Ketley, 2001). The role of some of the virulence factors in the pathogenesis of C. jejuni has been briefly explained below:

i. Flagella: Research has shown that a functional flagellum is essential for chemotaxis, adhesion and invasion in humans (Wassenaar and Blaser, 1999). Poor adhesion and invasion associated with aflagellar mutants further confirm the role of flagella in colonization and internalization (Yao, et al., 1993; Ziprin, et al., 1999).

ii. Outer membrane protein, Cad F: Cad F is one of the extensively studied Campylobacter adhesins (Krause-Gruszczynska, et al., 2007; Dasti, et al., 2010). It has been proposed to bind to the host fibronectin (also called fibronectin binding protein) and facilitate colonization (Ziprin, et al., 2001).

iii. Lipooligosaccharide (LOS): LOS is an essential component of outer membrane of Gram negative cell wall. It has been proposed to possess endotoxic activity and may also play a role in immune evasion (Wassenaar and Blaser, 1999; Young, et al., 2007). Also, molecular mimicry between lipooligosaccharide structures of C. jejuni and neuronal
gangliosides of the host can lead to autoimmune disorders such as Guillain-Barré syndrome and Miller Fisher syndrome (Yuki, et al., 2004; Komagamine and Yuki, 2006).

iv. **Capsule:** The polysaccharide capsule of *C. jejuni* may also play a role in immune evasion, adhesion and invasion of epithelial cells (Young, et al., 2007).

v. **Lipoprotein A (JlpA):** Jlp A is a surface exposed lipoprotein which mediates adhesion to intestinal epithelial cells (Butzler and Oosterom, 1991).

vi. **Periplasmic or membrane associated protein (PEB 1):** PEB 1 is an adhesin located in the periplasm and is believed to play a role in adhesion to host cells (van Vliet and Ketley, 2001; Young, et al., 2007).

vii. **Cytolethal distending toxin (Cdt):** Almost all strains of *C. jejuni* and *C. coli* possess cdt genes and secrete cytolethal distending toxin, which may cause cytotoxicity (van Vliet and Ketley, 2001). Cdt may also stimulate production of IL-8 in humans and thereby induce inflammation (Dasti et al., 2010). Cdt may also play a role in immune evasion and in inducing immune tolerance (Dasti, et al., 2010).

viii. **Fimbriae like structures:** Some researchers indicate that *C. jejuni* may express fimbriae-like appendages when grown in the presence of bile salts, which may also play a role in adhesion (van Vliet and Ketley, 2001)

Apart from the virulence factors described above, certain other factors such as major outer membrane proteins, P95 and *Campylobacter* invasive antigens (Cia) may also play a role in *Campylobacter* colonization and disease production (Konkel, et al., 2001). However, further research is needed to elucidate the exact mechanism by which *Campylobacter* produce disease in humans.
1.4 **CAMPYLOBACTER IN POULTRY**

1.4.1 Prevalence of *Campylobacter in poultry*

*Campylobacter* occurs as a commensal in the GI tract of poultry and is reported to be prevalent in 70-100% of poultry flocks (Peterson, 1994). A baseline study in Europe revealed that about 83% of poultry flocks and 98% of broiler carcasses were positive for *Campylobacter* (FSAI, 2011). Many case control studies demonstrated that a very high percentage of retail chicken (more than 70%) sold in Europe and North America have been contaminated with *Campylobacter* (Stern and Line, 1992; Zhao, et al., 2001). The high prevalence on retail poultry could be due to cross contamination during processing (Skirrow, 1990). The percent prevalence of *Campylobacter* in poultry flocks and on retail poultry products is shown in Tables 2 and 3 respectively. Suzuki and Yamamoto (2009) reviewed the literature on *Campylobacter* contamination in retail poultry meats and poultry products from all over the world and concluded that at least 50% of retail poultry sold in most countries has been contaminated with *Campylobacter*. Studies by Luangtongum and associates (2006) showed even higher risks associated with organic chicken and turkeys compared to conventionally raised birds.

*Campylobacter* has also been isolated from other birds such as pigeons, black birds, starlings and sparrows (Smibert, 1978; Skirrow, 1982). Even though *Campylobacter* is considered nonpathogenic to poultry, earlier reports suggest that *C. jejuni* may be the causative agent of vibrionic hepatitis (Dekeyser, et al., 1972; Shane, 1992). In case of Ostriches, *C. jejuni* and *C. coli* can cause Infectious Hepatitis, a disease similar to Vibrionic hepatitis and is characterized by brilliant green urates and severe necrotic hepatitis (Stephens, et al., 1998).
1.4.2 Colonization and transmission of *Campylobacter* in poultry

*Campylobacters* are reported to colonize the GI tract of poultry and domestic animals. It has been demonstrated that large numbers of *Campylobacter* (up to $10^9$CFU/mL) colonize in the ceca, especially in the cecal crypts in avian species (Beery, et al., 1988). Apart from the GI tract, they are also reported to colonize in some extra intestinal regions such as liver, spleen, gall bladder, thymus and reproductive tract (Newell and Fearnley, 2003; Cole, et al., 2004; Cox, et al., 2005).

1.4.2.1 Horizontal transmission

*Campylobacter* naturally colonizes the ceca of healthy chickens by 2-4 weeks of age (Shane 1992; Jacobs-Reitsma, et al., 1995; van Gerwe, et al., 2009). Initial protection from *C. jejuni* colonization, mainly during the first few weeks has been attributed to the presence of maternal antibodies (Shane 1992). Subsequently poultry may acquire these organisms from contaminated fomites, untreated water, rodents, flies and free living birds (Altekruse, et al., 1999; Corry and Atabay, 2001; Peterson, 1994). Once a single bird has been colonized, they shed the organisms in feces, contaminate the litter, feed, water and within a few weeks, entire flock will be colonized (Stern, et al., 2001a; Newell and Fearnley, 2003). Van Gerwe’s research group (2009) developed a mathematical model to quantify the rate of horizontal transmission in a broiler flock. According to this model for every colonized bird 2.37 new birds will get colonized per day.

1.4.2.2 Vertical transmission
Vertical transmission of *Campylobacter* from hen to chick is still controversial. Many researchers have demonstrated the presence of *Campylobacters* in various segments of male and female reproductive tracts of poultry (Camarda, et al., 2000; Cox, et al., 2002a, 2002b, 2005; Cole, et al., 2004).

Contradictory to earlier studies, some researchers studied the possibility of vertical transmission of *Campylobacter* in poultry and their research findings indicate that vertical transmission of *Campylobacter* is very unlikely (Callicott, et al., 2006; Shane, 1992). Further research needs to be done to strongly establish the possibility of vertical transmission in chickens.

1.4.3 USDA Regulations

President Obama’s Food Safety Working Group (FSWG), formed in 2009, made some recommendations to improve the U.S food safety system (USDA-FSIS, 2010). In an attempt to reduce the prevalence of *Salmonella* and *Campylobacter* in poultry products, the Food Safety Inspection Service (FSIS) developed the 3rd edition of compliance guide for the industry with recommendations for preharvest control of *Salmonella* and *Campylobacter* (USDA, 2010). FSIS also revised the performance standards for *Salmonella* and proposed the first ever performance standards for *Campylobacter* for chilled carcasses at young chicken and turkey slaughter establishments (USDA-FSIS, 2010). The new performance standards were announced on 10th May 2010, estimating that within 2 years of implementing the new standards, the number of illnesses due to *Salmonella* and *Campylobacter* could be reduced by 26,000 and 39,000 respectively every year (USDA-FSIS, 2010). The new standards were proposed based on the
information from the young chicken and turkey baseline studies (USDA-FSIS, 2008; USDA-FSIS, 2009). After careful analysis of the responses received during the 60 day comment period, starting July 2011, FSIS started implementing the new standards (USDA-FSIS, 2011). FSIS Inspection program personnel (IPP) will collect and analyze samples from processing facility as per the procedure described in the baseline surveys (USDA-FSIS, 2008; USDA-FSIS, 2009). For any chicken processing plant to pass the *Salmonella* standards, the number of positives should not exceed 5 in a 51 sample set. In case of turkey processing plants the number of *Salmonella* positives should not be more than 4 in 56 sample set. For *Campylobacter*, the IPP will collect 1mL samples for direct plating and 30 mL enriched samples, which will be plated to detect low levels of contamination (NACMCF, 2007). For chicken processing plants, 1mL samples were directly plated for both quantitative and qualitative purposes. The 1mL sample results can detect up to 1 CFU/mL, whereas 30 mL enriched sample will be plated only if the 1 mL samples fail to detect any *Campylobacter*. Results from 30 mL samples are indicative of low levels of contamination with detection limit as low as 0.03 CFU/mL. For any chicken processing plant to pass *Campylobacter* standards, a maximum of 8 positives out of a 51 sample set from 1mL results are allowed. For turkey processing plant, 1 mL and 24 mL samples will be collected as per the procedure described by the Food Safety Advisory Committee (NACMCF, 2007). For any turkey processing plant to fulfill the *Campylobacter* standards, a maximum of 3 positives from 56 sample set is allowed. Although performance standards are based on 1mL results, the larger portion of the samples are still collected as they serve as a measure for the industry performance. If any establishment fails to meet the performance standards in 3 consecutive tests, FSIS will suspend inspection services and that suspension will remain until the establishment takes corrective action and submits a written assurance of the actions being taken.
Once FSIS has finished testing 90% of eligible establishments for two sets, the names of the establishments that do not meet the standards will be posted (USDA-FSIS, 2010).

1.5CONTROL OF CAMPYLOBACTER IN POULTRY

1.5.1 Postharvest control strategies

Many epidemiological studies report a high prevalence of *Campylobacter* on broiler carcasses (up to 98%) throughout the world. Poultry processing involves steps like bleeding, scalding, defeathering, evisceration, washing and chilling. Each of these steps may be associated with changes in the prevalence of *Campylobacter* counts on broiler carcasses (Guerin, et al., 2010). In conventional processing plants, broilers carcasses are immersed in large chilling tanks, which may serve as a potential source of cross-contamination for broiler carcasses (Wempe, et al., 1983). It has been estimated that proper handling can reduce carcass contamination to <4 log CFU/ carcass (Lindqvist and Lindblad, 2008). Most studies demonstrate that processing reduces *Campylobacter* prevalence on broiler carcasses. However, carcasses harboring high numbers of *Campylobacter* before processing also showed higher numbers after chilling. It has been proposed that preharvest interventions may be more effective in reducing *Campylobacter* prevalence on broiler carcasses (Rosenquist, et al., 2003).

1.5.2 Preharvest control of *Campylobacter* in poultry

Since poultry are the predominant sources of human infections many research findings indicate that reduction of *Campylobacter* in the vehicle i.e., poultry would greatly reduce the risk of human infections (Allos, 2001). This was further supported by the findings of Rosenquist and
his colleagues (2003), who developed a risk model to study how different intervention strategies in poultry production and processing play a role in reducing the risk of human infections. The findings indicate that a 2 log reduction of \textit{Campylobacter} on chicken carcasses will result in reduction in human infections by 30 times (Rosenquist, et al., 2003). According to this risk model, attempts to reduce cross contamination at slaughter and during food handling in kitchens showed a limited effect on reducing human incidence (Rosenquist et al., 2003). This emphasizes the importance of reducing \textit{Campylobacter} in poultry and thereby reducing human incidences (Allos, 2001; Lin, 2009). Below are some of the preharvest control measures that may be used as potential strategies to reduce human infections.

