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Microbiota Metabolic Product Deoxycholic Acid Prevents Campylobacter jejuni Chicken Colonization through Modulating Ceca Anaerobes

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Microbiota Metabolic Product Deoxycholic Acid Prevents *Campylobacter jejuni* Chicken Colonization through Modulating Ceca Anaerobes

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ABSTRACT

Campylobacter jejuni is a prevalent infectious enteritis mainly foodborne from chickens. Despite of reducing *C. jejuni* food contamination dramatically decreases the occurrence of campylobacteriosis, few effective approaches are available for the bacterial reduction in chickens. The aim of this study was to use microbial metabolic product deoxycholic acid (DCA) to reduce *C. jejuni* chicken colonization. Broiler chicks were fed 0 or 1.5 g/kg DCA, lithocholic acid (LCA), or urodeoxycholic acid (UDCA) in diets or orally gavaged with cholic acid (CA, 1.5g/kg body weight). Birds were also transplanted with DCA modulated anaerobes (DCA-Anaero) or aerobes (DCA-Aero). Birds were infected with 10^9 CFU/bird human clinical isolate *C. jejuni* 81-176 or chicken isolate *C. jejuni* AR101 and were weighed or sacrificed to enumerate cecal *C. jejuni* colonization levels. *C. jejuni* was culture in broth with various concentrations of DCA, CA, and taurocholic acid (TCA). DCA modulated microbiota were analysis by real time PCR at phylum level. Notably, *C. jejuni* 81-176 was readily colonized intestinal tract at 10^5 CFU/g cecal digesta at d16 and reached an almost plateau of $2.8x10^7$ CFU/g cecal digesta at d21. Remarkably, DCA excluded *C. jejuni* cecal colonization at 100, 99.997, and 100% at 16, 21, and 28 days of age. DCA also improved chicken growth performance of body weight gain compared to infected control birds (1.45 vs. 1.29 kg/bird) at d28. Interestingly, DCA failed to inhibit *C. jejuni* 81-176 *in vitro* growth. Neither chicken ages of infection nor CA, LCA or UDCA altered *C. jejuni* AR101 chicken colonization level, while DCA reduced 91% of the bacterium in chickens at d28. Notably, DCA diet induced a distinct microbiota composition of phyla firmicutes (82.7.1 vs. 98.8%) and bacteroidetes (16.9 vs. 0.8%) compared to infected control birds. Importantly, DCA-Anaero attenuated 93% of

C. jejuni colonization at d28 compared to control infected birds ($1.79x10^6$ vs. $2.52x10^7$ CFU/bird). In conclusion, DCA shapes microbiota composition against *C. jejuni* colonization in chickens, suggesting a bidirectional interaction between microbiota and microbial metabolites. Simultaneously reconstituting both microbiota and microbial metabolites may render better therapeutic effect against enteritis or pathogen colonization.

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DEDICATION

I would like to dedicate this work to my parents who inspired and encouraged me to achieve my goals.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

1.1 Introduction

Food-production animals are critical food sources to humans because animal products contain valuable and balanced protein as well as many other essential micronutrients such as iron, zinc, and vitamins B-12 and A (WHO, 2003). The fast growing population along with the urbanization has increased the consumption of animal products (Broglia and Kapel, 2011). It has been estimated that the consumption of animal products will be increased to 376 million tons per year by 2030 due to rising human population and globalization (Dhama et al., 2013). Broiler chicken meat is more cost effective than beef because the cost of one kg of red beef meat is three to four times the cost of broiler meat (Wahyono and Utami, 2018). Unfortunately, animal products often carry zoonotic pathogens that can reach humans and cause foodborne diseases. Foodborne pathogens include various bacteria such as *Salmonella* Typhimurium, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium perfringens,* helminths such as *Taenia solium* and protozoa such *Toxoplasma gondii* and *Cryptosporidium parvum* (Dhama et al., 2013). Despite various food safety strategies, 47.8 million foodborne sickness cases in the United States were recorded in 2013 which included 127,839 hospitalizations and 3,037 mortalities (Scallan et al., 2013). *Campylobacter jejuni* (*C. jejuni*) is one of the most common causes of human gastrointestinal illnesses in the development countries (Bronzwaer et al., 2009). Campylobacteriosis is mainly caused by poor preparation of chicken meat or consumption of contaminated meat (Tam et al., 2009). Many surveys revealed that the bacterial loads of *Campylobacter* were high in the retail chicken meat. For instance, one survey in Washington D.C. found that more than 70% of broiler meat samples contained

Campylobacter (Zhao et al., 2001). It was estimated that there are one million campylobacteroisis cases occur in the United States every year (Scallan et al., 2011), which cause the economic loss of $1.7 billion (Batz et al., 2012).

1.2 *Campylobacter jejuni*

1.2.1 History of *Campylobacter jejuni*

Theodor Escherish was the first scientist who acknowledged the presence of *Campylobacter* with a spiral shaped bacteria in stool samples of neonates with diarrhoea in 1886 (Escherich, 1886; Samie et al., 2007; Skirrow and Butzler, 2000). Two British veterinary surgeons reported several novel "Vibrio" species in a range of animals in 1906 and a new bacterial specie *Vibrio foetus ovid* was isolated from aborted lamb foetuses and their dams as early as 1909 (McFadyean and Stockman, 1909). A bovine strain of Vibrio spp. was isolated in 1938 from a milk-borne disease affecting humans and causing an outbreak in the USA (Levy, 1946). In 1947, Vincent isolated this organism (Vincent, 1947). Consequently in the USA, Vibrio spp. were isolated from 11 different patients who had gastroenteritis (King, 1957). Among the 11 isolates, seven strains were *V. fetus*, and the other four were closely related species, titled 'related vibrios'. Based on carbohydrate fermentation and DNA guanine-cytosine (GC) content, King reported that "related vibrios" groups were differentiated into two groups of Vibrio spp. (Sebald and Veron, 1963). The "related vibrios" group bacteria have less GC content and were assigned to a new genus *Campylobacter* indicating its 'curved rods' morphology. Since then, the knowledge on

Campylobacter has expanded and many *Campylobacter* genomes are now available in GenBank.

Campylobacter genus contains 29 species and 12 subspecies and belongs to proteobacteria, which also comprises *Helicobacter*, *Arcobacter* and *Wolinella* (On, 2001; Pitkänen and Hänninen, 2017). The *Campylobacter* genus included a group of closely related organisms including *C. upsaliensis, C. helviticus, C. jejuni*, *C. coli*, *C. lari*, and *C. curvisus* and most of them are known to colonize the gastrointestinal tracts of a variety of host species (Wassenaar and Newell, 2000). Wasssenaar and colleague also acknowledged that *C. jejuni* and *C. coli* of the *Campylobacter* spp. are the most important zoonotic human's enteric pathogens in industrialised countries. *C. jejuni* is the major pathogen of campylobacteriosis and is responsible for 95% of cases, whereas *C. coli* accounts for only 5% (Pitkänen and Hänninen, 2017). Clinical manifestations caused by different *Campylobacter* spp. are very similar (Gillespie et al., 2002). Various epidemiological studies have treated campylobacteriosis caused by different *Campylobacter* spp. as a single clinical entity because of lack of differential diagnostic tools (Siemer et al., 2005).

1.2.2 Morphology and biochemical characteristics

Campylobacter spp. are gram-negative curved rods, motile, non-spore forming bacteria (Lee and Newell, 2006). The size of *Campylobacter* spp. members ranged from 0.2 to 0.8 μm wide and 0.5 to 5.0 μm long and dividing cells attaching together often show spiral forms (Hansson, 2007). *C. jejuni* is highly motile bacterium due to the presence of a

flagellum at one end or both ends of the cell. The motility is characterised by rotating movement to rapid corkscrew-like motion, which requires use of their unipolar or bipolar flagella (Konkel, 2001). The bacterium is highly adaptive to the bird intestine, reflected by their adaptation to grow at varied temperature range of 30-44°C but an optimum growth is seen at of 42°C (Nielsen, 2010). *C. jejuni* requires oxygen concentration in a range of 3 to 15% and 3-5% carbon dioxide and so it is microaerophilic. Having relatively small genomes (1.6 – 2.0 megabases), *C. jejuni* establishes long term associations with their hosts and biochemically the bacterium is catalase and oxidase positive and urease negative (Parkhill et al., 2000). *C. jejuni* hydrolyses hippurate, but *C. jejuni* subsp. *doylei* varies in its capability to hydrolyse hippurate (Vandamme et al., 1992), hence various other additional biochemical tests are used to differentiate *Campylobacter* spp.

1.2.3 Virulence factors of *Campylobacter jejuni*

While some *Campylobacter* spp. have been studied and fully sequenced, their likely pathogenicity, host association, population diversity, and epidemiology are still not clear. When colonizing the intestine, *C. jejuni* expresses numerous virulence factors and establishes an infection. Below are emphasised virulence factors which are important for successful infection and host interaction (Table 1.1).

1.2.3.1 *C. jejuni* flagellin, motility, adherence, and invasion

Motility is important for any organism to establish an active infection. The existence of flagellin is essential for the bacteria to initiate and expand colonization (Wassenaar et al., 1993; Nachamkin et al., 1993). *C. jejuni* infiltrate the mucus barrier of host intestine by its polar flagella at one or both ends which form cork-screw movement and allow them to enter into mucus (Szymanski et at., 1995). The flagellum has two sub units *flaA* and *flaB* endangered to antigenic variation and phase variation (Guerry et al., 1991). Apart from these two proteins several other proteins are also involved in flagellar expression (Parkhill et al., 2000).

Adhesion to the host cells is the first step for pathogens successfully infecting the host, followed by colonization and invasion (Pizarro-Cerda et al., 2006). CadF on *C. jejuni* is an important adhesion molecule. CadF mediates adherence in *C. jejuni* by binding to fibronectin on the intestinal epithelial cells, and facilitates the bacterial colonization in chickens (Konkel et al., 1997). Another essential *C. jejuni* adherence molecule is JlpA which adheres with heat shock protein on epithelial cells (Jin et al., 2003).

Once *C. jejuni* is able to infiltrate the barrier and have entered into the mucus layer, it requires to adhere to the surface for long term foundation and invasion. Biopsies studies of early *C. jejuni* invasion into intestine showed that the bacterium has the ability to invade gut tissue cells (Van Spreeuwel et al., 1985; Oelschlaeger et al., 1993).

The bacteria enter through interrupted tight junctions of epithelial cells (Grant et al., 1993; Bras et al., 1999). Here either it can reinvade the epithelial cell, replicate and induce apoptosis or be phagocytised by macrophages (Kiehlbauch et al., 1985). If *C. jejuni* is able to reinvade into intestinal epithelial cells, it exists within a membrane bound

compartment. Over the time of its residence, it acquires a metabolic state which renders an uncultivable status of this bacterium (Kiehlbauch et al., 1985; Humphrey et al., 1986; Russell et al., 1994). This state will possibly be intermittent by a conditions of oxygen limitation, and indicate that *C. jejuni* becomes oxygen sensitive or alters its respiration mode inside the epithelial cells (Watson and Galan, 2008).

1.2.3.2 Biofilm formation and quorum sensing

Biofilms are defined as multicellular layers of bacteria embedded within a matrix of extracellular polymeric substances to persist in unfavourable environment (Donlan and Costerton, 2002). Among the strategies to resist, biofilm is a life-style known to protect bacteria from various environmental stresses, antimicrobial agents and also increased bacterial resistance to host immune response (Donlan and Costerton, 2002; Chmielewski and Frank, 2003). Inside the biofilm, bacteria is protected from disinfectants and antimicrobials and become more resistant compared to their planktonic counterparts (Fux et al., 2005). The formation of biofilm may significantly increase the survive ability of bacteria to unfavourable environment, such as the formation of biofilm by *C. jejuni* in response to oxygen exposure (Murphy et al., 2006). *C. jejuni* strains have been reported to form different types of biofilm characterized as a structure attached to a surface, a pellicle formed at the surface of the liquid, or aggregates floating in the liquid culture (Joshua et al., 2006). Quorum sensing (QS), a cell-to-cell communication system helps in various *C. jejuni* virulence factors such as biofilm formation regulation (Reeser et al., 2007), autoagglutination (Jeon et al., 2003; Guerry et al., 2006), swarming motility (Jeon et al., 2003), transcription of cytolethal discending toxin (CDT) (Snelling et al., 2005),

and colonization of chickens and sensitivity to hydrogen peroxide (Quinones et al., 2009).

