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The role of microbiota on *Campylobacter jejuni* colonization and growth performance in broiler chickens

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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Abstract

The successful poultry production at modern era comes from the vertical integrated industrialization, which has fundamentally changed how animals have been living for millions of years. Antimicrobial growth promoters have been used to sustain the efficient industrialized animal production, driving antimicrobial overuse and resistance. Because of the increasing pressure from consumer's concerns and government regulations, it is urgent to develop antimicrobial free alternatives as growth promoters in poultry production, but few effective antimicrobial alternatives are currently available. *Campylobacter jejuni* is one of the worldwide prevalent foodborne bacterial pathogens mainly transmitted from poultry. However, few mechanisms are available on why *C. jejuni* colonizes chickens. In the first chapter, I have presented an overview of current knowledge on microbiota, chicken growth and *C. jejuni* infection.

In the second chapter, we aimed to investigate the mechanism of transplanting microbiota on *C. jejuni* chicken colonization. Mouse specific pathogen free (SPF) microbiota was cultured on Brain Heart Infusion agar (BHI) and collected as SPF-Aerobe and SPF-Anaerobe. Birds raised on floor pens were colonized with 10^8 CFU/bird SPF-Aerobe and SPF-Anaerobe at d 0 and infected with 10^9 CFU/bird *C. jejuni* chicken isolate AR101 at d 12. Birds were sacrificed at d 21 and 28 to enumerate *C. jejuni* cecal colonization on selective *Campylobacter* plates. The results show both SPF- Aerobe and SPF-Anaerobe microbiota reduced *C. jejuni* chicken colonization compare to infected birds at d 21 and 28, Also, we found that SPF-mouse microbiota was able to colonize in chicken gut by modulating chicken microbiota at phylum level. Furthermore, SPF-mouse microbiota prevented *C. jejuni* growth in *vitro*.

In the third chapter, we investigated investigate the effect of transplanting microbiota on the bird growth performance. Mouse SPF stool was cultured on Brain Heart Infusion (BHI) agar

under anaerobic or aerobic condition and collected as SPF-Aerobe and SPF-Anaerobe microbiota. Day-old birds were tagged, weighed, and randomly assigned to 8 pens with 15 birds/pen. The birds were orally gavaged with PBS (3 pens), 10^8 CFU/bird SPF-Aerobe (2 pens) or SPF-Anaerobe (3 pens). The feed intake and individual bird weight were measured at d 0, 14, 21 and 28. The broiler chickens were euthanized at d 14, 21 and 28. Intestinal digesta was collected to measure nutrient and bacteria levels. Notably, SPF-Aerobe and SPF-Anaerobe significantly increased body weight gain by 18% and 12% during d 0 to d 14, respectively, compared to the negative control. No significant difference of feed intake was observed among the groups. SPF-Aerobe significantly reduced periodic feed conversion ratio compared to the negative control by 20% during d 21 to 28. Both SPF-Aerobe and SPF-Anaerobe microbiota reduced accumulative feed conversion ratio compared to negative control by 18% and 14% respectively, during d 0-28. SPF microbiota increased the levels of macro-nutrients of gross energy, protein and fat in the digesta of the small intestine compared to the negative control. SPF-Anaerobe transplantation increased phylum *Bacteroidetes* but reduced *Firmicutes* in the digesta of small intestine and ceca compared to the negative control.

In conclusion, microbiota was able to reduce *C. jejuni* chicken colonization and to improve feed efficiency and early bird body weight gain. The results suggest that microbiota reconstitution in chickens could be used an effective antibiotic alternative to reduce foodborne pathogen *C. jejuni* and to improve poultry productivity.