1.5.2.1 Biosecurity

Research has shown that adopting biosecurity measures such as washing hands, wearing protective clothing, clean foot ware, cleaning and disinfection of houses, provision of clean water, restricting the movement of vectors and farm personnel will reduce the prevalence of many infections in livestock and poultry (Shane, 1992; Vandeplas, et al., 2008). Intervention studies done by researchers emphasize that implementation of strict on-farm biosecurity measures can potentially reduce the colonization of \textit{Campylobacter} in broiler flocks (Gibbens, et al., 2001; Katsma, et al., 2007). However, these measures can only reduce prevalence and do not guarantee complete elimination of \textit{Campylobacter} from poultry (van de Giessen, et al., 1998; Vandeplas, et al., 2008). Also, practical difficulties and costs associated with implementation of improved biosecurity measures further limits the application of this strategy to control \textit{Campylobacter} in broilers (van de Giessen, et al., 1998; Fraser, et al., 2010). A study conducted by Fraser and group (2010) indicated that implementation of biosecurity measures are strongly
influenced by the costs associated with adoption of such measures. Hermans and colleagues (2011c) reviewed various intervention strategies to control *Campylobacter*, and concluded that biosecurity measures can potentially reduce colonization, but do not eliminate *Campylobacter* from poultry.

### 1.5.2.2 Bacteriocins

Bacteriocins are antibacterial proteins or peptides produced by bacteria that can kill or inhibit the growth of other closely related bacteria (Cotter, et al., 2005; Florey, 1946; Florey et al., 1949; Jacob et al., 1953; Cleveland, et al., 2001; Joerger, 2003; Montville and Kaiser, 1993; Stern, et al., 2006). The first recorded evidence of bacterial antagonism was reported in 1877 (Pasteur and Joubert, 1877). Pasteur and Joubert (1877) reported that co-inoculating anthrax bacterium with some “common bacteria” can potentially reduce or inhibit the growth of anthrax bacillus. Later research suggested that the inhibition in the presence of “common bacteria” could be due to production of bacteriocins by these bacteria (Tagg, et al., 1976). Jacob and his colleagues (1953) first proposed the name bacteriocin to the antibacterial proteins of bacterial origin.

Earlier research on bacteriocins emphasized bacteriocins produced by gram negative bacteria (eg. colicins, Nisin etc.), especially the members of *Enterobacteriacea* family. However, both gram negative and gram positive bacteria have been reported to produce bacteriocins. It has been proposed that bacteriocins act by adsorbing to the surface receptors of susceptible bacteria and inhibit their growth by altering the membrane potential, inducing pore formation and causing leakage of cellular contents (Daw and Falkiner, 1996; Cleveland, et al., 2001). Some bacteriocins were reported to produce inhibitory action by interfering with the
protein or nucleic acid biosynthesis (Foulds, 1971). Contrary to the conventional belief that bacteriocins affect the growth of closely related bacteria, some bacteriocins have been reported to possess a broad spectrum of activity against a wide range of organisms. For example, bacteriocins like nisin Z, produced by Lactococcus lactis; enterocins (E-760) produced by Enterococcus spp. and reuterin produced by Lactobacillus reuteri have demonstrated broad spectrum activity against a wide range of bacterial pathogens (Rodriguez, et al., 2003; Kuwano, et al., 2005; Corr, et al., 2007; Line, et al., 2008; Svetoch, et al., 2011; Messaoudi, et al., 2012). Research showed that oral administration of bacteriocins produced by gram positive bacteria such as Bacillus circulans, Paenibacillus polymyxa (B 602) and Lactobacillus salivarius produced a significant reduction in Campylobacter colonization in broilers (Stern, et al., 2005, 2006; Svetoch, et al., 2005; Svetoch and Stern, 2010). Similarly, Cole and co-workers (2006) demonstrated that feeding purified bacteriocins in microencapsulated form can reduce C. coli to below the detection limit in turkeys. Unfortunately, the narrow spectrum of activity, susceptibility to proteolytic enzymes of the gastrointestinal tract and possible development of resistance to bacteriocins may limit the use of bacteriocins to reduce intestinal pathogens in poultry (Connerton and Connerton, 2005; Lin, 2009). Recent studies conducted by Hoang and group (2011a, 2011b) studied the in vitro and in vivo stability and development of resistance to bacteriocins. These studies reported that there is a limited possibility for Campylobacter to develop resistance against the tested bacteriocin. These experiments were conducted under controlled conditions with one strain of Campylobacter and one bacteriocin. However further research should be done to explain the role of complex interactions occurring in commercial settings, the influence of selection pressure and possible emergence of mutants resistant to the bacteriocins.
Further, bacteriocins need to be chemically characterized and should be approved by the FDA for use in foods. So far nisin is the only bacteriocin approved by the U.S. Food and Drug Administration for use in cheese products as an antimicrobial (21 CFR 184.1538). Though manufacturing companies can attribute the GRAS status to any new bacteriocin, they should be able to scientifically prove the efficacy and justify the application of such bacteriocin (Connerton and Connerton, 2005). Additionally, the costs associated with extraction and purification of bacteriocins further limits their use in poultry industry.

1.5.2.3 Bacteriophages

Bacteriophages are viruses that infect and kill bacteria (Duckworth and Gulig, 2002; Doyle and Erickson, 2006). Bacteriophages were first discovered independently by two European scientists Federick Twort (1915) and Félix d’Herelle (1917). Bacteriophages act by recognizing specific cell surface receptors of bacteria, inject their DNA into the host, and take over host cell machinery for rapid multiplication and release by lysis of the host bacterium (Duckworth and Gulig, 2002; Doyle and Erickson, 2006). Two types of bacteriophages exist in nature, temperate and lytic phages. Of these two phage types, lytic phages were often used for therapeutic purposes. Lytic phages are preferred over temperate phages, as they are highly specific, can be readily isolated from the same environment as the host and can effectively kill the host bacteria (Duckworth and Gulig, 2002; Doyle and Erickson, 2006).

d’Herelle in his book ‘The bacteriophage and its behavior’ described the therapeutic potential of bacteriophages in treating many diseases (d’Herelle, 1926). Later, many researchers successfully demonstrated the use of phages to control pathogens such as *E. coli* (Barrow, et al.,
Campylobacter specific phages have also been isolated and studied for their efficacy in reducing Campylobacter colonization in broiler chickens (Atterbury, et al., 2003; Loc Carrillo, et al., 2005; Wagenaar, et al., 2005; El-Shibiny, et al., 2009; Carvalho, et al., 2012). Wagenaar and associates (2005) studied both prophylactic and therapeutic efficacy of phages in reducing Campylobacter colonization in broiler chickens. These studies concluded that prophylactic treatment using bacteriophages will only delay colonization but not reduce Campylobacter in comparison to the control. On the other hand, therapeutic use of bacteriophage showed up to 3 log reduction in Campylobacter for a few days following administration. Several other studies also reported similar reduction in Campylobacter for first few days following phage therapy (El-Shibiny, et al., 2009; Carvalho, et al., 2010). These studies indicate that therapeutic use of bacteriophages, especially on the day before slaughter would be more practical and effective in reducing Campylobacter counts on chickens and thus could be an effective strategy to reduce the risk to humans (Wagenaar, et al., 2005).

Despite the successful demonstration of phages to reduce many enteric pathogens, possible development of phage resistance, transmission of antibiotic and/or virulence genes between bacteria limit the potential use of bacteriophages in controlling bacterial diseases (Duckworth and Gulig, 2002; Janež and Loc-Carrillo, 2013). Since phages themselves are foreign to the body, it is very likely that an immune response against phages may render them ineffective (Duckworth and Gulig, 2002). Moreover, narrow host range (due to high specificity) and consumer acceptability further limit the use of bacteriophages in food animals (Janež and Loc-Carrillo, 2013).
1.5.2.4 Organic acids

Organic acids are weak acids that are widely distributed in nature. Organic acids are GRAS and approved for use in foods intended for both human and animal consumption (Theron and Rykers, 2010; FSIS, 2013). Organic acids are known to possess antibacterial and antifungal properties and are extensively used in food preservation to increase the shelf life (Dibner and Buttin, 2002; Theron and Rykers, 2010). Organic acids are also known to have the capacity for a growth promotion effect and are extensively used in pig and poultry production (Patten and Waldroup, 1988; Dibner and Buttin, 2002). They are known to possess antimicrobial activity against many foodborne pathogens (Chaveerach, et al., 2002; Dibner and Buttin, 2002; Ricke, 2003; Jarquin, et al., 2007). Many researchers successfully demonstrated that inclusion of organic acids in drinking water can reduce pathogens such as *E. coli*, *Salmonella enterica*, *Campylobacter*, *Staphylococcus aureus* (Byrd, et al., 2001; Chaveerach, et al., 2002, 2004a; Parker, et al., 2007). Many authors demonstrated the efficacy of organic acids on *Salmonella in vitro* and *in vivo* (Van Immerseel, et al., 2003; Johny, et al., 2009). Earlier studies from our laboratory successfully demonstrated that prophylactic and therapeutic supplementation of 0.7% caprylic acid, a medium chain fatty acid, in feed produced a 2-3 log reduction in *Campylobacter* (Solis de Los Santos, et al., 2008a, 2008b, 2009, 2010). However, the results were not repeated when a sodium salt of caprylic acid was supplemented in feed or water (Metcalf, et al., 2011; Hermans, et al., 2010, 2012). Contradictory results reported by various researchers and failure of encapsulated organic acids to show consistent reduction (Van Immerseel, et al., 2004) further emphasize the need to develop novel and effective strategies that can consistently reduce *Campylobacter* colonization in broiler chickens.
1.5.2.5 Plant extracts

With the increasing consumer preference towards natural products, many researchers focused on using plant based products as potential alternatives to antibiotic growth promoters (Gauthier, 2003). Plant extracts, mainly essential oils obtained from various sources such as bay, cinnamon, clove, garlic, oregano, peppermint, rosemary, sage, thyme and many more have been extensively studied for their antimicrobial properties (Deans and Ritchie, 1987; Cosentino, et al., 1999; Dorman and Deans, 2000; Friedman, et al., 2002; Burt, 2004; Gill and Holley, 2004; Prabuseenivasan, et al., 2006; Si, et al., 2006; Cox and Markham, 2007; Kollanoor Johny, et al., 2010; Brenes and Roura, 2010; Stefanakis, et al., 2013; Venkitanarayanan, et al., 2013).

Extensive research has been done on plant essential oils and many researchers demonstrated the in vitro efficacy of these compounds against the common foodborne pathogens (Friedman, et al., 2002; Kollanoor Johny, et al., 2010; Upadhyay, et al., 2013; Upadhyaya, et al., 2013). Plant essential oils are generally regarded as safe (GRAS) and are approved for use in food animals by the FDA (21CFR182.20, FDA, 2013). However, many factors may influence the in vivo efficacy of essential oils. Factors such as chemical composition of essential oils, concentration of active compound, pH, composition and moisture content of feed may affect the efficacy of essential oils (Shelef, et al., 1984; Juven, et al., 1994; Tassou, et al., 1995; Gauthier, 2003; Si, et al., 2006; Santiesteban-Lopez, et al., 2007). Essential oils such as eugenol and trans-cinnamaldehyde showed a significant reduction in Salmonella colonization in broiler chickens (Kollanoor-Johny, et al., 2012). However the same compounds, eugenol and trans-cinnamaldehyde did not demonstrate a similar reduction in Campylobacter colonization in broiler chickens (Metcalf, 2008; Hermans, et al., 2011a). Studies conducted in our laboratory with other plant extracts such
as thymol, carvacrol, cranberry extract did not demonstrate significant reductions in *Campylobacter* colonization (Arsi, 2011; Woo-Ming, 2012). Even though plant derived compounds showed promising results in inhibiting *Campylobacter in vitro*, further studies are needed to develop an ideal dose-delivery system of an appropriate compound to produce significant reduction in *Campylobacter* in broiler chickens.