1.2.3.3 Cytolethal distending toxin and haemolysin

C. jejuni secretes cytolethal distending toxin (CDT) which is a protein with subunits of CdtA, CdtB and CdtC. These three units together block cell division by arresting G2-phase cell cycle (Lara-Tejero and Galan, 2001). Two units of CdtA and CdtC bind to the cell surface and facilitate the delivery of the active subunit CdtB. CdtB uses its DNase-I-like activity to cleave dsDNA molecules at G1 and G2 stage (Whitehouse, 1998). The toxin also provides the bacterium an evasion from host immune response. In addition, a few studies have reported *C. jejuni* haemolytic activity (Arimi et al., 1990) by which the bacterium is able to haemolysed the red blood cell membrane, and liberated heme is used as iron source (Reid et al., 2008). *C. jejuni* has two genes associated with haemolytic activity including *ceuE* and *pldA*. *CeuE* functions as a periplasmic binding protein and is a part of an ABC transport system. *pldA* is a gene encoding an outer membrane phospholipase A protein and has been studied in *C. coli*, and found its contribution in cell-associated haemolysis (Grant et al., 1997).

1.3 Campylobacteriosis

1.3.1 Campylobacteriosis in humans

C. jejuni is the major cause of campylobacteriosis in human, followed by *C. coli*, *C.upsaliensis*, *C. lari* and *C. fetus* (Acheson and Allos, 2001). Consumption of undercooked chicken meat or contaminated food is the main sources of campylobacteriosis

disease in humans (Skirrow and Benjamin, 1980a; Skirrow and Benjamin, 1980b). Both tamed (Blaser et al., 1980) and wild animals (Kapperud and Rosef, 1983) are work as reservoirs for the bacteria. The other modes of transmission comprise of contacting with faecal material, exposing from animals (specifically ruminants), or entertaining swimming (Mullner et al., 2009). In addition, contaminated water (Richardson et al., 2007), milk (Robinson and Tones, 1981), seafood (frost, 2001), vegetables, and fruits (Pielaat et al., 2014) are considered to be vehicles of the bacteria to humans and other animals. A dose as few as 800 colony forming unit (CFU) is able to induce the illness in humans (Black et al., 1988). Other studies were able to report the illness with 360 CFU (Hara-Kudo and Takatori, 2011). It's very important and critical to understand the comparative influence of each transmission pathway to decide a protective and effective control measurement.

1.3.2 Campylobacteriosis clinical symptoms and complications

The incubation period ranges from 2-5 days, but has been reported up to 10 days (Butzler, 2004). In human, manifestations are varied including acute gastroenteritis, cramping, abdominal pain, fever, and more or less, vomiting and headaches (Snelling et al., 2005). Campylobacteriosis is usually a self-limiting disease and the patients may recover with no medical intervention (Rosenquist et al., 2003). However, some patients may develop several complications. Guillain-Barre Syndrome (GBS) is common in post-infectious complications and causes flaccid paralysis affecting peripheral and cranial nerves in severe cases (Pithadia and Kakadia, 2010). Miller Fisher syndrome is variant of GBS, causes a non-paralytic condition with inability to move the eyes and nonreactive pupils (Fisher, 1956). Reactive arthritis is also noted in association with *C. jejuni* post-

infection (Nielsen, 2010). In addition, *Campylobacter* spp. have been related to other gastrointestinal manifestations such as inflammatory bowel diseases (IBD), Barrett's esophagus, and colorectal cancer, and in rare case extragastrointestinal manifestations such as bacteremia, lung infections, brain abscesses, and meningitis (Man, 2011).

1.3.3 Risk factors for campylobacteriosis

Although consumption of contaminated poultry products and other foods is the major source of campylobacteriosis, other risk factors contribute to the infection. Traveling is an important risk factor. In the United States, international travel was responsible of 18% of *Campylobacter* infections between 2005 and 2011 (Ricotta et al., 2014), and domestic travel in the USA increased the incidence of the infection in 2008 (OR: 2.5; 95%CI= 1.4–4.6) (Denno et al., 2009).

Other pathogen infections often lead to the susceptibility to campylobacteriosis in humans. In developing countries, *Campylobacter* infection is frequently associated with other enteric pathogens infection such as *E. coli* and *Rotavirus*, but it was rare in developed countries (Coker et al., 2002). HIV patients are highly susceptible and show severe illness comparing with non-HIV patients (Tee and Mijch, 1998). Living in rural areas where farm animal density is high increase the incidence of the disease (Arsenault et al., 2012).

In addition, studies found that sex and age are associated with *Campylobacter* infection in humans. By using FoodNet data on Campylobacteriosis infection incidence in the United States between 1996 and 1999, Samuel et al. (2004) found that campylobacteriosis was higher in males of all ages. In Canada, Green et al. (2006) found the highest rate of *Campylobacter* infection to be among 0–4 years old and 20–39 years old, with slightly

higher rates occurring in males (Green, 2006). However, the reason behind such high incidence in males compared to females is not fully understood (Friedman, 2000). In Germany, the study conducted by Fitzenberger et al. (2010) corroborates this pattern: campylobacteriosis incidence being highest among those under five years of age (61 cases/100,000), and those aged 15–44 years (56 cases/100,000) (Fitzenberger, 2010). Fernandez et al. 2008 found that children suffering from malnutrition were more frequent carriers of *Campylobacter* spp. (31.4%) than normal children (9.9%) in Chile.

1.3.4 Treatment and antibiotic therapy

Although in normal illness most of campylobacteriosis related diarrheal infection are self-limiting, certain cases require antibiotic treatment such as erythromycin (macrolide antibiotic) or ciprofloxacin (fluoroquinolones) (Balfour and Faulds, 1993). Multidrug-resistant (MDR) *Campylobacter* in humans (Kaakoush et al., 2015) as well as in domesticated animals (Qin et al., 2011) has raised concerns about the usage of antibiotics. In recent years, a number of studies report the increasing number of patients infected with strains resistant to erythromycin and ciprofloxacin (Engberg et al., 2001), and such individuals may have worse clinical outcomes (Helms et al., 2006) such as Guillain-Barre Syndrome (GBS), Miller Fisher syndrome, intestinal haemorrhage (Chamovitz et al., 1983), toxic megacolon (McKinley and Carlton, 1980). Recently, a few studies have been conducted to prevent and treat camylobacteriosis using antibiotics alternative. mTOR pharmacological inhibitor is able to prevent and treat campylobacteriosis in mice (Sun et al., 2012). Microbial metabolic product deoxycholic acid prevents and treat *C. jejuni*-induced colitis in mice (Sun et al., 2018).

1.4 Preventing *C. jejuni* colonization in chickens

1.4.1 *C. jejuni* colonization in chickens

Poultry has been concluded as the main source of infection (Friedman et al., 2000). In 2002, surveys in the United States and in the United Kingdom, 68%–83% of broiler carcasses contained the bacteria (Jorgensen et al., 2002). Cattles, chickens, and sheep carry the *C. jejuni* bacteria as asymptomatic carriers (Newell and Fearnley, 2003; Devane et al., 2005), where pigs and sheep have been identified as reservoirs of *C. coli* (Parsons et al., 2010; Nesbakken et al., 2003).

Chickens are considered as the natural host of *C. jejuni* (Yogasundram et al., 1989). Stern and colleagues in their one year survey study reported that approximately 90% of the flocks were positive to *C. jejuni* in the United States (Stern et al., 2001). In the UK, another survey during 2014 to 2015 showed that more than 72.5% of the broiler chicken carcasses at the retail stores were contaminated with *C. jejuni* (FSA, 2014). In Italy, a survey reported that 60% of the broiler chickens were carrying the pathogen (Comin et al., 2014). During 2009, 76% of broiler chickens meat carried *Campylobacter* at the retail stores in France (Guyard-Nicodème et al., 2015). This high load on broiler meat is due to the fact that *C. jejuni* bacteria spread rapidly between the birds (Newell and Fearnley, 2003), and the infected birds show no signs of illnesses (Lee and Newell, 2006). In addition, cross contamination between the negative and positive flocks can occur at the processing level and leads to increase the spreading the bacterium (Skirrow, 1990)

C. jejuni is ubiquitous at the area surrounding the farms (Newell and Fearnley, 2003). Most broiler flocks become infected with *C. jejuni* at 2 to 3 weeks of age (Evans and Sayers, 2000). Ringoir and colleagues proposed that the susceptibility to the pathogen in 2 day old chicks was higher than 2 weeks old chicks (Ringoir et al., 2007). In agreement with this, Cawthraw and Newell found that 1 day old broiler chicks were more susceptible to the pathogen in comparing with 1 week old chicks (Cawthraw and Newell, 2010). Horizontal transmission of the bacteria between the birds occurred in a very fast manner, and the majority of birds in the same flock become positive within days (Shreeve et al., 2000). Once the infection occurs, *C. jejuni* is able to proliferate in a high rate and reaches levels as high as 10^9 CFU in the ceca (Wassenaar et al., 1993). Beside the cecum, *C. jejuni* colonizes the mucus layer of the small intestine, and can reach to the liver and the spleen of the infected birds (Newell and Fearnley, 2003).

Infected birds shed the bacteria in feces and contaminate the surrounding area such as the litter, feed and water (Newell and Fearnley, 2003). Worker activities at the farm, rodents, flies, and other farm and wild animals are important vectors of the bacteria (Newell and Fearnley, 2003), since infected birds can shed high numbers of the bacteria and contaminate the soil, water sources, dust, building surfaces, and the air that surrounds the farm area (Kaakoush et al., 2015). *C. jejuni* can survive up to 6 days in chicken feces which can contaminate the environment when used as fertilizer (Ahmed et al., 2013).

It appears that the incidence of the infection in free-range chickens is significantly higher than the intensively raised chickens, and can reach as high as 100% of the flock (Heuer et al., 2001). Heuer and colleagues suggested that the abundance of the pathogen in the

environment which causes more exposure to the chickens is the reason of the higher positivity of such chickens. One study found that the prevalence of *Campylobacter* species in organic broilers chickens is three times higher than in conventionally raised broilers (54.2% versus 19.7%) (Rosenquist et al., 2013).

The ability of *Campylobacter*-positive lying hens to pass *C. jejuni* to their offspring through eggs is still controversial (Newell and Fearnley, 2003). The late infection ages of chicks supports the fact that broiler chicks gain *C. jejuni* from the environment rather than parent flocks, comparing with vertical transmission in *Salmonella* spp., in which chicks become infected at day one of age (Newell and Fearnley, 2003). Studies have been able to isolate *C. jejuni* from both male (Cox et al., 2001) and female reproductive systems (Camarda et al., 2000). In addition, other studied were able to infect chicken eggs with *C. jejuni*. For instance, in one study, the eggs were exposed to a suspension contained *C. jejuni*. The results showed that 4% of the eggs were infected, indicating the possibility of transmitting the bacteria to the eggs from feces (Allen and Griffiths, 2001). Nonetheless, *C. jejuni* remains in the egg shells and do not contaminate the inner contents of the eggs (Niell et al., 1985). No study have found strong evidence for the vertical transmission of the bacteria (Petersen et al., 2001; Sahin et al., 2003; Callicott et al., 2006). More research need to be conducted to strongly prove whether the vertical transmission is possible or not. Furthermore, poultry is also an important reservoir of other *Campylobacter* species, such as *C. upsaliensis*, and *C. concisus* (Kaakoush et al., 2014), as well as *C. lari* (Hariharan et al., 2009).

1.4.2 Preventions of *C. jejuni* chicken colonization by biosecurity, bacteriophage, vaccination, and probiotics

Controlling *Campylobacter* in poultry represents an immense challenge to the agriculture and food industries (Newell et al., 2010) as these bacteria are well adapted to avian species such that they may be considered commensal organisms of poultry. Following are some of current prevention approaches.