DEDICATION

This work is dedicated to my family

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CHAPTER I

Literature Review

1.1 Introduction

Animal meat is an important component of human diet that provides a range of micro and macro nutrients like fats, vitamins, minerals, and proteins. Meat is generally rich in proteins and so, can fulfill most of the human body's protein requirements (Ahmad et al., 2018). Meat consumption is expected to increase by 14% in the next decade. The poultry industry, which plays a critical role in food security and nutrition around the world, is among the fastest-growing subsectors in agriculture, particularly in developing nations. According to the Food and Agriculture Organization (FAO) of the United Nations, the estimated poultry meat produced in 2020 was around 333.7 million (FAO, 2020). Poultry is also expected to be a predominant source of meat and proteins in the future, accounting for 41% of the total meat consumption in the year 2030 (OECD-FAO, 2021). Chickens, along with turkeys, are the most widely bred and consumed poultry species on earth, accounting for roughly 36% of the total protein consumption (FAO, 2014; OECD-FAO, 2021). Currently, the major chicken meat producing countries are the United States and Brazil (USDA, 2020). The chicken meat sector is expected to grow further in future mainly due to the factors like population and income level growth and increasing urbanization. (FAO, 2020). For meat to be considered acceptable for consumption, it must meet some quality parameters. Factors that affect meat quality include its physical, biochemical, sensory, and microbial properties (Rodríguez-Carpena et al., 2011). The microbial content of meat is of paramount importance for food safety as presence of pathogenic bacteria can cause foodborne illnesses, thus, meat can function as a vector for spread of zoonotic diseases. Bacterial species such as *Salmonella, Campylobacters*, *Listeria Monocytogenes*, *Escherichia coli* (*E. coli)*, and

Clostridium are major foodborne pathogens and thus, their presence in meat is a cause of great concern regarding safe consumption of such meat (Dhama et al., 2013). The chicken meat often serves as a vector of zoonotic diseases to humans including campylobacteriosis, a foodborne infection caused by bacterium *Campylobacter jejuni* (*C. jejuni*) that infects around 33 million people each year (Havelaar et al., 2015; WHO, 2020).

C. jejuni is a prominent and significant zoonotic pathogen that acts as a source of severe and frequent intestinal infections among humans. The situation is most critical in the industrialized nations, where *C. jejuni* is recognized as the most prevalent causative agent of foodborne illnesses (Sheppard et al., 2012). The utmost reported origin of human *Campylobacter* infections are the farm animals, where the pathogen is most widely spread. These animals carry the pathogen without any visible signs. More specifically, poultry is the primary cause of human *C. jejuni* intestinal infections that account for approximately 80% of all campylobacteriosis infections worldwide (Hazards, 2010). This bacterial specie is believed to normally inhabit the intestinal mucosa of chicken, which spreads to humans by eating contaminated chicken meat (Hermans et al., 2011). *C. jejuni* infections are typically self-limiting that barely last for a few days. So, in most cases, antibiotic treatment is usually not required. Macrolides, like erythromycin, are currently widely regarded as the best treatment option for the *C. jejuni* infections, while fluoroquinolones serve as an alternative. Nevertheless, antimicrobial resistance in *Campylobacter* strains is becoming a widely recognized problem that is a major concern for infection treatment (Guyard-Nicodème et al., 2015). The rising incidence of antibiotic resistant *C. jejuni* infections in human renders clinical therapy of the diseases caused by this bacterium more challenging. Antibiotic resistance can lengthen sickness and complicate the treatment of bacteremia patients (Snelling et al., 2005).

C. jejuni naturally infects poultry by the fecal–oral pathway. This is followed by the establishment of this bacterium in the birds' intestinal tract with a significant bacterial presence in the caeca (approximately 10^9 colony-forming units (CFU)/g of cecal contents) (Sahin et al., 2015). Once *C. jejuni* infects and establishes itself in a single chicken, it can quickly spread to the whole flock, and within a matter of few days, the entire flock can be infected. The ceca and small intestines act as the primary areas of colonization, with the deep muscles, fabricius bursa, liver, spleen, and thymus also acting as the infection sites to a lesser extent. Despite high infection rates and speed of colonization, *C. jejuni* mainly remains apathogenic in chicken (Awad et al., 2015; Chaloner et al., 2014; Cox et al., 2005; Humphrey et al., 2015). However, this notion has been challenged by many studies that sprung up during the last few years reporting detrimental effects of *C. jejuni* that include impairment of the chicken's intestinal mucosa, increased intestinal permeability and reduced food absorption in the intestines (Awad et al., 2016; Awad et al., 2018). Antibiotics have been traditionally used at sub-therapeutic and therapeutic levels to promote growth and welfare of chicken. However, due to the rising antimicrobial resistance, the use of antibiotics in livestock production is receiving increased scrutiny and discouragement (Elhadidy et al., 2018)