### 1.5.2.6 Vaccination

Vaccination has been considered as one of the most effective strategy to control pathogenic infections. Unfortunately, no commercial vaccines are available to control *Campylobacter* infections in poultry. *Campylobacter* naturally colonizes and survives as a commensal in the G.I tract of poultry. It has been suggested that factors essential for colonization (flagellin, CadF, CiaB, major outer membrane proteins, lipopolysaccharide), could be potential targets for a vaccine (Ziprin, et al., 2001; de Zoete, et al., 2007; Lin, 2009). Research has been done to develop vaccines using killed whole cell vaccines, live attenuated vaccines, flagellum based vaccines and recombinant vaccines have shown limited success in controlling *Campylobacter* colonization (de Zoete, et al., 2007; Hermans, et al., 2011b; Laniewski, et al., 2013). Some researchers attempted to develop vaccines by expressing *Campylobacter* antigens on attenuated *Salmonella* strains with varying degrees of success (Wyszyńska, et al., 2004; de Zoete, et al., 2007; Laniewski, et al., 2013).

Recent studies reported promising results with some degree of success in developing a vaccine to protect against *Campylobacter* infections (Layton, et al., 2011; Annamalai, et al.,
2013). However, further research needs to be done to develop a vaccine that is cost effective and practical to administer under commercial settings.

1.6 PROBIOTICS AND PREBIOTICS FOR THE PREHARVEST CONTROL OF CAMPYLOBACTER IN POULTRY

1.6.1 Probiotics

The word probiotic in Greek means “for life” and the beneficial effects of probiotics on human health has been widely reported (Gibson and Fuller, 2000; Salminen, et al., 2010; Salim, et al., 2013; Serban, 2013). During early 1900’s researchers such as Tissier (1906) and Metchnikof (1907) published on the beneficial effects of probiotic bacteria on human health. Metchnikoff in his book “The Prolongation of Life: Optimistic studies” explained that milk fermenting microbes produce lactic acid, making the environment hostile for growth of pathogens. Lilly and Stillwell (1965) first used the term ‘probiotic’ to describe the growth promoting substances secreted by a protozoan that stimulates the growth of another. Since then, many scientists redefined probiotics as, “organisms and substances that contribute to intestinal microbial balance” (Parker, 1974); or “foods that contain live bacteria which are beneficial to health (Salminen, et al., 1998). The widely accepted 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).

The exact mechanisms by which probiotic bacteria confer health benefits are unclear. However, several researchers proposed the possible mechanism of action of probiotics (Fuller, 1989; Fooks, et al., 1999; Salminen, et al., 2010). Accordingly probiotics may produce
beneficial effects by competing with the pathogens for binding sites, nutrients, or by producing antimicrobial compounds (Fuller, 1989). Also, it has been reported probiotics may possibly improve barrier functions, prevent food allergies and may possibly play a role in prevention and treatment of GI tumors (Fuller, 1989; Ouwehand, et al., 2005; Sanders and Marco, 2010; Serban, 2013).

A wealth of research has been done on probiotics and it has been suggested that an ideal probiotic should meet the following criteria: 1) the probiotic should contain viable cells that are nonpathogenic and nontoxic; 2) should resist the hostile conditions prevailing in the stomach and small intestine before reaching the target site; 3) should provide protection from pathogens by immune modulation and competitive inhibition (Fuller, 1989; Chateau, et al., 1993; Gibson and Fuller, 2000). Over the years researchers demonstrated the in vitro and in vivo efficacy of several probiotics to inhibit enteric pathogens such as Salmonella, Campylobacter, and E. coli (Fooks and Gibson, 2002; Chaveerach, et al., 2004b; Santini, et al., 2010). However, orally provided probiotics may not be maintained in the GI tract for longer periods and, hence, it has been suggested that continuous supply of probiotics may be needed to produce the desired effect (Kaur, et al., 2002). To overcome this limitation, some researchers exploited the phenomenon of competitive exclusion.

1.6.2 Competitive exclusion (CE)

The concept of competitive exclusion was first proposed by Nurmi and Rantala. Nurmi and Rantala (1973) successfully demonstrated the protective effect of undefined cultures against Salmonella infections in young chickens. As per the Nurmi concept, day of hatch birds are
inoculated with adult microflora, which then colonize in the GI tract and offer protection against pathogens that they may encounter in future (Nurmi and Rantala 1973; Pivnick and Nurmi 1982; Nurmi, et al., 1992; Schneitz, et al., 1992;). Competitive exclusion products compete with pathogens by occupying their ecological niche and by making the luminal environment hostile via the production of volatile fatty acids (increase luminal pH) and antibacterial substances such as bacteriocins (Barnes, et al., 1980; Pivnick and Nurmi 1982; Nurmi, et al, 1992; Corrier, et al., 1994; Mead, 2000). Subsequently, several researchers demonstrated the effectiveness of undefined cultures to control *Salmonella* in chickens (Aho, et al., 1992; Palmu and Camelin, 1997). With the raising concerns about the safety of undefined cultures, later research mainly focused on developing defined cultures to ensure safety and effectively protect the host health from pathogens (Impey, et al., 1982; Schoeni and Wong, 1994; Bielke, et al 2003).

Competitive exclusion phenomenon was mainly developed to protect young chickens from *Salmonella* infections. Later, the concept has been extended to protect against many enteric pathogens in other species (Hakkinen and Schneitz, 1996; Soerjadi, et al., 1981; Soerjadi-Liem, et al., 1984; Hume, et al., 1998a, 1998b; Stern, et al., 2001). Most of the CE products with efficacy against *Salmonella* failed to demonstrate similar effect against *Campylobacter* (Stavric and D’Aoust, 1993; Mead, 2000). To protect against *Campylobacter*, researchers developed CE products with efficacy against *Campylobacter* (Aho, et al., 1992; Schoeni and Doyle, 1992; Schoeni and Wong, 1994; Stern, et al., 2001b; Ghereeb, et al., 2012; Aguiar, et al., 2013). However, most of the competitive exclusion products developed against *Campylobacter* demonstrated efficacy in vitro, but showed limited success in vivo (Santini, et al., 2010; Robyn, et al., 2012; Aguiar, et al., 2013). This reiterates the need to develop novel products or strategies
to inhibit *Campylobacter* colonization in poultry. One possible strategy is to supplement dietary prebiotics along with the probiotic bacteria.

### 1.6.3 Prebiotics

A prebiotic is a “non-digestible food ingredient that beneficially affects the host by improving its intestinal microbial balance” (Gibson and Roberfroid, 1995). Food ingredients should meet the following criteria to be classified as a prebiotic: 1) should not be metabolized in the stomach or small intestine and reach the large intestine intact 2) should be selectively metabolized by one or a limited number of beneficial bacteria and stimulate their growth and/or activity in the lower intestine; and 3) should modulate the gut microbiome and induce beneficial effects on host health (Gibson and Roberfroid 1994; Gibson and Fuller 2000). It has been indicated that prebiotic supplementation in feed is cheaper, less risky and can selectively enhance the growth of beneficial microflora and thereby protect the host from enteric pathogens (Patterson and Burkholder, 2003; Mac Farlane et al., 2006). Reportedly non-digestible oligosaccharides such as inulin, lactulose, galactooligosaccharides, glucooligosaccharides, fructooligosaccharides (FOS), and soybean oligosaccharides are commonly used as feed supplements to improve the host health and performance. Even though, prebiotics alone can induce beneficial effects on host health, synbiotics (combination of probiotics and prebiotics) are proposed to be more efficacious than prebiotics or probiotics in producing the desired response (Serban, 2013).

### 1.8 REFERENCES


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Figure 1. Scanning electron micrograph of *Campylobacter* (Pead, 1979).
Table 1: Human incidence of campylobacteriosis/100,000 people from all over the world

<table>
<thead>
<tr>
<th>Country</th>
<th>Incidence/ 100,000</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Czech republic</td>
<td>195</td>
<td>Bardoň, 2013; Havelaar, et al., 2013</td>
</tr>
<tr>
<td>New Zealand</td>
<td>161.5</td>
<td>Sears, et al., 2011</td>
</tr>
<tr>
<td>Canada</td>
<td>30.2</td>
<td>CMAJ, 2007</td>
</tr>
<tr>
<td>Ireland</td>
<td>42.6</td>
<td>FSAI, 2011</td>
</tr>
<tr>
<td>United States</td>
<td>14.3</td>
<td>CDC, 2013b</td>
</tr>
<tr>
<td>Iceland</td>
<td>38.62</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Norway</td>
<td>61.07</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Switzerland</td>
<td>100.80</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Belgium</td>
<td>70.46</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Denmark</td>
<td>73.01</td>
<td>EFSA, 2013b</td>
</tr>
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<td>Finland</td>
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<td>Germany</td>
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<td>Hungary</td>
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<td>Ireland</td>
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<td>Lithuania</td>
<td>34.64</td>
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<td>Luxembourg</td>
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<td>United Kingdom</td>
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<td>Spain</td>
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<td>Slovenia</td>
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<td>Slovakia</td>
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<td>Netherlands</td>
<td>50.89</td>
<td>EFSA, 2013b</td>
</tr>
</tbody>
</table>
Table 2: Percent prevalence of *Campylobacter* in poultry flocks from various countries across the world.