Bacteriocins are toxic peptides produced by certain bacteria and are able to kill or prevent the growth of other bacteria (Cotter et al., 2013). Administration of bacteriocin-producing bacterium *Paenibacillus polymyxa* or bacteriocins to chickens infected with 10^8 CFU/bird of four different strains of *C. jejuni* (AL-22, BH-6, Bl-1, CL-11) at day one of age, reduces the bacterial colonization from $10^{4.6}$ -$10^{6.3}$ CFU to an undetectable limit at day 10 of age (Stern et al., 2005). However, peptide bacteriocins can be degraded by the host digestive system (Joerger, 2003) and it remains elusive how it evades intestinal digestion and prevents *C. jejuni* colonization. The development of bacterial resistance to bacteriocins is another concern for applying it to reduce *C. jejuni* chicken colonization (El-Shibiny et al., 2009).

Since farm workers are important vehicles for the bacterium dissemination, applying hygienic standards such as washing hands, using shoes covers, dipping the feet in disinfectant solutions before and after dealing with chickens have showed to reduce cross contamination between chickens (Newell et al., 2011). Applying chemicals such as organic acids, acidified sodium chlorite, trisodium phosphate, or UV light during primary processing, freezing or irradiating at the slaughterhouses has showed reduction in

Campylobacter numbers on chicken carcasses (Wensley and Coole, 2013). Its high cost and negative effects on the meat quality have reduced its applications (Saxena et al., 2013).

Bacteriophages are bacterial viruses that have been used to control infectious bacteria such as *Shigella dysenteriae* since 1919 (Chanishvili, 2012). Bacteriophages have also been studied for use in the biocontrol method of *Campylobacter* species. Bacteriophage therapy was able to reduce *Campylobacter* counts by approximately 2 log10 cfu/g in a study in 2010 (Carvalho et al., 2010). However, in two of three field trials, a phage cocktail of $\log_{10}$ 5.8-7.5 pfu/bird applied to birds of 31 to 36 days old fails to reduce *Campylobacter spp.* colonization at 42 days of age (Kittler et al., 2013). Even though these results are promising, *Campylobacter* quickly develops resistance against the bacteriophages and is able to grow and reach the same pretreatment numbers (Hermans et al., 2011).

Vaccination is the administration of a killed or attenuated pathogen or parts of it to trigger the host immune system. Many efforts have been done to develop effective vaccines against *C. jejuni*, such as flagellar proteins vaccine, attenuated *C. jejuni* vaccine, and killed *C. jejuni*, however, no vaccine has successfully prevented the bacteria from colonizing the chicken gut (Kaakoush et al., 2015). In a study, birds vaccinated with *C. jejuni* 81–176 nanoparticle-encapsulated outer membrane proteins at d 7 and 21 and challenged with 1×10^8 cfu *C. jejuni* 81–176 at 35 days of age were resistant to the infection (Annamalai et al., 2013). *C. jejuni* cecal counts in the vaccinated birds stay below the detection limit at 42 days of age in the groups that received the vaccine compared to 10^6 CFU/g feces in control birds. More research is needed in this field in order to develop an effective chicken vaccine against *C. jejuni*.

1.4.3 Preventions of *C. jejuni* chicken colonization by microbiota

The microbiota defines as the commensal, symbiotic and pathogenic microorganisms that colonize the gastrointestinal tract and other areas of human and animal bodies (Sender et al., 2016). Once hatched, the chicks acquire microbiota from the environment at the hatchery (Pedroso et al., 2005). Studies have showed that chicken embryos gain microbiota even before the hatching phase either directly from the oviducts of their mothers (Gantois et al., 2009) or indirectly from the environment through the pores in the eggshells (Roto et al., 2016). Comprehensive dataset analysis showed that 915 operational taxonomic units (OTUs) were presented in broiler gut (Wei and Morrison, 2013). They showed that thirteen phyla are present including Firmicutes (70%), Bacteroidetes (12.3%) and Proteobacteria (9.3%), which accounting for more than 90% of all the sequences. The other phyla are Actinobacteria, Cyanobacteria, Spirochaetes, Synergisteles, Fusobacteria, Tenericutes, and Verrucomicrobia. Additionally, 117 genera were described, mostly *Ethanoligenes, Clostridium, Ruminococcus, Lactobacillus, Desulfohalobium* and *Bifidobacteriu*.

Gut microbiota is important to the digestive system function, host metabolism, and host immune function (Ballou et al., 2016). Gut microbiota is also able to digest complex indigestible carbohydrates such as polysaccharides, oligosaccharides, and disaccharides and converts them to simple to sugars, which can be further fermented to short chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate (Pan and Yu, 2014). Chicken gut microbiota contributes crucial nutrients to the host such as ammonium, amino acids, and some vitamins (Pan and Yu, 2014). Chambers and Gong found that SCFAs increase the development and growth of enterocytes, which leads to bigger intestines and ceca in conventional broilers compared to germ-free boiler chickens (Chambers and Gong, 2011).

Competitive exclusion is an important strategy of the intestinal microbiota against intestinal pathogens (Gabriel et al., 2006). Attachment of the pathogens to the epithelial layer of the intestine is the first step of the pathogens in birds (Lan et al., 2005). The intestinal mucosa in healthy birds is covered by a layer of microbiota that can effectively block the attachment sites and competing with pathogens (Lawley and Walker, 2013). Microbiota are also able to produce substances that inhibit the growth of some pathogens, such as hydrogen peroxide (H_2O_2), diacetyl, bacteriocins and organic acids (Clavijo and Flórez, 2017). Organic acids such as lactic, acetic, or propionic acid, increase the acidity in the intestine and decrease the proliferation of the pathogenic bacteria (Blajman et al., 2015).

Nurmi and Rantala in 1973 first highlighted the relationship between susceptibility to Salmonella infection and the late development of the gut microbiota in chickens. Research have been actively conducted to they are effective in controlling *salmonella* and *C. jejuni* infection in chickens using chicken origin microbiota, the administration of microbiota failed to reach the same results in *C. jejuni* colonization in chickens (Mead, 2000). *C. jejuni* colonization influences cecal microbiota with reductions in the relative abundance of family Lactobacillaceae and the *Clostridium* cluster XIVa (Connerton et al., 2018). Microbiota metabolites Short-Chain Fatty Acids (SCFAs) are sensed by *C. jejuni* for discriminating different spatial areas of intestines to colonize ceca of chickens (Luethy et al., 2017). Interestingly, probiotics Bifidobacterium longum PCB133 and a xylo-oligosaccharide failed to reduce *C. jejuni* chicken colonization using plate enumeration (Baffoni et al., 2017).

1.4.4 Microbial metabolic product bile acids

Bile acids with steroidal structure are synthesized from cholesterol in the liver and secreted into the bile with cholesterol, phospholipids and bilirubin. Bile acids are biosynthesized by hepatocytes and secreted into the bile canaliculi and subsequently in the gall bladder (Thomas et al., 2008). The biosynthesize of primary bile acids in the liver can be accomplished by two different pathways. The main pathway, which is responsible for the production of up to 75% of the bile acids, is known as the classical or neutral pathway, where the cholesterol undergoes 7α-hydroxylation process in the presence of 7-α-hydroxylase (CYP7A1) (Thomas et al., 2008). The other pathway is called the alternative or acidic pathway which is catalyzed by the enzyme sterol-27-hydroxylase (CYP27A1) (Russell, 2003). The primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA) which are typically conjugated to glycine or taurine before secretion (Hofmann et al., 2010). In response to host meal, biles flow out of liver in the gall bladder, and are released into the upper intestine (Oelkers et al., 1997). In the host intestine, bile acids work as detergents which emulsify fats, and assist in digestion, absorption and excretion. In addition to the digestion function, there are other roles of bile acids in the host that have been recognized, in particular via their role as endogenous ligands for cell receptors such as the nuclear receptor farnesoid X receptor (FXR), and the G protein-coupled plasma membrane bile acid receptor TGR5 (Jia et al., 2018). Bile acids themselves act as signalling molecules and regulate their own synthesis, and control the synthesis of cholesterol in the body (Lu et al., 2000). As FXR and TGR5 agonists, bile acids participate in some physiological processes in the host including the modulation of lipid, glucose and energy homeostasis, and the regulation of their synthesis, conjugation and transport (Mullish et

al., 2018). After their function in digestion, more than 95 % of bile acids are reabsorbed and sent back to the liver via portal circulation (Pellicoro et al., 2007). The primary bile acids CA and CDA are regularly transformed to the secondary bile acids (deoxycholic acid and lithocholic acid, respectively) by colonic bacteria (Nagengast et al., 1995)
(Figure 1.1).

1.4.5 Bile Acids and microbiota interaction

The intestinal microbiota is composed of several thousands of species, which together maintain host gut physiology and homeostasis (Tremaroli and Bakhed, 2012). The composition of the gut microbiota is affected by bile acids (Kakiyama et al., 2013). Bile acids cause disruption of the bacterial cell membranes, damaging the DNA and alteration in the protein conformation within the bacteria (Urdaneta and Casadesus, 2017). Due to the detergent activity of the biles, and the lipophilic nature of bacterial membranes, they represent one of the main targets of bile (Kurdi et al., 2006). Furthermore, bile acids are able to chelate with some essential ions of the gut microbiota such as iron and form Fe2+– bile salt complexes (Symeonidis and Marangos, 2012). Complex and significant changes in the gut microbiome are observed when rats are fed bile acids (Islam et al., 2011). For example, Islam and colleague added 1.25 mmol/kg and 5 mmol/kg of CA into rat diet. CA diet increased rat gut phylum Firmicutes between 93-98% compared to 54% in control rats while *Bacteroidetes* and *Actinobacteria* in CA rat decreased (Islam et al., 2011). Studies have demonstrated that alteration in gut microbiota or “dysbiosis” by too much bile acids have been associated with chronic liver disease and cirrhosis (Ridlon et al., 2013). Cirrhosis patients show reduced levels of bile acids as well as alteration of the gut microbiome, often

associated with decreased abundance of 7α-dehydroxylating Gram-positives like *Lachnospiraceae*, *Ruminococcaceae*, and *Blautia* in feces (Kakiyama et al., 2013).

Bile acids are biotranformed or metabolized by gut microbiota. Briefly, the first stage of bile acid modification by the gut microbiota is from the enzymes named bile salt hydrolases (BSHs) (Mullish et al., 2018). These enzymes deconjugate the taurine or glycine groups from conjugated bile acids via a hydrolysis reaction, and therefore form the primary bile acids CA and CDCA (Mullish et al., 2018). The second enzymatic steps are the removal of the hydroxyl group of C-7 from the primary bile acids and convert them into secondary bile acids in a process called 7-α-dehydroxylation (Mullish et al., 2018).

In order for the gut microbiota to survive in such harmful effect of BAs, they have developed several resistance mechanisms. The enterobacterial common antigen (ECA), which is a family-specific glycolipid present in the outer layer of *Salmonella enterica* membrane, is an example of such adaptation. It is found that a mutant *Salmonella enterica* with an ECA synthesis gene is more sensitive to bile acids (Ramos-Morales et al., 2003). When bile acids insert into the cytoplasm of the bacteria, another adaptation mechanisms takes place by the active extrusion of the bile acids by efflux pumps (Piddock, 2006). The efflux system AcrAB–TolC is the best characterized mong the efflux systems in enterobacterial spp. (Nikaido et al., 2008). In addition, Begley et al found that the deconjugation process of the bile acids that occur in the presence of BSH in wild type *Lactobacillus amylovorus* and *Lactobacillus plantarum* made them more tolerant to the bile acids in comparison with mutant BSH of the same bacteria (Begley et al., 2005). The presence of bile triggers the expression of virulence gene expression in several pathogenic bacteria that infect the digestive tract (Sistrunk et al., 2016). For instance, primary bile

acids promote the germination in the spore-forming bacteria *Clostridium difficile* (Sorg and Sonenshein, 2010). In *Escherichia coli*, one example of bile resistant is the efflux pump mechanisms, such as AcrAB-TolC (Sengupta et al., 2014). In *C. jejuni*, the bile salts trigger the expression of the CmeABC efflux pump (consists of a single periplasmic protein (CmeA), an inner membrane transporter (CmeB), and a single outer membrane protein (CmeC)), and leads to increase the tolerance of the bacteria against bile acids (Lin et al., 2003). According to Lin et al, mutant CmeABC *C. jejuni* bacteria were unable to colonize chicken intestine (Lin et al., 2003).