Due to the ever-increasing cases of campylobacteriosis around the world including the United States, investigations into the use of therapeutics alternatives to antibiotics are urgently needed as the treatment failures using antibiotics continue to increase globally. Implementation of the biosecurity measures in poultry farms, vaccinations , treatment with phages, probiotics, and poultry carcass disinfection are among the most studied alternative intervention strategies (Alrubaye et al., 2019). Although these measures decrease *C. jejuni* bacterial counts, more effective alternative approaches are urgently required, as evidenced by the consistently high

overall campylobacteriosis incidences reported around the world (WHO, 2020). There is currently little information available on utilizing microbiota to inhibit *C. jejuni* colonization in poultry. Microbiota transplantation has been proven to be highly effective in treating recurring *Clostridium difficile* infections in humans (Buffie et al., 2015; Silverman et al., 2010). Recent findings suggest that some anaerobic bacteria protect *Il10*$^{-/-}$ mice from the intestinal inflammation caused by *C. jejuni*. Moreover, it has been found that wild type mice containing intact conventional microbiota are protected from colonization by *C. jejuni*, even after high peroral infection dosage of *C. jejuni* (Heimesaat et al., 2019b; Sun, Jia, et al., 2018). While fecal microbiota transplantation (FMT) is becoming more widely used in human therapeutic practice, it is yet to be embraced in livestock in the true sense (Heimesaat et al., 2019b).

1.2 *C. jejuni*

C. jejuni belongs to the genus *Campylobacter* which contains over 28 other species besides *C. jejuni*. A wide variety of animals like poultry, cattle, and sheep naturally carry *C. jejuni* in their intestine. Chickens are the primary reservoir of this bacterium though it is mostly found in older chickens and rarely in chickens younger than 2 weeks. It has been postulated that the maternal antibodies in young chicks are mainly responsible for conferring protection against *C. jejuni* colonization. In majority of cases, *C. jejuni* does not cause infection in chickens and resides in the chicken gastrointestinal tract (Nauta et al., 2009) as a commensal bacterium (Awad et al., 2018). However, the bacterial specie is pathogenic to humans as it is responsible for the 95% of all campylobacteriosis cases, a foodborne disease that causes enteritis (CDC, 2021). According to direct and indirect data from experimental infections and aquatic outbreaks, respectively, exposure to mere hundreds of *C. jejuni* cells is enough to cause disease. The primary route *C. jejuni* dissemination into human population is chicken meat. However, chicken and human fecal

contamination of food and water sources are also recognized as significant sources of *C. jejuni* transmission (Pitkänen & Hänninen, 2017).

1.2.1 History of *C. jejuni*

Campylobacters were discovered by Theodor Escherich, who in 1886, identified the presence of spiral-shaped bacteria in the neonates' diarrheic stool samples. He named the range of symptoms caused by these newly discovered bacteria in children as "*cholera infantum*" and summer complaint (Condran & Murphy, 2008; Escherich, 1886; Samie et al., 2007). Several morphologically same spiral or "Vibrio" bacteria were later isolated by McFadyean and Stockman from different animals in 1906. They also isolated a bacterial specie which they named *Vibrio foetus ovid* based on their isolation from aborted lamb fetuses and their mothers (McFadyean & Stockman, 1909). The bacterium was then renamed to *Campylobacter fetus* in 1963 (Sebald & Veron, 1963). Véron and Chatelain (1973) proposed a new genus called *Campylobacter* to include all these bacteria including the bacterium now called *C. jejuni* (Acheson & Allos, 2001). This classification was made after clinical microbiologists in Belgium successfully isolated these bacteria from the stools of diarrheal patients in 1972, following King's 1957 report on the isolation of "Vibrio" bacteria samples from the blood of children having diarrhea. Soon after this report, *Campylobacter* became increasingly recognized as a common human pathogen. This realization was made after several laboratories started routine isolation and identification of *Campylobacter* from the stool sample due to the advancements in selective growth media in the early 1970s (Kist, 1985; Tauxe, 1992). By the mid-1980s, various scientific publications established *Campylobacter* as one of the leading causes of diarrhea around the globe (Acheson & Allos, 2001).