<table>
<thead>
<tr>
<th>Country</th>
<th>Prevalence in poultry flocks</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>74.6%</td>
<td>(Jore, et al., 2010)</td>
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<tr>
<td>Iceland</td>
<td>59.5%</td>
<td>Jore, et al., 2010</td>
</tr>
<tr>
<td>Norway</td>
<td>74.1%</td>
<td>Jore, et al., 2010</td>
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<td>Sweden</td>
<td>81.8%</td>
<td>Jore, et al., 2010</td>
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<td>Netherlands</td>
<td>58.9%</td>
<td>Jore, et al., 2010</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>63.4%</td>
<td>Bardoň, 2013; EFSA, 2013b</td>
</tr>
<tr>
<td>Ireland</td>
<td>80.6%</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Spain</td>
<td>68.4%</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Slovenia</td>
<td>88-93%</td>
<td>Bardoň, 2013</td>
</tr>
</tbody>
</table>
Table 3: Percent prevalence of *Campylobacter* in retail poultry reported from different countries

<table>
<thead>
<tr>
<th>Country</th>
<th>Prevalence in retail poultry</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>United States</td>
<td>71.5%</td>
<td>Suzuki and Yamamoto, 2009</td>
</tr>
<tr>
<td>Canada</td>
<td>57.7%</td>
<td>Suzuki and Yamamoto, 2009</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>75%</td>
<td>Bardoň, 2013</td>
</tr>
<tr>
<td>Spain</td>
<td>75.8%</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Poland</td>
<td>82.7%</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Ireland</td>
<td>52.9%</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Hungary</td>
<td>41.3%</td>
<td>EFSA, 2013b</td>
</tr>
<tr>
<td>Argentina</td>
<td>92.9%</td>
<td>Suzuki and Yamamoto, 2009</td>
</tr>
<tr>
<td>Italy</td>
<td>80.0%</td>
<td>Suzuki and Yamamoto, 2009</td>
</tr>
<tr>
<td>Africa</td>
<td>73.1%</td>
<td>Suzuki and Yamamoto, 2009</td>
</tr>
<tr>
<td>Asia</td>
<td>60.3%</td>
<td>Suzuki and Yamamoto, 2009</td>
</tr>
<tr>
<td>New Zealand</td>
<td>89.1%</td>
<td>Suzuki and Yamamoto, 2009</td>
</tr>
<tr>
<td>Japan</td>
<td>58.8%</td>
<td>Suzuki and Yamamoto, 2009</td>
</tr>
<tr>
<td>Australia</td>
<td>100%</td>
<td>Suzuki and Yamamoto, 2009</td>
</tr>
</tbody>
</table>
Chapter 2

Effect of selected probiotic and prebiotic combinations in reducing *Campylobacter* colonization in broiler chickens.
Chapter 2

EFFECT OF SELECTED PROBIOTIC AND PREBIOTIC COMBINATIONS IN REDUCING CAMPYLOBACTER COLONIZATION IN BROILER CHICKENS.

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2.1 ABSTRACT

Campylobacter is one of the leading causes of foodborne illness worldwide and human illnesses are often associated with consumption of contaminated poultry or poultry products. One of the strategies to reduce Campylobacter colonization in poultry is by use of probiotics. One hundred seventeen GRAS (Generally Regarded as Safe) bacteria were isolated from healthy birds and evaluated for efficacy against Campylobacter, in vitro. A soft agar overlay technique was used to measure anti-Campylobacter activity of these isolates based upon the diameter of the zone of inhibition around the bacterial colony. Twenty six such isolates with in vitro efficacy against Campylobacter were tested in vivo in 3 separate trials. For the in vivo trials, bacterial isolates were administered orally to chicks on the day of hatch. On day 7, chicks were orally challenged with a 4 strain mixture of wild type C. jejuni (~10⁷ CFU/mL) and on day 14 cecal samples were collected for Campylobacter enumeration. Out of 26 isolates with in vitro efficacy, 3 isolates produced a reduction in cecal Campylobacter counts (1-2 logs). In an effort to improve the efficacy of these 3 isolates, two prebiotics (Fructooligosaccharide/FOS or
Mannanoligosaccharide/MOS) were added to the feed in follow up trials during the entire 14 day trials. None of the isolates nor the FOS doses tested (0.125%, 0.25% or 0.5%) or the combination of individual isolates with selected FOS doses reduced cecal Campylobacter counts. When the isolates were tested with MOS (0.04%, 0.08% and 0.16%), combination of isolate 3 with 0.04% MOS in feed demonstrated a 3-log reduction in cecal Campylobacter counts. The results of this study indicate that the selection and administration of bacterial isolates in combination with selected prebiotics may reduce enteric Campylobacter counts in preharvest poultry, however few treatments are effective.

**KEYWORDS:** Campylobacter jejuni, probiotic, FOS, MOS, chicken.

### 2.2 INTRODUCTION

Campylobacter is a leading cause of foodborne illness in the United States and across the world (CDC, 2010; WHO, 2011), with 400 million illnesses annually worldwide (Rollwagen, et al., 1993). Campylobacter is estimated to cause 0.8 million illnesses each year in the United States (Scharff, 2012) and cost $1.7 billion annually (Batz, et al., 2012; Hoffmann, et al., 2012). Two species, C. jejuni and C. coli are usually associated with Campylobacter illness in humans, of which the majority of illnesses are caused by C. jejuni (Butzler, et al., 1973; Phillips, 1995; Debruyne, et al., 2008; EFSA, 2013). Case control studies have demonstrated that the majority of human infections are associated with consumption or improper handling of poultry (Skirrow, 1982; Kapperud, et al., 2003; Stafford, et al., 2008; Danis, et al., 2009). Therefore, a reduction or elimination of Campylobacter from poultry would greatly reduce the human incidence (Rosenquist, et al., 2003). Many intervention strategies have been evaluated in an effort to control Campylobacter in poultry, but none have been successful in eliminating this bacterium.
(Wagenaar, et al., 2005; Cole et al., 2006; de Zoete, et al., 2007; Solis de lo Santos et al., 2008, 2009; Vandeplas, et al., 2008; Santini, et al., 2010; Hermans, et al., 2011). In this study we attempted to reduce/inhibit enteric Campylobacter colonization in chickens by developing a probiotic culture. For this we collected and identified bacterial isolates which are Generally Regarded as Safe (GRAS, FDA, 2013b). Isolates with GRAS status are recognized as safe to immediately administer to birds without the cost and delays associated with regulatory approval (FDA, 2013a). Probiotics are “live microorganisms which when administered in adequate amounts can confer beneficial effects on host health” (Fuller 1989). Probiotics proved to be an effective strategy to control pathogens such as Salmonella, E. coli and Listeria (Soerjadi, et al., 1981; Impey, et al., 1982; Hakkinen and Schneitz, 1996; Hume, et al., 1998a, 1998b; Stern, et al., 2001; Fooks and Gibson, 2002; Bielke, et al 2003; Zhang et al., 2007b).

The objective of this study was to develop probiotic isolates that can inhibit Campylobacter colonization in broilers chickens. Studies were conducted to isolate GRAS bacteria from healthy chickens and evaluate their efficacy to inhibit Campylobacter in vitro. The isolates with in vitro anti-Campylobacter activity were selected and tested in vivo. Follow up studies were conducted to enhance the in vivo efficacy of selected isolates by additionally supplementing a prebiotic in the feed.

2.3 MATERIALS AND METHODS

2.3.1 Isolation and identification of probiotic bacteria

Probiotic bacteria were isolated from the cecal contents of healthy birds of different age groups according to the procedure of Aguiar and co-workers (2013). Briefly, the procedure involves collection of ceca from healthy chickens under aseptic conditions. Cecal contents were
then squeezed into sterile tubes and diluted with Buffed Phosphate Diluent (BPD). Up to 3 dilutions were made using BPD (1:10, 1:100 and 1:1000) and 100µL of each dilution was plated on blood agar plates (BAP, Difco™ Becton Dickinson and company, MD). Another set of dilutions were plated on deMan, Rogosa and Sharpe Agar (Difco™ Becton Dickinson and company, MD). Both the plates were incubated at 37°C for 24 hours. Isolated colonies from each plate were picked and streaked again on fresh BAP and incubated as described above. This procedure was repeated until pure cultures were obtained. Initial identification of isolated colonies was done using Gram’s staining. Further identification was done using Biolog® system (Biolog®, Hayward, CA) as per the procedure previously described by our laboratory (Aguiar, et al., 2013). Glycerol stock solutions of the isolates, which were identified as GRAS by FDA (2013b), were prepared and stored at -80°C (Aguiar, et al., 2013; Bhaskaran, et al., 2011; Bielke, et al., 2003).

2.3.2 Screening for in vitro anti-Campylobacter activity of the isolates

The in vitro screening for anti-Campylobacter activity was done using a soft agar overlay technique (Gratia, 1936; Aguiar et al., 2013). The isolates were first grown in 5 mL of Tryptic soy broth (TSB, BBL® Becton Dickinson and Company, MD) and incubated at 37°C for 24 hours. From the broth 100µL of culture was inoculated in the middle of a Tryptic Soy Agar (TSA, Difco™ Becton Dickinson and company, MD) plate and incubated at 37°C for 24 hours. Simultaneously Campylobacter strains were grown on Campylobacter Enrichment Broth (CEB, Neogen corporation, MI) and 100µL of 24 hour old Campylobacter culture was added to 2 mL of soft agar (0.65% agar) and carefully overlaid on the TSA plate with probiotic isolate in the middle (Aguiar, et al., 2013; Zhang, et al., 2007a). The TSA plate with soft agar was incubated
under microaerophilic conditions at 42°C for 48 hours. The isolates with in vitro anti-
Campylobacter activity show a clear zone of inhibition around the probiotic colony (Figure 1).
The bigger the diameter of the zone, the greater the inhibitory activity. The isolates with the greatest inhibitory activity against Campylobacter were selected for evaluation of in vivo efficacy against Campylobacter colonization in broiler chickens.

2.3.3 *In vivo* studies

2.3.3.1 Experimental animals and housing

For all the in vivo studies, day of hatch commercial broiler chicks were weighed at the beginning and at the end of each trial. Chicks were raised on floor pens with pine shavings and *ad libitum* access to both feed and water for the 14 day trial period.

2.3.3.2 Experimental design

During the first 3 trials, 26 isolates were evaluated for their in vivo efficacy against Campylobacter. In trials 1 and 2, a total of 100 birds were randomly divided into 10 treatment groups (n=10/treatment) with a Campylobacter control group (Campylobacter, no isolate) and 9 groups testing the efficacy of single bacterial isolate (a total of 18 separate isolates were tested, 9 in trial 1, another 9 in trial 2). In the third trial, a total of 90 birds (n=10/treatment) were randomly divided into 9 treatment groups (1 Campylobacter control group and 8 groups, each receiving single bacterial isolate). From these 3 trials, 3 isolates (Isolate 1, 2 and 3) with in vivo efficacy against Campylobacter were selected and tested in trials 4 and 5.

In trial 4, a total of 160 birds were divided into 16 treatment groups with 10 birds per treatment. Treatments include a Campylobacter control (Campylobacter, no isolate, 0% FOS), isolate controls (Campylobacter, Isolate 1, 2 or 3, 0% FOS), selected doses of
Fructooligosaccharides/FOS (BOC Sciences, NY), 0.125%, 0.25%, 0.5% in feed, and the combination of individual isolate with selected dose of FOS.

For trial 5, the experimental design was similar to trial 4 (total 160 birds with 16 treatments, n=10/treatment) except Mannanoligosaccharide/MOS (Actigen™, Alltech, Inc, KY) was fed to birds instead of FOS. In trial 5, treatment groups include a Campylobacter control (Campylobacter, no isolate, 0% MOS), isolate controls (Campylobacter, isolate 1, 2 or 3, 0% MOS), MOS controls (0.04%, 0.08% and 0.16%) in feed, and the combinations of MOS and individual isolates.

2.3.3.3 Bacterial Strains and dose

During each trial, all treatment groups, except for the Campylobacter control, were orally gavaged with 0.25 mL of specific bacterial isolate containing approximately 1x10⁷CFU/mL of bacterial isolate on day 1. On day 7, all the birds were orally gavaged with a 4 strain mixture of wild type Campylobacter containing approximately 1x10⁷CFU/mL organisms. On day 14, all the birds were euthanized and ceca were collected for Campylobacter enumeration.

2.3.3.4 Campylobacter enumeration

Cecal Campylobacter counts were determined as per the procedure described in earlier studies (Bhaskaran, et al., 2011; Aguiar, et al., 2013). Ceca were collected on day 14 and the contents were serially diluted (1:10) with BPD. Each dilution was then plated on Campylobacter Line Agar (CLA, Line, 2001) and incubated under microaerophilic conditions for 48 hours at 42°C. Suspected Campylobacter colonies were confirmed by latex agglutination test (LATEX:
CAMPY [jcl]™ Test Kit, Scimedx corporation, NJ) and further confirmation was done with api® Campy (bioMérieux Vitek, Inc, MO).