1.4.6 Bile Acids on health and diseases

Beside their known digestive function as emulsifier to lipids, more attention has been raised about bile acids function as signalling molecules in the body which may lead to develop drugs for chronic diseases such as obesity, diabetes and atherosclerosis. (Thomas et al., 2008). In addition, bile acids are prescribed to patients suffer from deficiency in bile acids production (Hofmann, 1999). FDA approved deoxycholic acid for the treatment of submental fat to reduce facial fullness or convexity through disrupting adipocytes (Sykes et al., 2017). Another example is the usage of urodeoxycholic acid to treat patients with congenital abnormal bile acids (Hofmann, 1999), patients with primary biliary cirrhosis (Pares et al., 2006), and gallstone (Montini et al., 1994).

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APPENDIX

Table 1.1 Virulence genes of *C. jejuni*

Adhesin	**In vitro cell line**	**Important for**	**Reference**
CadF	Int407	chicken	Monteville et al., 2003; Konkel et al., 1997
FlpA	Int407	chicken	Konkel et al., 2010
CapA	Caco-2	chicken	Ashgar et al., 2007
JlpA	HEp-2	chicken	Jin et al., 2001, Flanagan et al., 2009
PEB1	HeLa	mouse	Kervella et al., 1993
PEB4 (surA)	Int407	mouse	Asakura et al., 2007
Cj1496c	Int407	chicken	Kakuda and DiRita, 2006
MOMP	Int407	chicken	Moser et al., 1997
CPS	Int407, Caco2	chicken; Mouse	Bacon et al., 2001, Jones et al., 2004
LOS	Int407	chicken	Fry et al., 2000, Müller et al., 2007,

CadF: Campylobacter adhesion to fibronectin, FlpA: fibronectin-like protein A, CapA: Campylobacter
adhesion protein A, JlpA: jejuni lipoprotein A, PEB1 and 4: protein Pei, Ellison and Blaser 1 and 4,
MOMP: Major outer membrane protein, CPS: Capsular polysaccharide, LOS: Lipooligosaccharide, ND:
Not determined

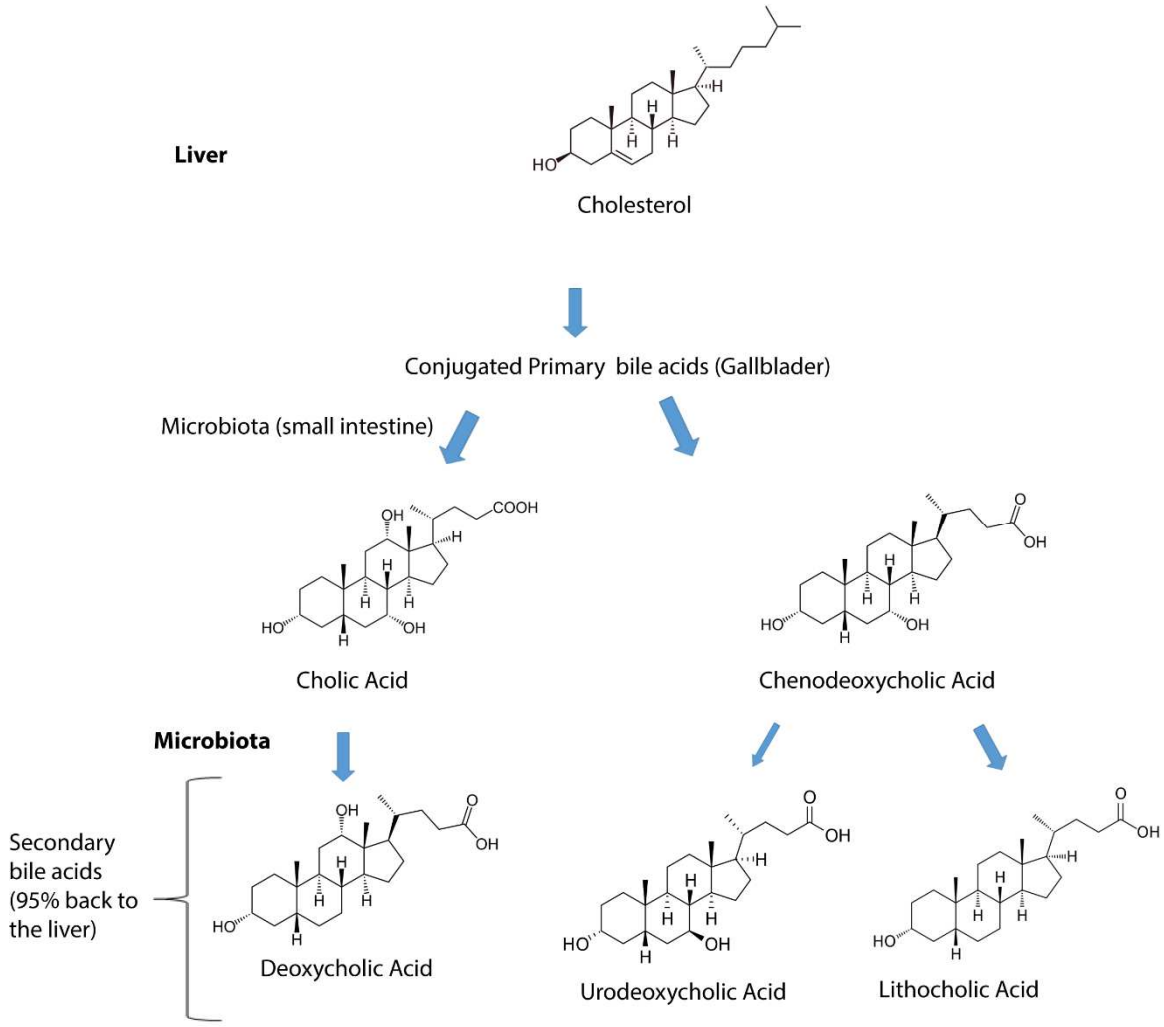

Figure 1.1 Flow chart demonstrates the structures of primary and secondary bile acids with their synthetic pathways.

CHAPTER II

MICROBIAL METABOLITE DEOXYCHOLIC ACID SHAPES MICROBIOTA AGAINST *CAMPYLOBACTER JEJUNI* CHICKEN COLONIZATION

2.1 Abstract

Campylobacter jejuni is a prevalent infectious enteritis mainly foodborne from chickens. Despite of reducing *C. jejuni* food contamination dramatically decreases the occurrence of campylobacteriosis, few effective approaches are available for the bacterial reduction in chickens. The aim of this study was to use microbial metabolic product deoxycholic acid (DCA) to reduce *C. jejuni* chicken colonization. Broiler chicks were fed 0 or 1.5 g/kg DCA, lithocholic acid (LCA), or urodeoxycholic acid (UDCA) in diets or orally gavaged with cholic acid (CA, 1.5g/kg body weight). Birds were also transplanted with DCA modulated anaerobes (DCA-Anaero) or aerobes (DCA-Aero). Birds were infected with 10^9 CFU/bird human clinical isolate *C. jejuni* 81-176 or chicken isolate *C. jejuni* AR101 and were weighed or sacrificed to enumerate cecal *C. jejuni* colonization levels. *C. jejuni* was culture in broth with various concentrations of DCA, CA, and taurocholic acid (TCA). DCA modulated microbiota were analysis by real time PCR at phylum level. Notably, *C. jejuni* 81-176 was readily colonized intestinal tract at 10^5 CFU/g cecal digesta at d16 and reached an almost plateau of $2.8x10^7$ CFU/g cecal digesta at d21. Remarkably, DCA excluded *C. jejuni* cecal colonization at 100, 99.997, and 100% at 16, 21, and 28 days of age. DCA also improved chicken growth performance of body weight gain compared to infected control birds (1.45 vs. 1.29 kg/bird) at d28. Interestingly, DCA failed to inhibit *C. jejuni* 81-176 *in vitro* growth. Neither chicken ages of infection nor CA, LCA or UDCA altered *C. jejuni* AR101 chicken colonization level, while DCA reduced 91% of the bacterium in chickens at d28. Notably, DCA diet induced a distinct microbiota composition of phyla firmicutes (82.7.1 vs. 98.8%) and bacteroidetes (16.9 vs. 0.8%) compared to infected control birds. Importantly, DCA-Anaero attenuated 93% of

C. jejuni colonization at d28 compared to control infected birds ($1.79x10^6$ vs. $2.52x10^7$ CFU/bird). In conclusion, DCA shapes microbiota composition against *C. jejuni* colonization in chickens, suggesting a bidirectional interaction between microbiota and microbial metabolites. Simultaneously reconstituting both microbiota and microbial metabolites may render better therapeutic effect against enteritis or pathogen colonization.

2.2 Introduction

Campylobacter jejuni asymptomatically colonizes in poultry gut but is one of the prevalent food borne pathogens in the developed countries. Antibiotic resistant *C. jejuni* has been reported in children and adults in the USA and worldwide (1-5). More than 13 campylobacteriosis cases per 100,000 population were recorded in 2014 in the USA, which represents a 13% increase compared to 2006-2008 and is higher than the combined incidences by the following 8 bacterial pathogens (6). A total of 1.3 million individuals are afflicted by the disease, resulting in 76 deaths every year (7). Furthermore, *C. jejuni* post infectious complications are important and include arthritis (8), the neurodegenerative disorder Guillian-Barré Syndrome (9), Irritable Bowel Syndrome (10) and Inflammatory Bowel Diseases (IBD) (11, 12). Clinical symptoms of campylobacteriosis include abdominal cramps, watery to bloody diarrhea, fever and gastrointestinal inflammation (13). At the cellular level, the intestinal tract of *C. jejuni*-infected patients display infiltration of immune cells such as neutrophils, crypt abscesses and presence of fecal leukocytes (14).

Although antibiotics treatment has a marginal benefit (1.32 days) on the duration of symptoms (15), it is the only current available therapy for patients with severe campylobacteriosis or for those at high risk for severe disease (16). However, increasing in antimicrobial resistance (17), prompts the need for immediate and sustainable counter-actions from agricultural industry to medical field. Antimicrobial resistance presents as the emergence of multiple drug-resistant microbes or “superbugs”. More recently, McGann and colleagues reported a “superbug” of an *Escherichia coli* strain resistant to the last resort antibiotic, Colistin, in the USA (18). Misuse or overuse of antimicrobial

agents in medical and agricultural practice is contributing to exacerbating the episodes of emerging antimicrobial resistant microbes (17). Hence, an effective and sustainable solution is to find antimicrobial alternatives in agricultural industry and medical field.

Tremendous efforts have been put forward to prevent campylobacteriosis by reducing *C. jejuni* contamination in animal food sources, particularly broiler chicken meat. The intervention approaches include on-farm biosecurity measures (19), vaccines (20), probiotics (21), phages (22), and post-slaughter decontamination of poultry carcasses (23). Reducing carcass *Campylobacter* counts by 2 log is estimated to decrease a 30-fold in human campylobacteriosis (24). Although those measures to prevent *C. jejuni* contamination have achieved some success, improvement is needed, as evidenced by the relative consistent rate of campylobacteriosis incidences in the Morbidity and Mortality Weekly Report at CDC infectious disease database from January, 1996 to June, 2017 (25).