1.2.2 Morphology and biochemical characteristic

C. jejuni are gram-negative curved rods, motile, and non-spore forming morphologically, and signifies its presence with highly motility due to presence of polar flagellum at one or both ends of the cell. Size varies from 0.2 to 0.8 μm wide and 0.5 to 5.0 μm long. The daughter cells are often attached together showing spiral forms. The bacterium has a preference for poultry such as chicken as the body temperature of these birds is ideal for its growth as evidenced by its ability to grow best at 42ºC. However, temperature between 30 and 44ºC can also support the growth of this bacterium (Nielsen et al., 2010). *C. jejuni* is microaerophilic and requires an oxygen concentration in range from 3 to 15% and 3 to 5% carbon dioxide. Biochemically, these bacteria are catalase and oxidase positive and urease negative, and can hydrolyse hippurate (Hansson, 2007). *Campylobacter* species can have unipolar or bipolar flagella that allow motility in the bacterial species. *C. jejuni* grows slowly; initial isolation of these bacteria from stool samples takes 72–96 hours, while isolation from blood samples requires even more time (Murray et al., 2008). The bacterium requires a balanced amount of nitrogen, 3-15% oxygen concentration, and carbon concentration of 3-5% (Murray et al., 2008; Nielsen, 2010). *C. jejuni* cannot ferment carbohydrates, so they take energy by reducing carboxylic acids and amino acid substrates. It can reduce nitrate and fumarate to succinate. The bacterium is biochemically oxidase and catalase-positive, while its urease-negative has the property to establish a long-term association with its host (Vandamme et al., 1992).

C. jejuni is a slow growing and fastidious microorganism that requires specific set of laboratory conditions for its isolation. In the food samples, presence of other bacteria that are fast growing limits isolation of *C. jejuni.* Therefore, enrichment of food samples is required to allow small number of *C. jejuni* cells to proliferate and be used for the isolation of this bacterium. Normally, peptone water is used for this purpose (Gharst et al., 2013). The sample containing

enriched bacterial colonies is then plated on selective media for the isolation. The selective medium can be blood-based such as the media developed by Bolton and Robertson (1982) or a blood-free medium which contains charcoal, cefoperazone, and deoxycholate and is called CCD agar based on the initials of these three components (Bolton et al., 1984). Once plated, the bacterial plates are incubated at microaerophilic conditions and at higher temperatures preferably 42ºC (Gharst et al., 2013).

1.2.3 *C. jejuni* chicken colonization

The rate of *Campylobacter* spread in poultry is much higher than in any other animal. It has been shown that after colonizing the gastrointestinal tract (Nauta et al., 2009) of one bird, rapid transmission occurs horizontally, after which, the whole flock can acquire *C. jejuni* infection within few days (Horrocks et al., 2009; Shreeve et al., 2000). Because of such high spread in chickens, *C. jejuni* can be found ubiquitous in the environment surrounding chickens (Newell et al., 2003). Infection originates primarily in the ceca and small intestine, with secondary infections occurring in the deep muscles, fabricius bursa, liver, spleen, and thymus. Despite high infection rates and speed of colonization, chickens remain asymptomatic in overwhelmingly majority of cases (Awad et al., 2018). Though *C. jejuni* and similar species are nearly ubiquitous inhabitants of avian digestive tracts, numerous studies suggest that they have poor survivability outside of these environments. It has also been widely observed that *C. jejuni* metabolic capacities are limited in comparison to other digestive tract microbial occupants like *Bacteroidetes* and *E. coli*, so *C. jejuni* and similar species have been labelled as metabolically fastidious (Murphy et al., 2006). This lifestyle indicates that *C. jejuni* cannot withstand changes in the pH and oxygen levels, osmotic, temperature conditions, and limited nutritional availability. *C. jejuni* is a thermophilic bacterium belonging to *Campylobacter* species that grows best between 37°C and 42°C, and does

not replicate below 30°C (Penner, 1988) . It was proposed that the pathogen's failure to proliferate at the reduced temperatures could be attributed to a lack of cold-shock proteins (Hazeleger et al., 1998). However, *C. jejuni* can respire and generate ATP even in lower temperatures like 4°C, allowing it to perform its metabolic activity for longer time periods at low temperatures. Because of this reason, refrigerated chicken meat contaminated with *C. jejuni* can also serve as the cause of infections as the bacterium can grow at refrigeration temperatures (Bhaduri & Cottrell, 2004).