2.3.4 Statistical Analysis

Data were analyzed using PROC GLM procedure of SAS (SAS, 2011). Campylobacter colonies were enumerated and the counts for CFU/mL were then log transformed before analysis to achieve homogeneity of variance (Byrd, et al., 2003). Treatment means were partitioned by least square means analysis and a probability of P < 0.05 was considered for statistical significance.

2.4 RESULTS

For this study 117 GRAS bacteria were isolated from healthy chickens. Of these 117 isolates, 48 isolates inhibited Campylobacter in vitro, as determined by the soft agar overlay technique (data not shown). When 26 such isolates with greatest inhibitory activity in vitro were tested in vivo, only 3 isolates (isolates 1, 2 and 3) showed approximately a 1-2 log reduction in Campylobacter compared to the control (Figure 2). In the last two trials utilizing both bacterial isolates and FOS or MOS in the feed, none of the individual isolates nor the FOS or MOS treatments by themselves showed any reduction in Campylobacter (Tables 1 and 2). However, in trial 5, the treatment group which received isolate 3 and 0.04% MOS in feed showed a 3 log reduction of Campylobacter compared to the control group (Table 2). Even though FOS treatments did not reduced Campylobacter, increases in body weight gain were seen with 0.125% FOS and its combinations with the 3 isolates, combination of 0.25% FOS and isolate 2, 0.5% FOS and the combinations of 0.5% FOS and isolate 3 as compared with the birds fed
control diet (Table 3). However, MOS treatments or its combinations with probiotic isolates did not show an improvement in body weight gain compared to the control (Table 4). Additionally, birds fed 0.16% MOS and its combination with isolate 1 and 2 did not achieve as much gain as the controls.

2.5 DISCUSSION

Campylobacter naturally colonize in high numbers (up to $10^9$ CFU/mL) in the mucosal crypts of the avian ceca (Beery, et al., 1988). Isolating GRAS bacteria from the chicken ceca and orally inoculating such bacteria into the poultry early in life (day 1) may initiate colonization of the ceca and inhibit the colonization of Campylobacter. The exact mechanism by which the probiotic bacteria produce beneficial effects is not fully understood. It has been proposed that probiotic bacteria may inhibit pathogens by competing for adhesion sites and nutrients (Pivnic and Nurmi, 1982; Callaway, et al., 2008). Other mechanisms by which they produce beneficial effects include production of volatile fatty acids and antimicrobial substances (Barnes, et al., 1980; Nurmi, et al., 1992; Stavric, 1992; Corrier, et al., 1994; Callaway, et al., 2008). In the present study, when 26 GRAS isolates with in vitro Campylobacter activity were tested in vivo, only 3 isolates showed a 1-2 log reduction in Campylobacter (Figure 2). However, these isolates reduced Campylobacter only in 1 out of 3 trials. In follow up trials, the 3 efficacious GRAS isolates were supplemented with a prebiotic in order to enhance their in vivo effect. Studies have demonstrated that prebiotics, used alone or in combination with probiotics, provide beneficial effects on host health by selectively stimulating the growth and/or activity of beneficial microflora (Gibson and Roberfroid 1995; Gibson and Fuller 2000). We selected one of the most studied prebiotic compounds, FOS for our initial study (Bailey, et al., 1991; Gibson and Fuller,
Fructooligosaccharides are non-digestible carbohydrates present in many plant sources such as wheat, onion, banana, garlic and chicory (Niness, 1999). Research has demonstrated that FOS can induce beneficial effects in the host by selectively promoting the growth of *Bifidobacteria* and *Lactobacillus* and decreasing the numbers of pathogens such as *Clostridia* and *E. coli* (Janardhana, et al., 2009; Kim, et al., 2011). Also, it has been shown to produce immunomodulatory effects by producing short chain fatty acids (SCFA) like butyrates (Janardhana, et al., 2009). However, our studies with FOS did not demonstrate a reduction in *Campylobacter* in the ceca of broiler chickens. Similar inconsistencies on the efficacy of FOS supplementation on *Salmonella* colonization have been reported (Chambers, et al., 1997; Fukata, et al., 1999; Oyarzabal and Conner, 1996; Ten Bruggencate, et al., 2003). It is possible that the birds raised under controlled conditions may not have the same microflora as commercial poultry which would efficiently utilize FOS and produce the target effect in the host. Also, there are reports that higher than optimum doses of FOS in the diet are associated with increased gas production and poor performance (Kim, et al., 2011). Since, we could not bring about the desired reduction in *Campylobacter* colonization using FOS in the diet, we evaluated another potential prebiotic compound, MOS, in our next study.

Mannanoligosaccharide is derived from the cell wall of yeast, *Saccharomyces cerevisiae* (Miguel, et al., 2004). Research has shown that dietary inclusion of MOS has immunomodulatory effects and promotes growth in swine and poultry (Waldroup, et al., 2003; Miguel et al., 2004; Parks, et al., 2005; Baurhoo, et al., 2009; Chee, et al., 2010a, 2010b). Research findings indicated that MOS selectively binds to Type I fimbriae of Gram negative bacteria and thereby prevents their adhesion to mucus (Spring, et al., 2000; Baurhoo, et al., 2009). This selective adhesion of MOS may decrease intestinal colonization of pathogens and
increase mucosal excretion of pathogens such as *E. coli* and *Salmonella*. Studies reported supplementation of MOS in feed have been associated with a selective increase in numbers of *Bifidobacteria* and *Lactobacillus spp.* and a decrease in numbers of enteric pathogens like *E. coli* and *Salmonella* (Baurhoo, et al., 2007a; Baurhoo, et al., 2007b; Fernandez, et al., 2002; Swanson, et al., 2002). In the present study, supplementation of MOS alone did not show a reduction in *Campylobacter*. However, birds receiving isolate 3 along with 0.04% MOS in the feed showed a 3 log reduction in *Campylobacter* (Table 2). The exact mechanism by which MOS can reduce *Campylobacter* is not known. However, it may act indirectly by promoting the growth of probiotic bacteria, which in turn may be responsible for the reduction of *Campylobacter* in the broiler ceca.

Contrary to other research reports, our studies with selected doses of MOS did not show an improvement in growth performance. Also, supplementation of higher doses of MOS (0.16% in feed) did not demonstrate any additional advantage in reducing *Campylobacter* or improvement in growth performance. A few other studies in poultry have also reported that supplementation of MOS alone in the feed did not show a growth promotion effect (Fritts and Waldroup, 2003; Yang, et al., 2008; Santos, et al., 2013). Factors like dietary interactions, optimal probiotic and prebiotic combinations and the length of study period may influence the outcome of the results. It is also possible that these 14 day trials do not provide long enough exposure of all the gut microflora to the FOS/MOS to produce measurable changes in growth performance.

2.6 CONCLUSION
The selected probiotic isolates when administered individually, did not show a consistent reduction in *Campylobacter in vivo*. However, the combination of a probiotic isolate and a prebiotic may have potential as a strategy to reduce cecal colonization of *Campylobacter*. Further studies will be undertaken to combine isolates and continue to evaluate the different combinations of probiotic and prebiotic compounds.

### 2.7 REFERENCES


early colonization by *Salmonella* Enteritidis in chicks is improved by higher dietary threonine levels. J. Appl. Microbiol. 114:1158-1165.


Zhang, G., L. Ma, and M. P. Doyle. 2007a. Potential competitive exclusion bacteria from poultry inhibitory to *Campylobacter jejuni* and *Salmonella*. J. Food Prot. 70:867-873.

Figure 1. Soft agar overlay showing a clear zone of inhibition of *Campylobacter* around the probiotic colony\(^1\) (Photograph by K. Arsi, taken on 12 October, 2011 at John W. Tyson building, Laboratory 324, Department of Poultry Science, University of Arkansas).

\(^1\)The probiotic isolate was grown on the middle of a Tryptic soy agar plate (represented by the dotted circle). The plate with the probiotic was overlaid with 2mL of soft agar containing *Campylobacter* (\(~10^6\) CFU/mL) and incubated under microaerophilic conditions at 42°C for 48 hours.

A lawn of *Campylobacter* can be seen on the agar plate with a clear zone of inhibition around the probiotic colony.
Figure 2: The probiotic isolates with *in vivo* efficacy against *Campylobacter*.1

1*Campylobacter* counts (Log CFU/mL) in the cecal contents of birds orally gavaged with probiotic isolates in comparison to control.

a, bTreatments without common variables differ significantly (*P* < 0.05)

All *Campylobacter* data were log_{10} transformed for statistical analysis.
Table 1: The effect of selected bacterial isolates, FOS doses and their combinations on cecal *Campylobacter* counts (log CFU/mL of cecal contents) in 14 day old broiler chicks (Mean ± SEM) during Trial 4.\(^1\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log CFU/mL (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter</em> Control</td>
<td>6.24 ± 0.71 a</td>
</tr>
<tr>
<td>Isolate 1</td>
<td>5.75 ± 1.01 a</td>
</tr>
<tr>
<td>Isolate 2</td>
<td>5.74 ± 0.88 a</td>
</tr>
<tr>
<td>Isolate 3</td>
<td>5.94 ± 0.55 a</td>
</tr>
<tr>
<td>0.125% FOS</td>
<td>8.48 ± 0.20 b</td>
</tr>
<tr>
<td>Isolate 1 + 0.125% FOS</td>
<td>7.62 ± 0.16 b</td>
</tr>
<tr>
<td>Isolate 2 + 0.125% FOS</td>
<td>7.87 ± 0.34 b</td>
</tr>
<tr>
<td>Isolate 3 + 0.125% FOS</td>
<td>8.46 ± 0.13 b</td>
</tr>
<tr>
<td>0.25% FOS</td>
<td>8.42 ± 0.24 b</td>
</tr>
<tr>
<td>Isolate 1 + 0.25% FOS</td>
<td>8.31 ± 0.25 b</td>
</tr>
<tr>
<td>Isolate 2 + 0.25% FOS</td>
<td>8.65 ± 0.21 b</td>
</tr>
<tr>
<td>Isolate 3 + 0.25% FOS</td>
<td>8.65 ± 0.11 b</td>
</tr>
<tr>
<td>0.5% FOS</td>
<td>8.03 ± 0.31 b</td>
</tr>
<tr>
<td>Isolate 1 + 0.5% FOS</td>
<td>8.08 ± 0.20 b</td>
</tr>
<tr>
<td>Isolate 2 + 0.5% FOS</td>
<td>7.88 ± 0.19 b</td>
</tr>
<tr>
<td>Isolate 3 + 0.5% FOS</td>
<td>8.65 ± 0.16 b</td>
</tr>
</tbody>
</table>

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

\(^1\) Chicks were orally challenged on day 7 with 0.25 mL of approximately \(1 \times 10^7\) CFU/mL of a 4 strain mixture of wild type *Campylobacter jejuni* (Total 160 birds with 16 treatments, \(n=10/\text{treatment group}\))