Sparse information is currently available on using microbiota to prevent *C. jejuni* colonization in poultry. Microbiota transplantation has shown tremendous success against recurrent *Clostridium difficile* infection (26). The production of secondary bile acids by specific bacteria are attributed to inhibit *C. difficile* colonization and infection (27). Bile acids at 3-30 mM in the human small intestine (28), are the byproducts of cholesterol and biotransformed from conjugated, to unconjugated primary bile acids, and to secondary bile acids. Majority of bile acids (>95%) are effectively absorbed in intestine (29). Primary bile acids of cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from cholesterol in hepatocytes and conjugated with glycine or taurine (30). In the intestine, the conjugated primary bile acids are deconjugated by bacterial bile salt

hydrolase (BSH) and further altered by microbiota to produce secondary bile acids including lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA). Deconjugating enzyme BSH is present in all major bacterial divisions and archaeal species in the human gut including members of genus *Lactobacillius*, *Bifidobacteria*, *Clostridium*, and *Bacteroides* (29, 31-33). Secondary bile acid producing bacteria consist of a small population of genus *Clostridium*, including *C. scindens*, *C. hiranonis*, *C. hylemonae* (Clostridium cluster XVIa), and *C. sordelli* (Clostridium cluster XI) (29). Besides emulsification of lipid for digestion, bile acids are implicated in various signaling pathways including nuclear receptors of farnesoid X receptor (FXR) (34), pregnane X receptor (PXR) (35), and vitamin D receptor (VDR) (36), as well as G protein-coupled receptors of G-protein-coupled bile acid receptor (TGR5) (37) and sphingosine-1-phosphate receptor 2 (S1P2) (38). The bile acid-metabolizing bacteria such as *Lactobacilli* and *Bifidobacteria* are probiotics and they enhance health by promoting host immune homeostasis (39). Besides bile acids influence host response, their level is associated with microbial community dynamics in the gut (40). Bile acids directly inhibit gut microbes (41) and indirectly modulate microbiota through FXR-induced antimicrobial peptides (42). Mice fed CA have increased class Clostridia (70 vs. 39%) compared to control mice, and genus *Blautia* (including *Ruminococcus spp.* and Clostridium cluster XIVa) expands from 8.3 to 55-62% (43). Bile acids, particularly secondary bile acid DCA, has been reported in association with a variety of chronic diseases, such as obesity, diabetes, and colorectal tumorigenesis (44, 45). Only very recently, new evidences shed light on the beneficial property of secondary bile acids in health and diseases, such as gut motility (46) and *C. difficile* infection (27). We found

that specific pathogen free (SPF) *Il10*$^{-/-}$ mice resisted against *C. jejuni* 81-176 induced colitis, while the mice were susceptible to campylobacteriosis after treated with antibiotic clindamycin which kills bile acid metabolizing bacteria (47). 16S rDNA sequencing, bioinformatics, and HPLC/MS analysis showed that clindamycin depleted all secondary bile acids, particularly DCA. Furthermore, anaerobe metabolite DCA prevents and treats *C. jejuni*-induced colitis in ex-Germ free mice (47). However, it remains unclear whether DCA regulates *C. jejuni* chicken colonization and transmission.

In this study, we hypothesized that DCA prevented *C. jejuni* chicken colonization. Our data indicate that DCA indeed prevented against chicken colonization of *C. jejuni* human clinical isolate 81-176 or chicken isolate AR101 and even improved growth performance. Subsequent mechanistic studies found that DCA modulated intestinal microbiota. Remarkably, DCA-modulated anaerobe attenuated *C. jejuni* chicken colonization. Thus, the action of DCA against *C. jejuni* chicken colonization represents a unique bidirectional interaction between microbiota and microbial metabolites. It would be more effective against enteritis or pathogen colonization through modulating both microbiota and microbial metabolites.

2.3 Material and Methods

Chicken experiment

The experiments were approved by the Institutional Animal Care and Use Committee of (IACUC) the University of Arkansas. Cohorts of 13 to 18 one-day-old broiler chicks per group obtained from Cobb-Vantress (Siloam Springs, AR) were neck-tagged and

randomly assigned to floor pens with a controlled age-appropriate environment. The birds were fed a corn-soybean meal-based starter diet during 0-10 days of age and a grower diet during 11-28 days of age. The basal diet was formulated as described before (48). Bird were fed diet supplemented with 0 or 1.5 g/kg DCA, LCA, or UDCA (all from Alfa Aesar, MA) from d 0 or gavaged daily with CA (30 mg/kg body weight) from d 4. Birds were infected with 10^9 CFU/bird human clinical isolate *C. jejuni* 81-176 at 14 days of age or chicken isolate AR101 at d 5, 10, or 14. AR101 was isolated at Poultry Health Laboratory at University of Arkansas-Fayetteville. Based on our findings, birds were mostly infected with *C. jejuni* AR101 at day 10 in order to have enough time for both *C. jejuni* colonization and microbiome maturation. Chicken body weight and feed intake were measured at 0, 14, 21, and 28 of age. Birds were sacrificed at 16, 21 or 28 days of age to collect cecal samples for enumerating *C. jejuni*. Cecal digesta samples were collected for DNA isolation or were ten-fold serially diluted with sterile PBS and cultured at 42 ^{0}C for 48 hours under microaerophilic atmosphere. Colonies were enumerated and CFU per gram was calculated.

Microbiota transplantation and *C. jejuni* colonization

Cecal digesta samples from 28 day old birds infected with *C. jejuni* 81-176 and fed 1.5g/kg DCA diet were used for isolating microbiota and transplantation. The samples were stored at -80 ^{0}C. The samples then cultured on BHI plates under aerobic or anaerobic conditions for 48 hours at 42°C. The resulted bacteria were labelled as DCA modulated aerobes (DCA-Aero) and DCA modulated Anaerobes (DCA-Anaero). Chickens were orally gavaged once with 10^8 CFU/bird DCA-Aero or DCA-Anaero at 0

days of age. At 10 days of age, the birds were infected with 10^9 CFU/bird chicken isolate *C. jejuni* AR101. Cecal digesta collected at day 21 and 28 of age were serially diluted and cultured for *C. jejuni* enumeration as described above.

***In vitro C. jejuni* growth with various bile acids**

The impact of various species of bile acids on *C. jejuni* growth was measured. Briefly, 10^3 CFU/tube *C. jejuni* 81-176 were inoculated into 5 ml *Campylobacter* enrichment broth in the presence of DCA, TCA, or AC at different concentrations of 0, 0.1, 1, 3, 5, 10 mM. The bacteria were cultured for 48 hours at 42 ^{0}C under microaerobic condition. *C. jejuni* growth was then measured by OD_{600} on NanoDrop spectrophotometer (Thermofisher).

Microbiota composition at phylum level

Cecal digesta samples were collected and DNA was extracted using bead beater disruption and phenol: chloroform separation as describe before (Sun *et al*., 2018). The levels of five phylum bacteria were determined using SYBR Green PCR Master mix (Bio-Rad) on a Bio-Rad 384-well Real-Time PCR System. The PCR reactions were performed according to the manufacturer's recommendation. The following gene primers (47, 49) were used: Universial 16S (Univ): 16s357F: 5'-CTCCTACGGGGAGGCAGCAA-3', 16s1392R: 5'-ACGGGCGGTGTGTRC-3'; α-proteobacteria: α682F 5'-CIAGTGTAGAGGTGAAATT-3', 908αR 5'-CCCCGTCAATTCCTTTGAGTT-3'; γ-proteobacteria: 1080γF 5'-TCGTCAGCTCGTGTYGTGA-3' γ1202R 5'-CGTAAGGGCCATGATG-3';

Bacteroidetes (Bact): 798cfbF 5'-5'-CRAACAGGATTAGATACCCT-3' cfb967R 5'-GGTAAGGTTCCTCGCGTAT-3'; Firmicutes: 928F-Firm 5'-TGAAACTYAAAGGAATTGACG-3' 1040 Firm R 5'-ACCATGCACCACCTGTC-3'; Actinobacteria: Act920F3 5'-TACGGCCGCAAGGCTA-3' Act1200R 5'-TCRTCCCCACCTTCCTCCG-3'. The relative fold change of each phylum in one sample were normalized to Universal 16S gene using ΔCt method. For example, the relative fold of bacteriodetes in one sample was calculated as $2^{(Ct_{Bact} - Ct_{Univ})}$. The percentage of each phylum was then calculated as the phylum relative fold divided by the sum folds of all five phyla.

Statistical Analysis

Values are shown as mean ± standard error of the mean as indicated. Differences between groups were analyzed using the nonparametric Mann–Whitney *U* test performed or unpaired t-test with Welch's correction using Prism 7.0 software. Experiments were considered statistically significant if *P* values were <0.05.

2.4 Results

DCA prevents *C. jejuni* 81-176 cecal colonization in chickens

Secondary bile acid DCA prevents and treats *C. jejuni*-induced intestinal inflammation in germ free *Il10*$^{-/-}$ mice (Sun et al., 2018). Because chickens are the natural reservoir of *C. jejuni*, we then interrogate the hypothesis that DCA may modulate *C. jejuni* colonization in chickens. The birds fed diet supplemented with 1.5 g/kg DCA were orally infected

with a single dose of 10^9 CFU/bird human clinical isolate *C. jejuni* strain 81-176 at 14 days of age. *C. jejuni* colonization level was determined by collecting and culturing cecal digasta of the birds at 16, 21, and 28 days of age using *C. jejuni* selective medium. Notably, no *C. jejuni* in cecal disgesta was detected from birds without the bacterial infection, suggesting the clean facility at our chicken farm and the success of strict biosecurity measurement during our experiments. *C. jejuni* was readily colonized the intestinal tract at a level of 10^5 CFU/g cecal digesta at 16 days of age, only 2 days post infection (Figure 2.1). *C. jejuni* colonization level then increased more than 100 folds and reached an almost plateau of 2.8 x10^7 CFU/g cecal digesta at 21 days of age. Remarkably, DCA excluded *C. jejuni* cecal colonization at 100, 99.997, and 100% at 16, 21, and 28 days of age compared to the infected control birds, based on our detection limit of 10^2 CFU/g of the cecal digesta. These results suggest that the secondary bile acid DCA effectively reduces *C. jejuni* 81-176 colonization in the intestinal tract of broiler chickens.

DCA promotes bird growth performance

Increased level of secondary bile acids DCA has been associated with obesity, but the role of DCA on animal growth is unclear. To investigate the contribution of DCA on chicken growth, the bird growth performance of body weight gain was measured at 14 and 28 days of age with or without *C. jejuni* infection. Unlike the outcome of severe intestinal diseases when infecting to human or mice (14, 50), *C. jejuni* infection in chickens neither induced diseases nor reduced the bird growth performance on body weight gain compared to uninfected birds (Figure 2.2A). Remarkably, DCA promoted growth performance of body weight gain by 36.3 % at 14 days of age, compared to

control birds. The body weight gain of birds fed DCA diets posed 12.7% increase compared to infected control birds at 28 days of age (Figure 2.2B). Interestingly, *C. jejuni* colonization increased chicken body weight gain during d 14-28. These findings suggest that DCA promotes bird growth performance of body weight gain and *C. jejuni* colonization doesn't induce adverse effect on bird health.

Bile acids fail to inhibit *C. jejuni* 81-176 *in vitro* growth

Since DCA prevented *C. jejuni* colonization in chickens, we then examine whether DCA would directly inhibit *C. jejuni* growth. The bacterium was inoculated in *Campylobacter* enrichment broth in the presence of different concentrations (0, 0.1, 1, 3, 5, 10 mM) of bile acid DCA, CA and TCA and culture at 42°C for 48 hours under microaerobic condition. Interestingly, DCA at 0.1, 1, and 5 mM didn't alter *C. jejuni* growth, while DCA at 3 and 10 mM increased *C. jejuni* growth by 4.7 and 4.5 folds, respectively (Figure 2.3). Conjugated bile acid TCA and primary bile acid CA neither increased nor reduced *C. jejuni* in vitro growth. Because bile acid DCA didn't reduce but increased *C. jejuni* growth, it is impossible that DCA against *C. jejuni* chicken colonization results from direct inhibition of DCA on *C. jejuni* growth.

Bird ages of infection don't alter *C. jejuni* AR101 chicken colonization

Our long-term goal is to use microbiome to reduced *C. jejuni* transmission in chickens and to attenuated transmitted camplylobacteriosis using mouse model as reported before (47, 50). Hence, *C. jejuni* colonized and transmitted in chickens with microbiome treatment is required to be isolated for subsequently infecting *Il10*$^{-/-}$ mice. It was

problematic when *C. jejuni* 81-176 couldn't be isolated from DCA treated chickens as showed in Figure 2.1. Different *C. jejuni* strains show variable colonization ability in chickens (51) and chicken ages of infection impact *C. jejuni* chicken colonization (52). Based on current state of knowledge, chickens were infected at 5, 10, and 14 days of age with *C. jejuni* strain AR101 which was originally isolated from Poultry Health laboratory at University of Arkansas at Fayetteville. As showed in Figure 2.4, *C. jejuni* AR101 was able to colonize chicken ceca at 28 days of age. Interestingly, infecting AR101 at d 5, 10, or 14 didn't affect the bacterial chicken colonization ($6.73x10^6$, $2.4x10^7$, or $7.49x10^6$ CFU/g cecal digesta, respectively) (Figure 2.4). These data suggest that chicken ages of infection don't alter *C. jejuni* chicken colonization level. Based on these findings, in the following experiments, birds were mostly infected with *C. jejuni* AR101 at day 10 of age in order to have enough time for both *C. jejuni* colonization and microbiome development.