Initially thought of as a commensal bacterium in chicken, it was later discovered that *C. jejuni* is capable of invading the intestinal mucosa of chicken and spreading to internal organs (Lamb-Rosteski et al., 2008; Van Deun et al., 2008). Moreover, *C. jejuni* colonization in the chicken gut has been found to be associated with mucosal injury and increased intestinal permeability, implying that *C. jejuni* translocate through the transcellular and paracellular pathways. *C. jejuni* also alters host cellular activities by interacting with the intestinal epithelium and by disrupting Ca^{2+} signaling and nutritional absorption in chickens (Awad et al., 2016). The physiology and metabolic profile of *C. jejuni* infection in chickens is less known in comparison with other food-borne infections (Howlett et al., 2014). *C. jejuni* has a high requirement for non-carbohydrate derived carbon sources due to its low capacity to metabolize sugars and lack of phosphofructokinase enzymes (Parkhill et al., 2000). Previous research has indicated that the bacterium uses only a small number of TCA cycle intermediates and amino acids as essential carbon sources. The bacterial ability to utilize aspartate, glutamate, proline and serine is a characteristic of *C. jejuni* infections (Howlett et al., 2014). Investigations regarding the metabolic capabilities of *C. jejuni* and how these relate to the colonization of host species and virulence are just beginning to emerge to expand insight and offer possibilities for *C. jejuni* infection control. As a result, better knowledge of the pathogen’s metabolism and the impact of diverse substrates

on its physiology is critical in defining adaptability and colonization of this bacterium in different host niches, as well as pathogenicity (van der Hooft et al., 2018).

1.2.4 Virulence factors of *C. jejuni* in chickens

To successfully colonize in the GIT of chicken, *C. jejuni* has to make use of various physiological processes requiring many factors. Over the last three decades, a great insight about the several strategies that *C. jejuni* uses for colonizing chickens has been gained. The majority of genes that are important for colonization of *C. jejuni* are encoded in the plasmids like pVir plasmid, which makes it easy for these genes to spread among the bacterial populations (Hassan et al., 2019). To survive for an extended period of time, *C. jejuni* has acquired tolerance for the acidic pH and alkaline bile during transition through gut and cecal colonization (Beery et al., 1988). The movement of bacteria move through hostile organs to relatively safer environments is facilitated by the bacterial ability to move with the help of flagella and through chemotaxis (Hermans et al., 2011). Various studies have highlighted the crucial role of motility in successful and long-term colonization of chickens by *C. jejuni*. Motile *C. jejuni* colonize the chicken ceca for extended periods of time with much greater efficiency and larger numbers than non-motile mutants (Mertins et al., 2012; Morooka et al., 1985). When *C. jejuni* enters the target organs, it attaches itself to epithelial cells before colonizing them (Beery et al., 1988). The colonization of host is aided by a well-functional Quorum Sensing (QS) system present in *C. jejuni* just like in other bacteria. QS helps in different bacterial processes, including biofilm formation, CDT transcription (Jeon et al., 2003), autoagglutination, motility packing, colonization of animals, and in expression of many other virulence factors (Quiñones et al., 2009). *C. jejuni* uses QS to identify changes in host environment and microbial populations by creating signaling molecules such as autoinducer-2, or AI-2 (Bassler et al., 1994; Castillo et al., 2014). The *AI-2* gene which is induced by methylene

recycling pathway (Plummer, 2012) is primarily involved in motility, flagella expression, oxidative stress, and bacterial colony formation. Information about the environment and other *C. jejuni* cells obtained through QS is important for the formation of *C. jejuni* colonies in the host. Once the host is successfully colonized, *C. jejuni* can then form biofilms which help it to withstand any stress imposed by the host and the new environment. The biofilm is a defensive structure that provides a protective sheath to the bacteria under which they can reside in a relatively dormant state until favorable conditions return (Chmielewski & Frank, 2003; Murphy et al., 2006).