All *Campylobacter* data were log\(_{10}\) transformed for statistical analysis.
Table 2: The effect of selected bacterial isolates, MOS doses and their combinations on cecal *Campylobacter* counts (log CFU/mL of cecal contents) in 14 day old broiler chicks (Mean ± Standard Error of Mean) during Trial 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log CFU/mL (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter</em> Control</td>
<td>7.65 ± 0.43 a</td>
</tr>
<tr>
<td>Isolate 1</td>
<td>7.40 ± 0.45 a</td>
</tr>
<tr>
<td>Isolate 2</td>
<td>7.58 ± 0.35 a</td>
</tr>
<tr>
<td>Isolate 3</td>
<td>7.10 ± 0.30 a</td>
</tr>
<tr>
<td>0.04% MOS</td>
<td>7.40 ± 0.28 a</td>
</tr>
<tr>
<td>Isolate 1 +0.04% MOS</td>
<td>7.59 ± 0.24 a</td>
</tr>
<tr>
<td>Isolate 2 + 0.04% MOS</td>
<td>7.69 ± 0.27 a</td>
</tr>
<tr>
<td>Isolate 3 + 0.04% MOS</td>
<td>4.37 ± 0.90 b</td>
</tr>
<tr>
<td>0.08% MOS</td>
<td>7.01 ± 0.72 a</td>
</tr>
<tr>
<td>Isolate 1 + 0.08% MOS</td>
<td>7.56 ± 0.43 a</td>
</tr>
<tr>
<td>Isolate 2 + 0.08% MOS</td>
<td>7.02 ± 0.73 a</td>
</tr>
<tr>
<td>Isolate 3 + 0.08% MOS</td>
<td>7.58 ± 0.30 a</td>
</tr>
<tr>
<td>0.16% MOS</td>
<td>8.02 ± 0.11 a</td>
</tr>
<tr>
<td>Isolate 1 + 0.16% MOS</td>
<td>7.76 ± 0.33 a</td>
</tr>
<tr>
<td>Isolate 2 + 0.16% MOS</td>
<td>8.33 ± 0.15 a</td>
</tr>
<tr>
<td>Isolate 3 + 0.16% MOS</td>
<td>7.75 ± 0.24 a</td>
</tr>
</tbody>
</table>

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

^1^ Chicks were orally challenged on day 7 with 0.25 mL of approximately 1 x 10^7^CFU/mL of a 4 strain mixture of wild type *Campylobacter jejuni* (Total 160 birds with 16 treatments, n=10/treatment group). All *Campylobacter* data were log_{10} transformed for statistical analysis.
Table 3: The effect of selected bacterial isolates, FOS doses and their combinations on cecal *Campylobacter* counts (log CFU/mL of cecal contents) in 14 day old broiler chicks (Mean ± Standard Error of Mean) during Trial 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight gain in 14days</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter</em> Control</td>
<td>345.6 ± 18.5 b</td>
</tr>
<tr>
<td>Isolate 1</td>
<td>285.6 ± 6.6 a</td>
</tr>
<tr>
<td>Isolate 2</td>
<td>352.7 ± 15.6 bc</td>
</tr>
<tr>
<td>Isolate 3</td>
<td>260.6 ± 7.74 a</td>
</tr>
<tr>
<td>0.125%FOS</td>
<td>395.85 ± 12.16 de</td>
</tr>
<tr>
<td>Isolate 1 + 0.125%FOS</td>
<td>391.13 ± 4.49 cde</td>
</tr>
<tr>
<td>Isolate 2 + 0.125%FOS</td>
<td>416.2 ± 15.72 e</td>
</tr>
<tr>
<td>Isolate 3 + 0.125%FOS</td>
<td>400.85 ± 10.39 de</td>
</tr>
<tr>
<td>0.25%FOS</td>
<td>370.0 ± 19.6 bcd</td>
</tr>
<tr>
<td>Isolate 1 + 0.25%FOS</td>
<td>376.51 ± 11.41 bcde</td>
</tr>
<tr>
<td>Isolate 2 + 0.25%FOS</td>
<td>392.48 ± 13.15 cde</td>
</tr>
<tr>
<td>Isolate 3 + 0.25%FOS</td>
<td>369.0 ± 9.0 bcd</td>
</tr>
<tr>
<td>0.5%FOS</td>
<td>413.08 ± 12.4 e</td>
</tr>
<tr>
<td>Isolate 1 + 0.5%FOS</td>
<td>369.45 ± 27.6 bc</td>
</tr>
<tr>
<td>Isolate 2 + 0.5%FOS</td>
<td>378.48 ± 15.58 bcde</td>
</tr>
<tr>
<td>Isolate 3 + 0.5%FOS</td>
<td>396.64 ± 13.35 de</td>
</tr>
</tbody>
</table>

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

1In trial 4, a total of 160 birds were randomly divided into 16 treatment groups with 10 birds in each treatment. Chicks were weighed at the beginning and at the end of the study (day 14). The average body weight gain in grams is shown in the table.
Table 4: The effect of selected bacterial isolates, MOS doses and their combinations on body weight gain (grams) in 14 day old broiler chicks (Mean ± Standard Error of Mean) during Trial 5\(^1\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight gain in 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter</em> Control</td>
<td>416.6 ± 8.8 def</td>
</tr>
<tr>
<td>Isolate 1</td>
<td>377.4 ± 21.8 cdef</td>
</tr>
<tr>
<td>Isolate 2</td>
<td>395.1 ± 10.6 cde</td>
</tr>
<tr>
<td>Isolate 3</td>
<td>397.6 ± 13.1 cde</td>
</tr>
<tr>
<td>0.04% MOS</td>
<td>425.5 ± 5.4 ef</td>
</tr>
<tr>
<td>Isolate 1 + 0.04% MOS</td>
<td>406.9 ± 8.3 cdef</td>
</tr>
<tr>
<td>Isolate 2 + 0.04% MOS</td>
<td>394.3 ± 9.8 cde</td>
</tr>
<tr>
<td>Isolate 3 + 0.04% MOS</td>
<td>431.3 ± 10.6 f</td>
</tr>
<tr>
<td>0.08% MOS</td>
<td>414.5 ± 10.7 def</td>
</tr>
<tr>
<td>Isolate 1 + 0.08% MOS</td>
<td>410.3 ± 9.8 def</td>
</tr>
<tr>
<td>Isolate 2 + 0.08% MOS</td>
<td>420.7 ± 14.3 def</td>
</tr>
<tr>
<td>Isolate 3 + 0.08% MOS</td>
<td>387.5 ± 6.9 bcd</td>
</tr>
<tr>
<td>0.16% MOS</td>
<td>356.3 ± 13.0 ab</td>
</tr>
<tr>
<td>Isolate 1 + 0.16% MOS</td>
<td>375.1 ± 17.8 abc</td>
</tr>
<tr>
<td>Isolate 2 + 0.16% MOS</td>
<td>351.3 ± 22.8 a</td>
</tr>
<tr>
<td>Isolate 3 + 0.16% MOS</td>
<td>412.5 ± 7.4 def</td>
</tr>
</tbody>
</table>

\(a,b,c,d,e,f\) Means within columns with no common superscript differ significantly (P < 0.05).

\(^1\)In trial 5, a total of 160 birds were randomly divided into 16 treatment groups with 10 birds in each treatment. Chicks were weighed at the beginning and at the end of the study (day 14). The average body weight gain in grams is shown in the table.
Attn  University of Arkansas Graduate School

March 15, 2014

Dear Sir,

I attest that Komala Arsi was first author of the manuscript cited below and completed at least 51% of the work for the paper.


Yours Sincerely,

Dan J. Donoghue,
Professor,
Department of Poultry Science
POSC O-114
University of Arkansas
Fayetteville, AR 72701
Phone: (479) 575-2913
Email: ddonogh@uark.edu
February 9, 2012

MEMORANDUM

TO: Dr. Dan Donoghue

FROM: W. Roy Penney
Institutional Biosafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 08021

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 08021, "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
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DATE: September 14, 2010
SUBJECT: IACUC PROTOCOL APPROVAL
Expiration date: September 30, 2013

The Institutional Animal Care and Use Committee (IACUC) has APPROVED Protocol #11006-“TESTING THE EFFICACY OF PROBIOTIC CULTURES AGAINST CAMPYLOBACTER COLONIZATION IN CHICKENS”. You may begin this study immediately.

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cme/car

cc: Animal Welfare Veterinarian
Chapter 3

Evaluating route of inoculation, oral versus intra-cloacal, as a screening method of *in vivo* efficacy of anti-*Campylobacter* bacterial isolates in chickens
Chapter 3

EVALUATING ROUTE OF INOCULATION, ORAL VERSUS INTRA-CLOACAL, AS A SCREENING METHOD OF IN VIVO EFFICACY OF ANTI-CAMPYLOBACTER BACTERIAL ISOLATES IN CHICKENS

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3.1 ABSTRACT

Campylobacter is the leading cause of foodborne illness worldwide. It is commonly present in the intestinal tract of poultry and campylobacteriosis in humans is often associated with consumption of poultry or poultry products. Many strategies have been tried to eliminate Campylobacter from preharvest poultry with limited success. One strategy to reduce Campylobacter colonization in poultry is by the use of oral probiotics. Unfortunately, oral probiotics can produce variable results, possibly because they are destroyed passing through the acidic stomach of poultry. Encapsulation of isolates may overcome this problem but there is no assurance these isolates will have efficacy in the lower GI tract. Therefore, screening candidate isolates by directly placing them in the lower intestinal tract via cloacal inoculation may eliminate the time and expense of encapsulating ineffective isolates. Thus, the purpose of this study was to collect bacterial isolates with anti-Campylobacter activity in vitro and evaluate their efficacy in vivo upon either oral or intra-cloacal administration.
Bacterial isolates were collected from healthy birds of different age groups and evaluated for efficacy against *C. jejuni*, *in vitro*. Furthermore, the isolates with generally regarded as safe (GRAS) status were identified and subjected to motility enhancement. Earlier studies from our laboratory demonstrated that motility enhanced isolates reduced *Campylobacter* colonization in poultry more effectively than control isolates. Ten GRAS isolates demonstrating *in vitro* anti-*Campylobacter* properties and enhanced motility were evaluated after either oral or intra-cloacal inoculation into day of hatch chicks (n=10 birds/isolate). When isolates were dosed orally, only one isolate showed a 1 log reduction in cecal *Campylobacter* counts in 14 day old chickens. When these isolates were administered intra-cloacally, five of these isolates produced a 1-3 log reduction in cecal *Campylobacter* counts. These results support the strategy of evaluating the efficacy of potential probiotic isolates via cloacal inoculation prior to undergoing the effort of protecting isolates (e.g., encapsulation) for oral administration.

**KEYWORDS** *Campylobacter jejuni*, probiotic, GRAS, chicken, intra-cloacal.

### 3.2 INTRODUCTION

*Campylobacter* is one of the major causes of foodborne illness and is a public health concern throughout the world (WHO, 2011). Recent reports estimated that *Campylobacter* is the second most common cause of foodborne enteritis in the US with a reported number of 14.3 cases for every 100,000 people (CDC, 2013). *Campylobacter* infections in humans may range from a self-limiting illness to neurological or joint disorders such as Guillain-Barré syndrome and reactive arthritis (Altekruse, et al., 1999). Many sources such as contaminated water,
unpasteurized milk, fresh produce and contact with domestic animals have been reported to cause *Campylobacter* illness in humans (Robinson, et al., 1979; Skirrow, 1982; Kapperud, et al., 2003; Stafford, et al., 2008; Danis, et al., 2009). However, consumption or improper handling of poultry is considered the major source (>70%) of human infections (King, 1957, 1962; Skirrow, 1982; Stafford, et al., 2008; Danis, et al., 2009). Since poultry are considered the major risk for human infections, reducing *Campylobacter* in poultry would greatly reduce the human infections (Rosenquist, et al., 2003). Recent implementation of *Campylobacter* performance standards by USDA further emphasizes the need to control *Campylobacter* in poultry (USDA-FSIS, 2011).