Bile acids other than DCA don't reduce *C. jejuni* AR101 colonization in chickens

Since DCA reduced *C. jejuni* 81-176 colonization in chickens, it is possible that other bile acids also decrease the bacterial chicken colonization. To examine this hypothesis, birds were gavaged with PBS or CA (1.5 g/kg body weight, daily) from 4 days of age and then infected with *C. jejuni* AR101 once at 5 days of age. Interestingly, CA didn't significantly ($1.82x10^6$ vs. $6.73x10^6$ CFU/g, P=0.1) reduce AR101 chicken colonization (Figure 2.5) at day 28 of age, 23 days post infection. To assess if other secondary bile acids attenuated *C. jejuni* chicken colonization, birds were fed diets supplemented with 0 or 1.5 g/kg DCA, LCA, or UDCA from d 0. The birds were infected once with *C. jejuni*

AR101 at d10. Consistent with previous findings in Figure 2.1, DCA reduced *C. jejuni* AR101 colonization compared to infected control birds ($2.06x10^6$ vs. $2.39x10^7$ CFU/g), while LCA and UDCA failed to decrease AR101 chicken colonization ($2.05x10^7$ and $1.40x10^7$ CFU/g, respectively) (Figure 2.6). Together, these data suggest that only secondary bile acid DCA effectively reduce *C. jejuni* chicken colonization.

DCA modulates bird cecal microbiota

Relative abundance of phylum Firmicutes is dramatically expanded from 54 to 93% in intestine of rat fed primary bile acid CA (43). Since DCA neither inhibited *C. jejuni in vitro* growth nor induced intestinal diseases (histopathology, data not shown) in chickens, we then reasoned that DCA might modified microbiota against *C. jejuni*. To examine this hypothesis, cecal digesta from birds infected with *C. jejuni* 81-176 and/or fed DCA (Figure 2.1) were used to extract DNA. We selected these samples because *C. jejuni* wasn't detected in the samples and the samples would be used for following experiment of isolating microbiota and transplantation. Phylum specific primers were used to analyze the microbiota composition. Interestingly, *C. jejuni* infection didn't change microbial composition in microbiota of infected vs. uninfected birds (Figure 2.7). Remarkably, DCA reduced the phylum Firmicutes (82.7 vs. 98.8%) compared to infected control birds, while increased Bacteroidetes (16.9 vs. 0.8%). These results indicate that DCA is able to alter the chicken gut microbiota.

DCA modulated-anaerobes attenuates *C. jejuni* AR101 chicken colonization

Since DCA modulated chicken cecal microbiota, we then hypothesized that the altered microbiota might contribute to the reduction of *C. jejuni* in chickens. In our previous studies, we found that only anaerobes prevent *C. jejuni*-induced intestinal inflammation in mice (47). Based on these knowledge, cecal digesta from birds fed DCA and infected with *C. jejuni* 81-176 in Figure 2.1 were used to culture bacteria on BHI plates under aerobic or anaerobic conditions. The resulting bacteria were labeled as DCA-modulated aerobes (DCA-Aero) or DCA-modulated anaerobes (DCA-Anearo), respectively. To functionally dissect the role of these newly isolated microbiota, birds were gavaged once with DCA-Aero or DCA-Anaero microbiota at 0 day of age. The birds were then infected with 10^9 CFU/bird *C. jejuni* AR101 at 10 days of age. Consistent with previous observations, *C. jejuni* colonization in chicken ceca reached a plateau at a level of $2.80x10^7$ CFU/g cecal digesta at 21 days of age (Figure 2.8), or 11 days post infection. Importantly, DCA-Anaero significantly attenuated 93% of *C. jejuni* cecal colonization at day 28 of age compared to infected control birds ($1.79x10^6$ vs.$2.52x10^7$ CFU/bird), while DCA-Aero only reduced 34.46% of *C. jejuni* ($1.65x10^7$ vs. $2.52x10^7$) colonization. These findings suggest that DCA modulates microbiota and the modulation on anaerobes mainly contributes to the reduction of *C. jejuni* colonization in chickens.

2.5 Discussion

Although *C. jejuni* is one of the prevalent foodborne pathogens in developed countries, a paucity of information is available regarding reducing the pathogen in the main food animal source of chickens. Moreover, the microbiota and cellular events implicated in

host resistance/susceptibility to *C. jejuni* infection remain elusive (53, 54). Here we report that microbial metabolic product DCA prevented colonization of both human clinical isolate *C. jejuni* 81-176 and chicken isolate AR101 in chickens. Interestingly, bile acids of DCA, CA, or TCA failed to reduce *C. jejuni in vitro* growth. Furthermore, neither bird ages of *C. jejuni* infection at d 5, 10 or 14, nor other bile acids of CA, LCA or UDCA influenced the bacterial chicken colonization levels. Mechanistic studies revealed that DCA modified chicken cecal microbiota with increased phylum Bacteroidetes and reduced Firmicutes. Importantly, DCA-modulated anaerobes prevented *C. jejuni* chicken colonization. Altogether, these findings identified a novel mechanism that DCA shapes microbiota composition against *C. jejuni* colonization in chickens, suggesting a bidirectional interaction between microbiota and microbial metabolites.

A remarkable observation from our study is that DCA but not CA, LCA, or UDCA prevents *C. jejuni* colonization in chickens. The logic reasoning would be that the reduction would be through DCA directly impairing *C. jejuni* growth. DCA at 1.2 mM inhibits *C. jejuni* 81-176 *in vitro* growth after 12 hour incubation (55), but Lin and colleague found that the MICs of DCA and CA for *C. jejuni* 81-176 are 24 and 14 mM, respectively (56). Interestingly, DCA at 48 mM fails to reduce *C. jejuni* 43431 growth at 6, 22, 25 and 30 hours of incubation but not at 16 hours (57). Consistent with the latter reports, we found that conjugated bile acid TCA, primary bile acid CA, or secondary bile acid DCA from 0.1 to 10 mM didn't inhibit *C. jejuni in vitro* growth and DCA at high concentrations even increased the bacterial growth. In animals, DCA reduces *C. jejuni* induced intestinal inflammation in ex-Germ Free mice without altering *C. jejuni* colonization level in colon (47). These knowledge and data suggest that DCA reduces *C.*

jejuni colonization through mechanisms other than directly inhibiting the bacterial growth.

Comprehensive database analysis showed that chicken microbiota at phylum level is mainly comprised of 13 phyla including Firmicutes (70%), Bacteroidetes (12.3%), Proteobacteria (9.3%) , and other small proportion of Actinobacteria, Cyanobacteria, Spirochaetes, Synergisteles, Fusobacteria, Tenericutes, and Verrucomicrobia (58) (Wei et al., 2013). Our phylum level analysis of microbiota has found that birds fed DCA were colonized with reduced Firmicutes (82.7.1 vs. 98.8%) and increased Bacteroidetes (16.9 vs. 0.8%), which is associated with less *C. jejuni* colonization compared to control infection birds. Notably, this finding is consistent with a field survey report that birds from the farm with the highest *Campylobacter* counts are associated with the highest percentage of Firmicutes and the lowest percentage of Bacteroidetes, although microbiota composition is highly variable between inter- or intra-farms (59). Interestingly, the microbiota in mice fed CA expands phylum Firmicutes (54 to 99%), class Clostridia (70 to 39%), and genus Blautia (8.3 to 55-62%), in the expense of phylum Bacteroidetes (30 to 0.39%) (43). The levels of bile acids are associated with microbial community dynamics in the gut (40). Bile acids directly inhibit gut microbes (41) and ileal bacterial overgrowth through FXR-induced antimicrobial peptides (42). However, it remains elusive how various bile acid species differentially influence intestinal microbiota composition.

Based on the results of DCA altering microbiota, we hypothesized that the microbiota might play a role on protecting against *C. jejuni* chicken colonization. To functionally dissect the protection of the DCA-modulated microbiota, microbiota culture

and chicken microbiota transplant were performed. Indeed, DCA modulated anaerobes reduced *C. jejuni* chicken colonization, while DCA modulated aerobes failed to do so. Microbiota prevents against *C. jejuni* chicken colonization because *C. jejuni* colonizes at higher level in germ free or antibiotics pre-treated chickens compared to conventional birds (60). Specific members of microbiota play an important role against *C. jejuni* induced diseases. Microbiota diversity and the relative abundances of genera Dorea and Coprococcus in family Lachnospiraceae were higher in healthy travelers compared to individuals with campylobacteriosis (54). Mouse microbiota with higher level of genera *Clostridium XI*, *Bifidobacterium*, and *Lactobacillus* is associated with resistance to *C. jejuni* induced colitis (47). The three genera bacteria metabolize conjugated bile acids into secondary forms (29), which prevent campylobacteriosis in mice (47). Interestingly, probiotics *Bifidobacterium longum* PCB133 failed to reduce *C. jejuni* chicken colonization (61). We are processing the cecal samples to extract bacterial DNA to run 16S rDNA sequencing and we expect that we will find specific bacteria in DCA-modulated anaerobes responsible for protection against *C. jejuni* chicken colonization.

Finally, we found that *C. jejuni* colonization levels was independent on the ages of birds infected. It remains controversial whether chicken ages of inflection play any role on *C. jejuni* chicken colonization. *C. jejuni* is detected in 40% of broiler chicken flocks at 4 weeks of age and in 90% of flocks at 7 weeks of age (62). This infection pattern could be attributed to age-related resistance or the necessary timing for *C. jejuni* transmission within house. Birds from parent breeders colonized with high level *C. jejuni* strain 99/308 are resistant to 99/308 only at 8 days of age but not at 1 or 22 days of age (63), suggesting specific and limited protection from the breeders. However, Han and

colleagues showed that *C. jejuni* strains Lior6 and 0097 colonize less in birds of d 22 compared to d 1, after 14 days post infection (52). Because bird microbiota starts to assemble after hatch, the older birds in some farms might develop microbiota resistant to *C. jejuni*, as in Han and colleagues' report (52). Similarly, germ free mice transferred to SPF housing for 14 days resist against *C. jejuni* induced colitis (47). In those reports with no age difference on *C. jejuni* colonization, the resistant microbiota assembly might be blocked by strict biosecurity measurements or no available resistant microbiota in the environment.

In conclusion, the microbiota metabolite secondary bile acid DCA decreases *C. jejuni* counts and microbiota composition in the chicken intestine. At mechanistic level, DCA-modulated anaerobic microbiota may be responsible for protecting against *C. jejuni* colonization in chickens. These findings identified a novel mechanism that DCA shapes microbiota composition against *C. jejuni* colonization in chickens, suggesting a bidirectional interaction between microbiota and microbial metabolites. Simultaneously reconstituting both microbiota and microbial metabolites may render better therapeutic effect against enteritis or pathogen colonization.

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APPENDIX

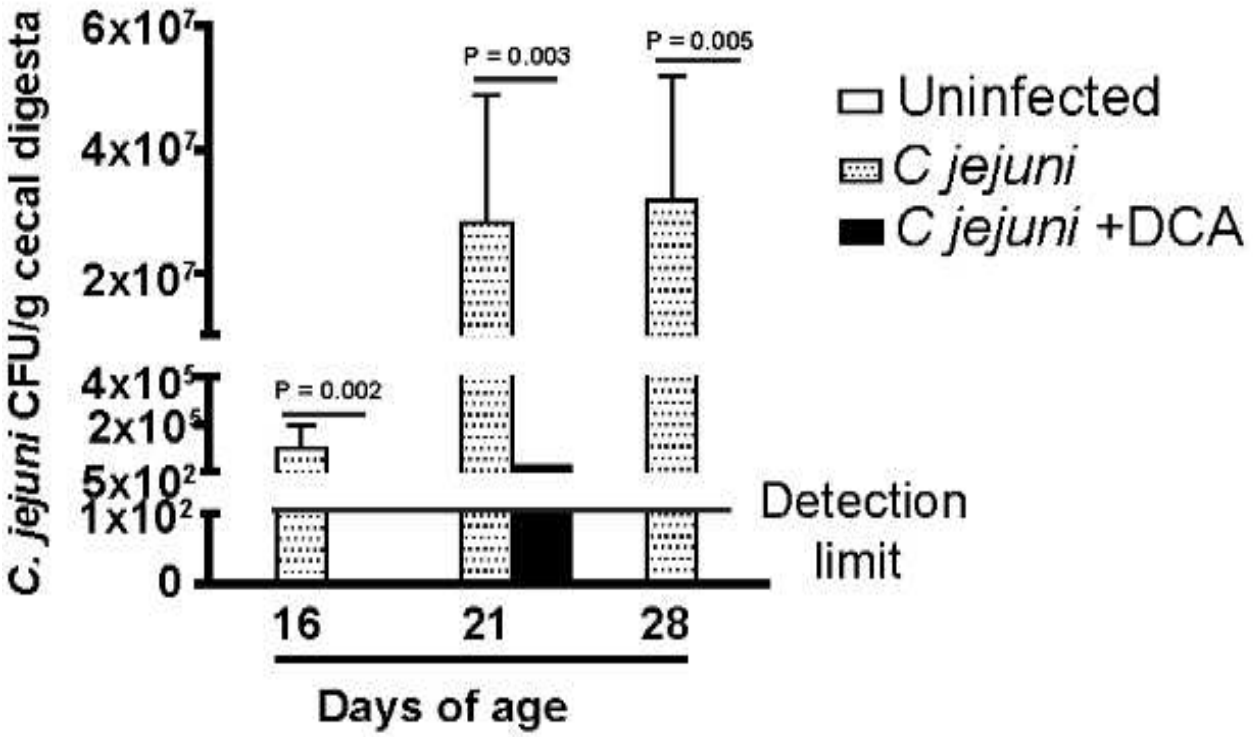

Figure 2.1 DCA prevents against *C. jejuni* 81-176 colonization in chickens.

Cohorts of 13 one-day-old broiler chickens per group were fed 0 or 1.5 g/kg DCA diets. The birds were infected with *C. jejuni* 81-176 at 14 days of age. Cecal digesta were collected at 16, 21, and 28 days of age, serially diluted, and cultured on *C. jejuni* selective medium at 42 ºC. *C. jejuni* was counted after 48 hours of culture. All graphs depict mean ± SEM. Significant if $P<0.05$.

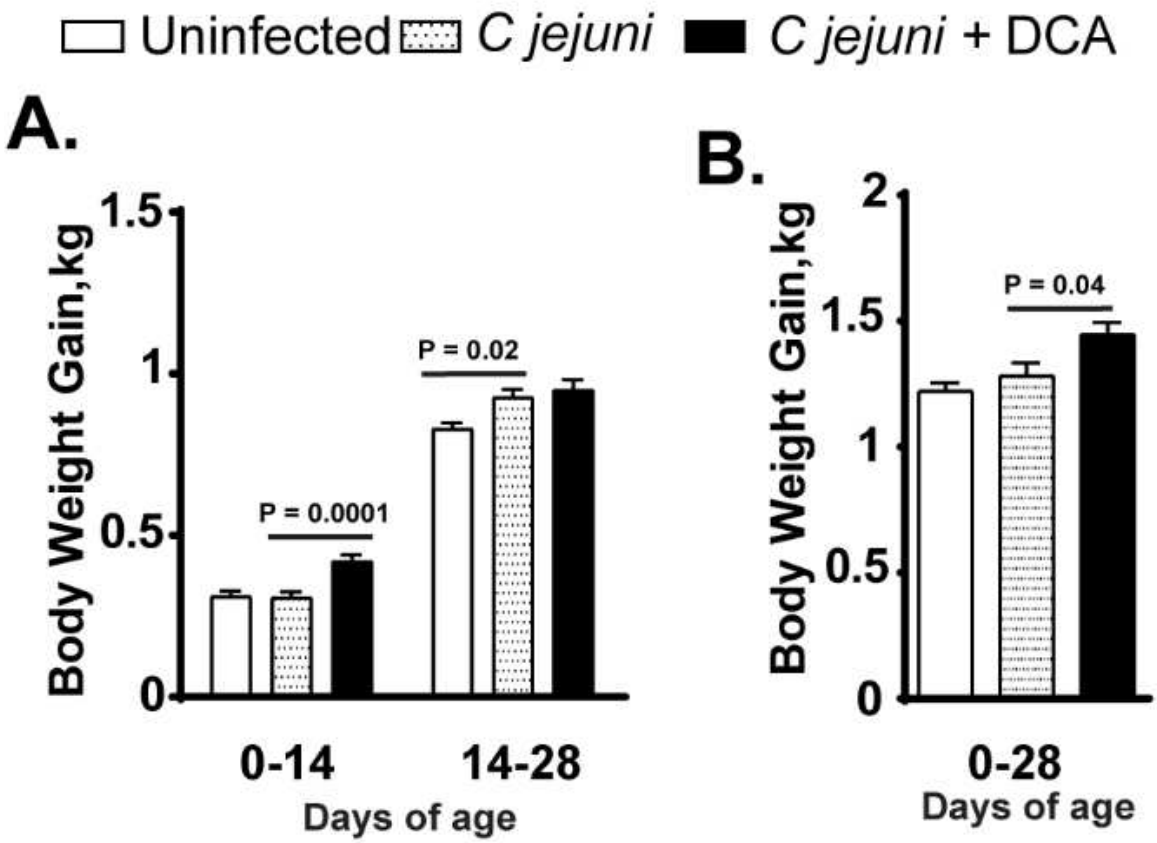

Figure 2.2 DCA promotes broiler chicken growth performance.

Birds were fed DCA diet and infected with *C. jejuni* as described in Figure 1. (A) Periodic bird growth performance of body weight gain. (B) Accumulative body weight gain at 28 days of age. All graphs depict mean ± SEM. Significant if $P<0.05$.

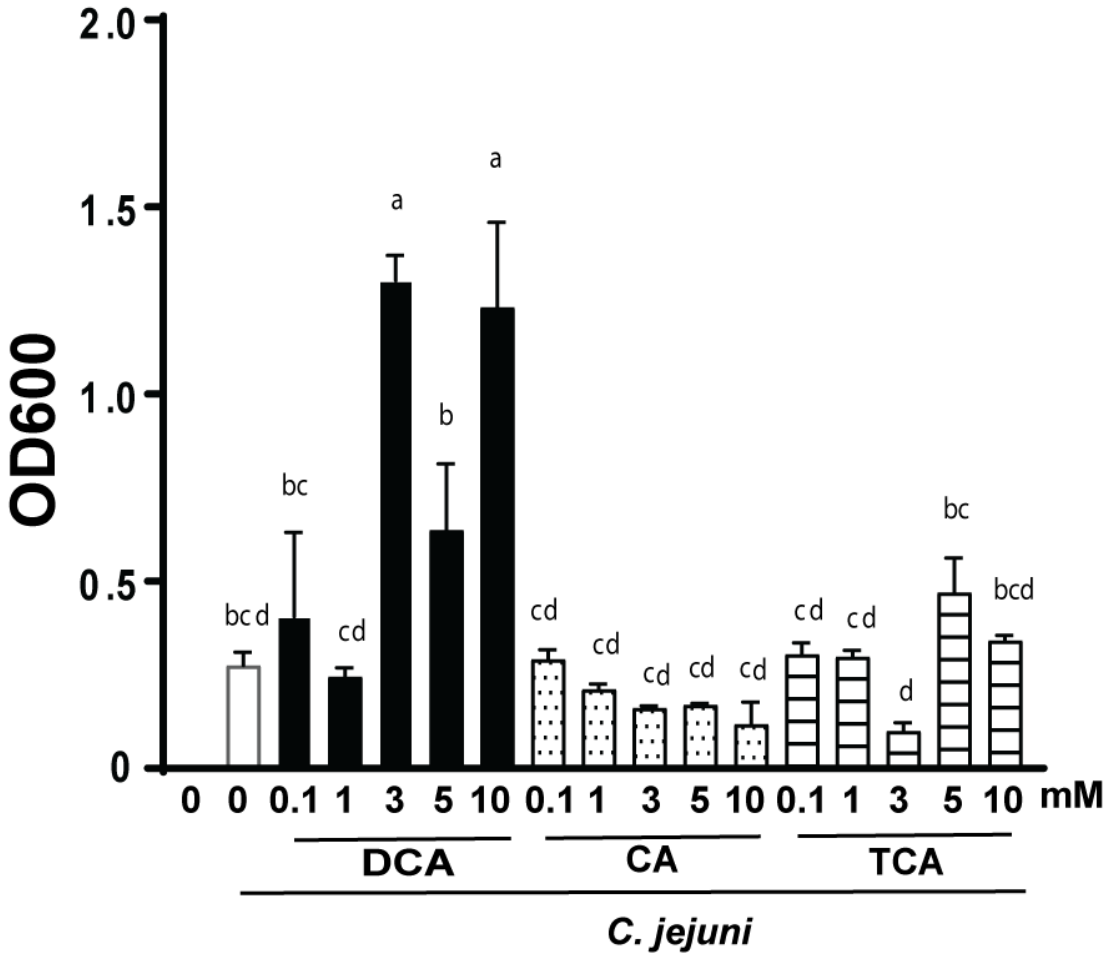

Figure 2.3 Bile acids fail to inhibit *C. jejuni in vitro* growth.

C. jejuni 81-176 at 10^3 CFU was inoculated into 5 ml *Campylobacter* broth under microaerophilic condition and cultured at 42 ºC for 48 hours. The broth was supplemented with various concentrations (0, 0.1, 1, 3, 5, and 10 mM) of secondary bile acid DCA, primary bile acid CA, or conjugated bile acid TCA.

Different letters represent significance ($P<0.05$) between treatments.

Figure 2.4 Chicken ages of infection doesn't influence the bacterial colonization levels.

Cohorts of 18 one-day-old broiler chicks per group were fed basal diet and orally gavaged with 10^9 CFU of *C. jejuni* AR 101 at 5, 10, or 14 days of age. At d 28, birds were humanely sacrificed and cecal samples were collected. Cecal digesta samples were serially diluted and cultured on *Campylobacter* selective media. Colonies were enumerated and *C. jejuni* colonization levels were calculated. All graphs depict mean ± SEM.

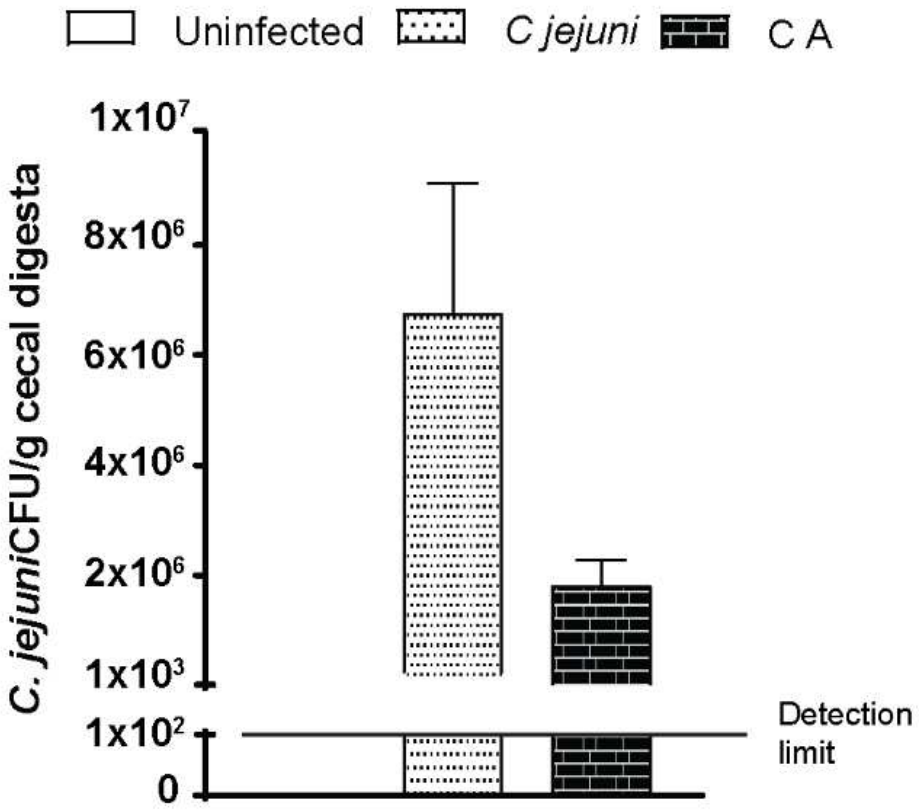

Figure 2.5 CA fails to prevent *C. jejuni* colonization in chickens.