Several strategies have been attempted over the years to control this bacterium in poultry (Wagenaar, et al., 2005; Cole, et al., 2006; Stern, et al., 2006; Solis de los Santos, et al., 2008, 2009). However, no single strategy has been effective in eliminating *Campylobacter* from poultry (Hermans, et al., 2011). One strategy to reduce *Campylobacter* colonization in poultry is by use of oral probiotics. Probiotics are “live microorganisms which when administered in adequate amounts can confer beneficial effects on host health” (Fuller, 1989). Earlier studies using probiotics to reduce *Campylobacter* colonization in broiler chickens demonstrated inconsistent results (Vandeplas, et al., 2008; Willis and Reid, 2008; Robyn, et al., 2013). One possible explanation for such inconsistent results could be due to failure of isolates to survive in the stomach acids and colonize the lower intestine to competitively inhibit *Campylobacter* (Ding and Shah, 2009). This hurdle can be overcome by microencapsulating the probiotics, which protects the probiotic isolates from the harsh conditions in the upper gastrointestinal (GI) tract and may successfully deliver them in the lower intestinal tract (Ding and Shah, 2009). Since microencapsulation procedures are costly and are often challenging (Rokka and Rantamäki, 2010), we attempted to develop a screening technique to identify potential candidates for
microencapsulation. For this study, GRAS bacteria were isolated from healthy broiler chickens. Isolates with GRAS status are recognized as safe to immediately administer to birds without the cost and delays associated with regulatory approval (FDA, 2013a). We also enhanced the motility characteristics of GRAS bacteria, as the recent reports from our laboratory demonstrated that motility enhanced isolates are more efficacious than unenhanced isolates in reducing Campylobacter colonization in broiler chickens (Aguiar, et al., 2013). The isolates which exhibited enhanced motility were then evaluated for their anti-Campylobacter activity in vitro. The motility enhanced isolates with in vitro efficacy against Campylobacter were administered both orally and intra-cloacally to day of hatch (day 1) birds to evaluate their efficacy against Campylobacter in chickens.

3.3 MATERIALS AND METHODS

3.3.1 In vitro studies

3.3.1.1 Isolation and identification of probiotic bacteria

Generally Regarded as Safe (GRAS) bacteria were isolated from the cecal contents of healthy broiler chickens of different age groups according to the procedure of Aguiar and co-workers (2013). Briefly, the procedure involved collection of ceca from healthy chickens under aseptic conditions. Cecal contents were then squeezed into sterile tubes and diluted with Butterfield’s Buffered Phosphate Diluent (BPD, Difco™ Becton Dickinson and company, Sparks, MD). Up to 3 dilutions were made using BPD (1:10, 1:100 and 1:1000) and 100µL of each dilution was plated on Blood Agar Plates (BAP, Difco™ Becton Dickinson and company, Sparks, MD) and incubated at 30°C for 24 hours. The isolated colonies were re-streaked on fresh Blood agar plates until pure cultures were obtained. Initial identification of isolated
colonies was done using Gram’s staining. Further identification was done using Biolog® system (Biolog, Inc. Hayward, CA) as per the procedure described (Biolog, 2004). Glycerol stock solutions of the isolates, which were identified as GRAS by FDA (2013b), were prepared and stored at -80°C (Bielke, et al., 2003).

3.3.1.2 Selection for motility enhancement

The process of selecting bacterial isolates for enhanced motility was described by Skaar et al., (1957) and according to their procedure, bacterial isolates can be motilized by growing the isolates on semi solid agar and selectively sub culturing the colony with greatest motility. In this study, selection for motility enhancement was done according to the procedure of Skaar and co-workers, with modifications as described by Aguiar and co-workers (2013). Briefly, the procedure involves growing the isolates in 5 mL of Tryptic Soy Broth (TSB, Difco™ Becton Dickinson and company, Sparks, MD) for 24 hours at 30°C. The isolates were then inoculated on Motility Test Media (BBL™ Becton Dickinson and company, Sparks, MD) with a sterile inoculation stick (VWR International LLC, Radnor, PA). The isolates were gently stabbed in the middle of the motility test plate and incubated at 30°C for 24 hours. Isolates exhibiting motility move from the point of stab towards periphery (Figure 1). To select for enhanced motility, the colony which migrated farthest from the initial point was picked using a sterile loop and inoculated into 5 mL of fresh TSB and incubated at 30°C for 24 hours. The procedure of selectively growing the colony that migrated farthest during each passage was repeated up to 10 times and the diameter during each pass was recorded. After the 10th passage, the isolates which demonstrated enhanced motility were identified using the Biolog® system and the glycerol stock solutions of such isolates were prepared and stored at -80°C (Aguiar, et al., 2013).
3.3.1.3 In vitro screening for anti-Campylobacter activity

The isolates with enhanced motility were further screened for anti-Campylobacter activity in vitro by using the soft agar overlay technique (Zhang, et al., 2007). For screening with soft agar overlay technique, a loop full of each isolate from the glycerol stock was inoculated into a separate tube containing 5 mL of TSB and incubated at 30°C for 24 hours. From the broth culture, 100 µL of each isolate was inoculated on a separate Tryptic Soy Agar (Difco™ Becton Dickinson and company, Sparks, MD) plate and incubated at 30°C. Simultaneously 2 passages of Campylobacter were also grown in Campylobacter Enrichment Broth (CEB, Acumedia® Neogen corporation, Lansing, MI) according to the procedure described in our earlier studies (Solis de los Santos 2008). A 100µL aliquot of Campylobacter culture was then added to 2 mL of soft agar at 45°C and then carefully overlaid on the TSA plate containing the isolate at the middle and incubated at 42°C for 48 hours. The isolates with efficacy against Campylobacter showed a clear zone of inhibition around the probiotic isolate (Figure 3). Additionally, we also tested the in vitro efficacy by co-culturing Campylobacter with individual isolate in 5 mL of TSB and incubate under microaerophilic conditions for 24 hours (Bhaskaran, et al., 2012). The cultures were then serially diluted and each dilution was plated on Campylobacter Line Agar (CLA, Line, 2001) and incubated at 42°C for 48 hours. Campylobacter colonies were enumerated and compared with control for in vitro screening. Isolates which demonstrated enhanced motility and reduced Campylobacter in vitro were selected and tested in vivo.

3.3.2 Animal studies

3.3.2.1 Experimental animals and housing
Day of hatch broiler chicks were obtained from a local commercial hatchery. Chicks were raised on floor pens with pine shavings and ad libitum access to both feed and water for the 14 day trial period.

3.3.2.2 Experimental design

Ten isolates with GRAS status demonstrating motility enhancement and anti-
Campylobacter properties in vitro were selected for in vivo testing. The treatments included a Campylobacter control (no isolate) and 20 treatments (n=10 birds/treatment), receiving one of the ten bacterial isolates on day of hatch either orally (10 treatments) or intra-cloacally (10 treatments). On day 7, all the birds were orally gavaged with 0.25mL of a 4 strain mixture of wild type of Campylobacter containing approximately $1 \times 10^7$ CFU/mL of organisms.

Before inoculating the isolates through intra-cloacal route, we performed a preliminary study to determine where the isolates would be deposited in the intestinal tract using this technique. For this study, 20 day of hatch chicks were inoculated with 0.25mL of a black food color (Americolor super black soft gel paste, Americolor™ Corporation, Placentia, CA) through the intra-cloacal route. A 3 inch long sterile stainless steel straight canula with a rounded tip (VWR International LLC, Radnor, PA) was lubricated and passed through the cloaca. The birds were held gently with one hand, and the canula was passed gently through the cloaca and 0.25mL of food color was deposited when 3/4th of the canula was inserted into the tract. No apparent stress was observed in the birds subjected to this procedure. The birds were euthanized immediately after depositing the dye, using carbon dioxide, and the intestinal tract was exposed to locate the site where the dye was deposited. This study was repeated twice (n=20 birds/study) to evaluate the consistency of this technique.
3.3.2.3 Bacterial strains and dose

On Day 1, each treatment group, except controls, were orally or intra-cloacally dosed with 0.25 mL of TSB containing at least $1 \times 10^7$ CFU/mL of an individual isolate. On day 7, all the birds were orally gavaged with a 4 strain mixture of wild type *Campylobacter* containing at least $1 \times 10^7$ CFU/mL organisms. On day 14, all the birds were euthanized using carbon dioxide gas and the ceca were collected aseptically for *Campylobacter* enumeration.

3.3.2.4 *Campylobacter* enumeration

Cecal *Campylobacter* counts were determined as per the procedure explained in our earlier studies (Bhaskaran, et al., 2011; Aguiar, et al., 2013). Ceca were collected on day 14 and the contents were serially diluted (1:10) with BPD. Each dilution was then plated on CLA and incubated under microaerophilic conditions for 48 hours at 42°C. Suspected *Campylobacter* colonies were confirmed by latex agglutination test (LATEX: CAMPY [jel]™ Test Kit, Scimedx corporation, Denville, NJ) and further confirmation was done with api® Campy (bioMérieux, Inc, Durham, NC)

3.3.3 Statistical analysis

Data were analyzed using PROC GLM procedure of SAS (SAS, 2011). *Campylobacter* colonies were enumerated and the counts for CFU/mL were then log transformed before analysis to achieve homogeneity of variance (Byrd, et al., 2003). Treatment means were partitioned by least square means analysis and a probability of $P < 0.05$ was considered for statistical significance.
3.4 RESULTS

In the preliminary study, birds inoculated intra-cloacally with dye demonstrated that the dye was deposited in the lower intestine (Figure 4). The dye in all birds consistently reached the lower intestine, including the ceca, the predominant site of *Campylobacter* colonization in poultry (Beery, et al., 1988), as well as depositing color through the ileum of the small intestine.

For this study 67 GRAS bacteria were isolated (all *Bacillus* spp.). Of the 67 isolates, only 10 isolates demonstrated both enhanced motility and anti-*Campylobacter* activity in the *in vitro* assays (Figure 2) and these isolates were tested *in vivo*. When orally dosed, only one isolate (Isolate 1) showed about a 1 log reduction in cecal *Campylobacter* counts compared to control (Figure 5). However, when these isolates were administered intra-cloacally, isolates 7 and 9 demonstrated a 1-2 log reduction in *Campylobacter* counts whereas isolates 6, 8 and 10 demonstrated a 3 log reduction in *Campylobacter* counts compared to the control (Figure 6).