Cohorts of 18 one-day-old broiler chickens were orally gavaged daily with CA or PBS from 4 days of age and infected with *C. jejuni* AR101 at d 5. All birds were sacrificed and cecal digesta were collected at 28 days of age, serially diluted, and cultured on *C. jejuni* selective medium at 42 ºC. *C. jejuni* was counted after 48 hours. All graphs depict mean ± SEM. Significant if $P<0.05$.

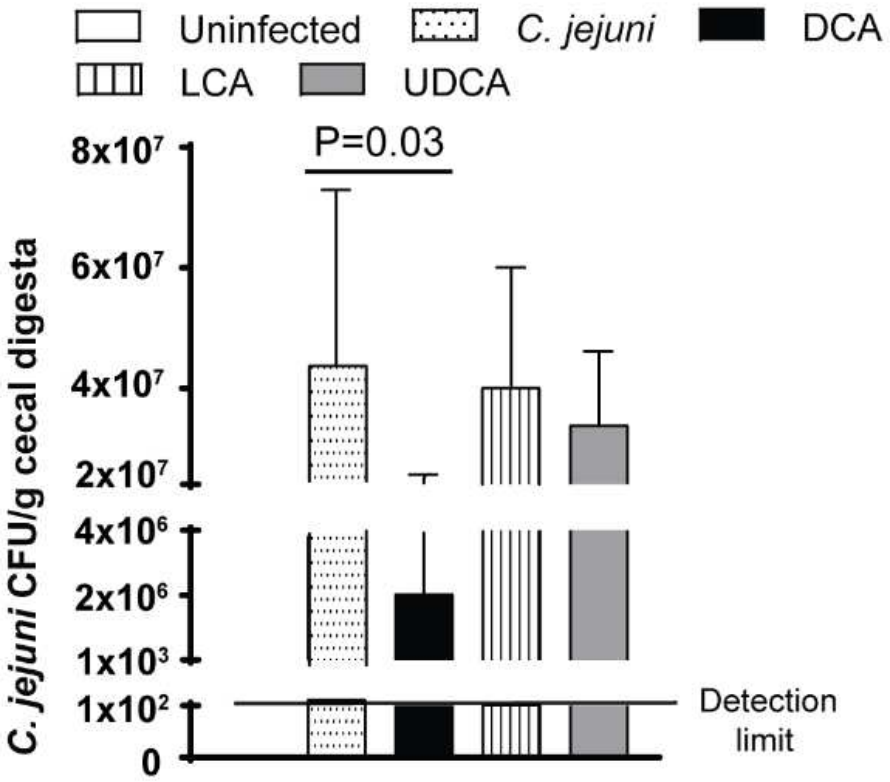

Figure 2.6 Other secondary bile acids fail to prevent *C. jejuni* colonization in chickens.

Cohorts of 18 chicks per group were fed diets supplemented with 0 or 1.5 g/kg of DCA, UDCA, or LCA. and were infected with *C. jejuni* AR 101 at day 10 of age. All birds were sacrificed and cecal digesta were collected at 28 days of age, serially diluted, and cultured on *C. jejuni* selective medium at 42 ºC. *C. jejuni* was counted after 48 hours. All graphs depict mean ± SEM. Significant if $P<0.05$.

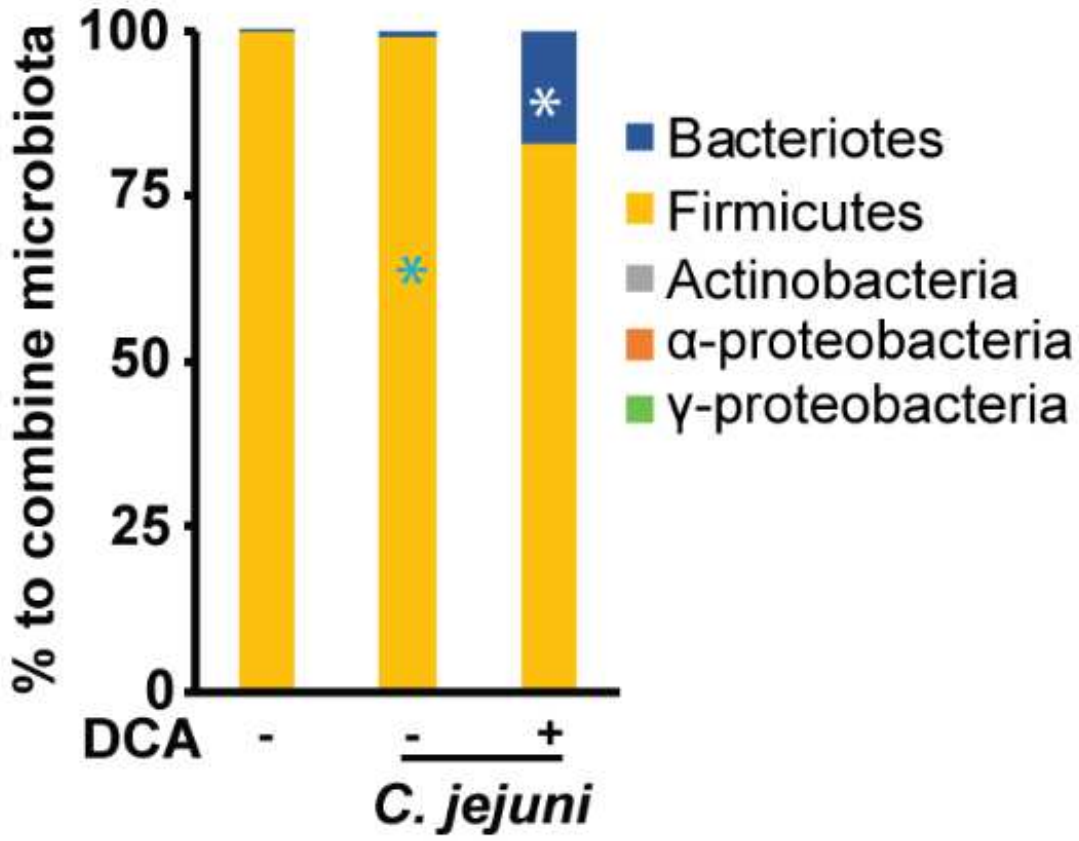

Figure 2.7 DCA modulates cecal microbiota.

Cohorts of 13 chicks per group were fed 0 or 1.5 g/kg DCA diets and infected with *C. jejuni* 81-176 as described in Figure 1. Cecal digesta samples were collected and DNA was isolated. Real time PCR was performed to calculate bacterial composition at phylum level. All graphs depict mean ± SEM. *, $P<0.05$.

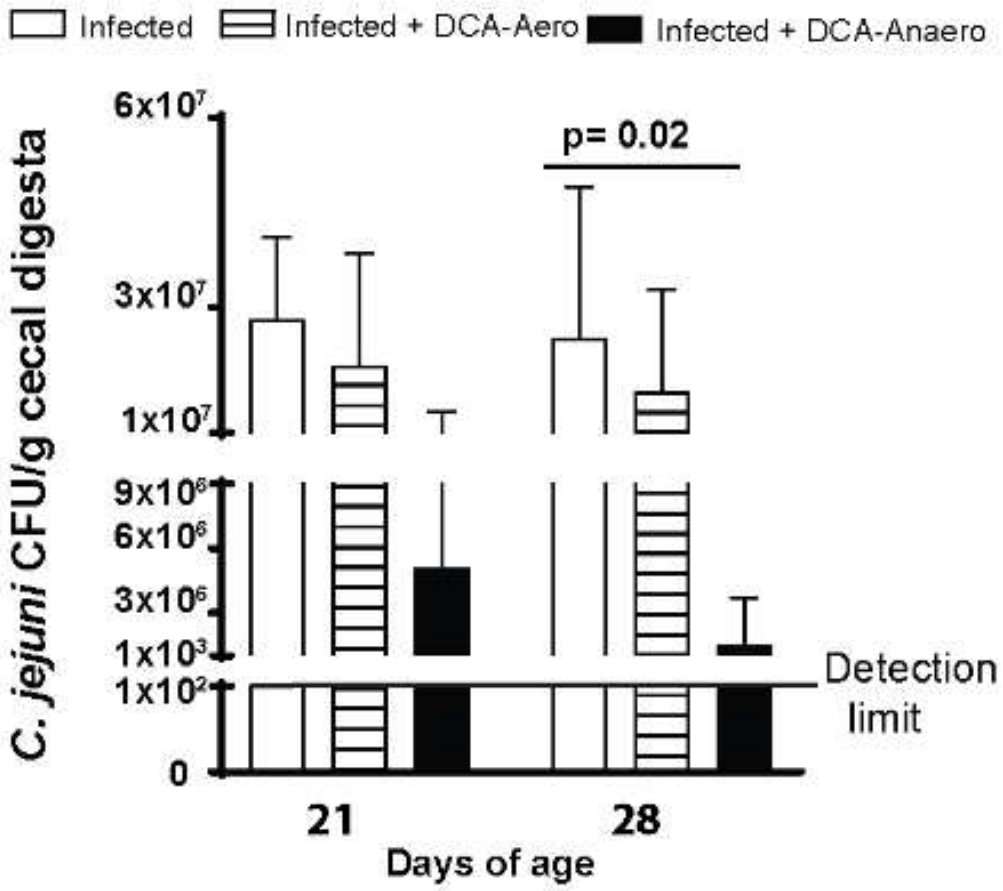

Figure 2.8 DCA-modulated microbiota attenuates *C. jejuni* colonization in chickens.

Cohorts of 18 broiler chickens were orally transplanted with 10^8 CFU/bird DCA-Aero or DCA-Anaero at d 0. The birds were then infected with 10^9 CFU/bird *C. jejuni* AR 101 at 10 days of age. Cecal digesta were collected at 21 and 28 days of age, serially diluted, and cultured on *C. jejuni* selective medium at 42 ºC. *C. jejuni* was counted after 48 hours. All graphs depict mean ± SEM. Significant if $P<0.05$.

CONCLUSION

Campylobacter jejuni is one of the prevalent food borne pathogens inciting enteritis in the developed countries. Poultry are the main reservoir of the pathogen, which infects human through either direct contact with infected birds or consumption of undercooked and contaminated chicken meat. Fecal microbiota transplantation of anaerobic microbiota or supplementation of anaerobic metabolic product deoxycholic acid (DCA) is able to prevent and treat *C. jejuni*-induced intestinal inflammation in ex-germ free *Il10*$^{-/-}$ mice. Based on the knowledge, we hypothesized that DCA prevented *C. jejuni* chicken colonization. We found that dietary DCA at 1.5 g/kg feed prevented *C. jejuni* human clinical isolate 81-176 and chicken isolate AR101. Interestingly, bile acids of conjugated primary bile acid taurocholic acid, deconjugated primary bile cholic acid (CA), and secondary bile acid DCA failed to inhibit *C. jejuni in vitro* growth. Furthermore, CA and other secondary bile acids lithocholic acid and urodeoxycholic acid didn't reduce *C. jejuni* AR101 chicken colonization. Analyzing microbiota at phylum level revealed that DCA reduced the relative abundance of phylum Firmicutes but increased Bacteroidetes compared to birds fed basal diet. Functionally examining the microbiota by transplantation showed that DCA-modulated anaerobes reduced *C. jejuni* AR101 chicken colonization compared to control birds. In conclusion, microbiota metabolic product DCA attenuates *C. jejuni* colonization level in chickens through modulating bird microbiota anaerobes. These findings may lay the ground work for exploring microbiota and its metabolic activities to control *C. jejuni* or other pathogens.