3.5 DISCUSSION

Scientists started recognizing the importance of *Campylobacter* as a zoonotic disease in the 1970s (Butzler and Skirrow, 1979; Skirrow, 1977). Since then, there has been an increased focus on controlling this human pathogen. Since the discovery of the beneficial effects of probiotics, researchers have successfully developed probiotic cultures to control many enteric pathogens in humans and domestic animals (Corrier, et al., 1994; Hakkinen and Schneitz 1999; Pascual, et al., 1999; Casey, et al., 2007). However, probiotics developed to reduce *Campylobacter* in poultry had inconsistent results (Mead, 2000; Robyn, et al., 2013). One possible reason for such inconsistencies could be due to failure of probiotic bacteria to survive gastric acidity and reach the lower intestine to colonize and compete with *Campylobacter*. 
For this study, motile enhanced GRAS bacterium were isolated from the cecal contents of healthy birds and evaluated for their ability to reduce *Campylobacter* colonization in chickens. Our lab recently demonstrated that motility enhanced isolates are more efficacious (Aguiar, et al., 2013), apparently because they are better able to reach the enteric crypts, the predominant site of *Campylobacter* colonization (Beery, et al., 1988). The bacterial isolates may also inhibit *Campylobacter* by competing for nutrients, adhesion sites or by releasing antibacterial substances such as bacteriocins and/or volatile fatty acids (Barnes, et al., 1980; Pivnic and Nurmi, 1982; Nurmi, et al., 1992; Stavric, 1992; Corrier, et al., 1994; Callaway, et al., 2008). In the current study, only 1 of 10 isolates given orally to chicks demonstrated a 1 log reduction in cecal *Campylobacter* colonization in chickens (Figure 5). However, when these isolates were administrated directly into the lower intestine, 5 isolates reduced cecal *Campylobacter* counts when compared with controls (Figure 6). This difference is possibly due to fewer bacterial isolates given orally surviving passage through the acidic conditions in the stomach (Ding and Shah, 2009). Ding and Shah (2009) also report that even acid tolerant strains such as *Lactobacillus* spp., show a significant reduction in viable bacteria on exposure to acidic conditions. All the bacterial isolates used in the current study belong to the genus *Bacillus*. Even though *Bacillus* spp. were widely used as probiotics in humans and domestic animals, usually it is the spore form of *Bacillus* that has demonstrated the anti-bacterial activity against pathogens such as *E. coli*, *Salmonella*, *Clostridium* and *Listeria monocytogenes* (Mazza, 1994; La Ragione et al., 2001, 2003; Sanders, et al., 2003; Barbosa, et al., 2005; Hong, et al., 2005). Interestingly, it has also been demonstrated that the vegetative cells of the *Bacillus* spp. are susceptible to the acidic conditions in the stomach (Barbosa, et al., 2005). Isolates need to reach the lower intestine in sufficient numbers to inhibit *Campylobacter*. It has been proposed that
microencapsulation can protect probiotic isolates from modification in the upper GI tract and allow release in the lower intestinal tract (Kailasapathy, 2002; Anal and Singh, 2007; Rokka and Rantamäki, 2010). Recent studies by Matthes and co-workers (2010) supported the potential of rectal administration of probiotics as a treatment strategy to control ulcerative colitis in humans. It has been proposed that the rectally administered probiotic isolates produce beneficial effects by reducing the inflammation, modify microflora and enhance the mucosal immune response in patients with ulcerative colitis (D'Incà, et al., 2011; Oliva, et al., 2012). The mechanism by which rectally administered probiotics produce beneficial effects is similar to oral probiotics, however, rectally administered probiotics do not encounter stomach acids and destruction in the upper intestinal tract (Fedorak, 2010). Based on these research reports and our results, we propose that some probiotic isolates can reduce *Campylobacter* colonization in broiler chickens, if protected during transit in upper GI tract and are available in the lower intestinal tract. Although it is not practical to administer probiotic isolates through intra-cloacal route in a commercial poultry setting, this study supports the testing of isolates intra-cloacally as a screening procedure to evaluate their efficacy against enteric *Campylobacter*. This screening procedure can eliminate the time and expense of protecting isolates (e.g., encapsulation) which may not have efficacy even if they reach the lower intestine.

### 3.6 CONCLUSION

We developed a screening procedure to test the probiotic isolates by administering them intra-cloacally to evaluate their efficacy against enteric *Campylobacter*. Potential probiotic isolates thus identified, can be combined and given in a protected form (encapsulated) for more practical and efficient reduction of *Campylobacter* in broiler chickens.
3.7 REFERENCES


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Zhang, G., L. Ma, and M. P. Doyle. 2007. Potential competitive exclusion bacteria from poultry inhibitory to *Campylobacter jejuni* and *Salmonella*. J. Food Prot. 70:867-873.
Figure 1. Comparison of an isolate exhibiting different degrees of motility before and after selection for enhanced motility\(^1\) (Photograph by K. Arsi, taken on 13 August, 2012 at John W. Tyson building, Laboratory 324, Department of Poultry Science, University of Arkansas).

\(^1\)Selected GRAS bacterial isolates were screened for enhanced motility characteristics by passing the isolates continually for up to 10 times. The isolates were inoculated on a motility test plate and during each passage, the colony which moved farthest from the initial point is selected and passed in TSB. The procedure was repeated up to 10 times and isolates which exhibited enhanced motility after 10 passages were selected and further screened for anti-\textit{Campylobacter} activity \textit{in vitro}.

In Figure 1, (A) represents motility exhibited by an isolate prior to selection for enhanced motility and (B) represents increased motility exhibited by the same isolate after selection for enhanced motility.
Selection for motility enhancement was done by growing the isolates in TSB at 30°C for 24 hours and then inoculating the cultures by stabbing on the middle of a motility test media plate and incubated for 24 hours. The motile isolates migrate from the point of stab towards periphery. The colony which migrates farthest from the initial point was selectively picked and inoculated in 5 mL of TSB. The procedure was repeated for up to 10 times and the diameter of dispersion from the initial point was recorded during passage.

The isolates that exhibited enhanced motility along with *in vitro* anti-*Campylobacter* activity are selected for the *in vivo* study. Motility enhancement of the isolates used in the *in vivo* study are shown in Figure 2.
Figure 3. Soft agar overlay showing a clear zone of inhibition in a lawn of *Campylobacter* around the probiotic colony¹ (Photograph by K. Arsi, taken on 12 October, 2011 at John W. Tyson building, Laboratory 324, Department of Poultry Science, University of Arkansas).

¹The probiotic isolate was grown on the middle of a Tryptic soy agar plate (with in the inner circle). The plate with the probiotic was overlaid with 2mL of soft agar containing *Campylobacter* (~10⁶ CFU/mL) and incubated under microaerophilic conditions at 42°C for 48 hours.

A lawn of *Campylobacter* can be seen on the agar plate with a clear zone of inhibition around the probiotic colony.
Figure 4. Demonstration of the site of deposition of the dye when broiler chicken administered with 0.25mL of a black food color through the intra-cloacal route\(^1\) (Photograph by K. Arsi, taken on 30 July, 2013 at Poultry Research Farm, University of Arkansas).

\(^1\)Day of hatch broiler chicks were administered 0.25mL of black food color (Americolor super black soft gel paste) through the intra-cloacal route using a well lubricated sterile straight canula. The site at which the dye has been deposited was demonstrated by exposing the intestinal tract and compared with an uninoculated control bird.

In Figure 4, (A) represent the lower intestinal tract of the uninoculated control bird and (B) represent the bird inoculated with 0.25mL of food color and demonstrating the black food color in the ileum.
Figure 5. Effect of oral inoculation of bacterial isolates (numbered 1-10) on cecal *Campylobacter* counts in 14 day old broiler chickens\(^1\).

\[\text{Selected isolates with enhanced motility and anti-}\text{*Campylobacter* activity in vitro}\]

\(\begin{array}{cccccccccccc}
\text{Control} & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 \\
\hline
\text{Campylobacter colonization (Log CFU/mL)} & 6 & 7 & 8 & 9 & 8 & 8 & 9 & 8 & 8 & 8 \\
\end{array}\]

\(^1\)Birds in each treatment group except control were orally dosed with 0.25 mL of TSB containing at least 1x10^7 CFU/mL of an individual isolate on day 1. All the birds were orally challenged on day 7 with 0.25 mL of a 4 strain mixture of wild type *Campylobacter jejuni* containing ~1 x 10^7 CFU/mL of *C. jejuni*.

All *Campylobacter* data were log\(_{10}\) transformed for statistical analysis.

\(^{a,b,c,d,e}\) Means within treatment groups with no common superscript differ significantly (P<0.05).
Figure 6. Effect of intra-cloacal inoculation of bacterial isolates (numbered 1-10) on cecal *Campylobacter* counts in 14 day old broiler chickens

Birds in each treatment group except control were intra-cloacally dosed with 0.25 mL of TSB broth containing at least 1x10^7 CFU/mL of an individual isolate on day 1. All the birds were orally challenged on day 7 with 0.25 mL of a 4 strain mixture of wild type *Campylobacter jejuni* containing ~1 x 10^7 CFU/mL of *C. jejuni*.

All *Campylobacter* data were log_{10} transformed for statistical analysis.

Means within treatment groups with no common superscript differ significantly (P<0.05).
Attn  University of Arkansas Graduate School

March 15, 2014

Dear Sir,

I attest that Komala Arsi was first author of the manuscript cited below and completed at least 51% of the work for the paper.


Yours Sincerely,

Dan J. Donoghue,  
Professor,  
Department of Poultry Science  
POSC 0-114  
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Phone: (479) 575-2913  
Email: ddonoghi@uark.edu
MEMORANDUM

TO: Dr. Dan Donoghue
FROM: W. Roy Penney
Institutional Biosafety Committee

RE: IBC Protocol Approval
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cme/car

cc: Animal Welfare Veterinarian
CONCLUSIONS

Ever since the discovery of the zoonotic potential of *Campylobacter* organisms, several epidemiological investigations have revealed an increased risk of human infections associated with improper handling and/or consumption of contaminated poultry products. Over the years several intervention strategies have been investigated to eliminate or reduce *Campylobacter* from preharvest poultry with limited success. In this project, we evaluated the efficacy of probiotic bacterial isolates in reducing *Campylobacter* colonization in 14 day old broiler chickens. When we administered the probiotic isolates individually, they did not demonstrate a consistent reduction in *Campylobacter*. However, one of the selected isolates demonstrated a significant reduction when a prebiotic (Mannanoligosaccharide at 0.04%) was supplemented in the feed. Even though our strategy of combining probiotics with prebiotics seemed to be an effective strategy to reduce cecal colonization of *Campylobacter*, oral probiotics can produce variable results, possibly due to destruction in the acidic environment of the stomach. This hurdle can potentially be overcome by microencapsulating the probiotics, which protects the probiotic isolates from the harsh conditions in the upper gastrointestinal (GI) tract and may successfully deliver them in the lower intestinal tract. However, encapsulation procedures are costly and there is no assurance that these isolates will have efficacy in the lower GI tract. For the second study, we developed a screening method to determine the efficacy of potential isolates by directly placing them in the lower intestinal tract via cloacal inoculation. Our results demonstrated that probiotic isolates showed a significant reduction in *Campylobacter* when given intra-cloacally compared to oral administration. Based on our results, we propose that some probiotic isolates can reduce *Campylobacter* colonization in broiler chickens, if protected during transit through the upper GI tract and therefore available in the lower intestinal tract.
Thus, intra-cloacal inoculation of probiotics can be used as a screening procedure to evaluate their efficacy against enteric *Campylobacter*. Potential probiotic isolates thus identified, can be combined and given in a protected form (encapsulated) for more practical and efficient reduction of *Campylobacter* in broiler chickens.