1.2.5 Persistence of *C. jejuni* in chickens

The body conditions of chickens are favorable for *C. jejuni* as high temperature, nutrient availability, and microaerophilic conditions in the chicken gut make it easy for the *C. jejuni* cells to persist in the chicken GIT (Burnham & Hendrixson, 2018). The colonization of chicken gut is still hostile for the *C. jejuni*. However, the tolerance to various stressors like pH and varying nutritional and oxygen concentration conditions aid in overcoming these impediments (Murphy et al., 2006). The whole list of genetic and regulatory processes used by *C. jejuni* for permanent and successful colonization and persistence in the chicken GIT are unknown (Awad et al., 2018). Chickens rarely show symptoms of disease from colonization by *C. jejuni*. Except in chickens that lack properly developed immune system, and absence of a strong immune response to the *C. jejuni* invasion reflects a long-term evolutionary adaptation of this bacterium in the chicken GIT (Awad et al., 2015; Burnham & Hendrixson, 2018). However, the absence of an early immune response against *C. jejuni* by chicken has been shown to allow the bacterium to spread into other organs like the liver. So, it is surmised that the chicken immune response effectively keeps *C. jejuni* population in check and resists colonization of the bacterium in other organs besides GIT (Vaezirad et al., 2017).

1.2.6 Antibiotics usage and antibiotic resistance of *C. jejuni*

Antibiotics are commonly used in poultry farming throughout the globe to enhance productivity and chicken health. Though, the beneficial effects of antibiotics in chicken growth and productivity are well established, their effect on altering the chicken microbiota ecology is also gaining traction. Antibiotic administration harms beneficial populations and promotes growth of harmful gut bacteria (She et al., 2018). It has been experimentally shown that chickens and mice treated with antibiotics are highly susceptible to *C. jejuni* colonization (Giallourou et al., 2018; Han et al., 2017). Recently, Han et al. (2020) has empirically confirmed that long-term antibiotic exposure in broiler chickens leads to reduction in diversity of intestinal microbiota, which makes it easier for *C. jejuni* to colonize the chicken intestine. This colonization in reduced gut microbial diversity environment results in development of disease symptoms like diarrhea, intestinal damage and infiltration of heterophil into the intestine.

The effects of extended exposure of antibiotics are not limited to chicken health only. Numerous scientific studies over the years have shown that antibiotic resistance in the farm animal bacteria can spill over to environment and humans through contact and consumption of animal products (Lu et al., 2021). The injudicious and overuse of antimicrobials select for antibiotic resistance (ABR) genes in bacteria which are shown to be transferred to humans and the environment (Marshall & Levy, 2011). The evidence for the selection of ABR genes in bacteria and their spread to humans was established long time ago. For example, by using chickens fed with a diet containing tetracycline, a study in 1976 revealed that almost all of the chicken's intestinal flora contained tetracycline-resistant microbes within a week of eating the diet which spread to the 31% of all the farm personnel six months after initiating the experiment (Levy et al., 1976). The spread of ABR costs the economy substantial amounts each year because less effective antibiotics lead to higher medical costs (Salim et al., 2018). According to an estimate from the

European Union (EU), over 25,000 individuals die each year from diseases caused by drug-resistant bacteria, costing the healthcare system €1.5 billion. Furthermore, around 90% of antibiotics given to cattle are excreted into the environment, potentially causing contamination (Cogliani et al., 2011). These public health safety concerns have led many countries to ban or restrict the use of antibiotics in the animal feed. The World Health Organization (WHO) has recommended that the usage of AGPs which are also utilized frequently in human medicine, be completely stopped until risk studies are completed (WHO, 2012). According to the European Food Safety Authority (EFSA) report on antimicrobial resistance (Djenane et al.) among zoonotic and human indicator bacteria, high level of resistance against medically important antibiotics like ciprofloxacin and tetracycline is an immediate public health concern (EFSA, 2020).

In general, the regions where overuse of antibiotics is common, higher prevalence of antibiotic resistant *C. jejuni* strains is expected than the regions with low antibiotic usage in animal farming (Norström et al., 2007). Studies have reported that regular and injudicious use of fluoroquinolones (one of the most common antibiotics used in chicken farming) increased resistance in chicken and human *C. jejuni* isolates (Wieczorek & Osek, 2013). This not only increases transmission of *C. jejuni* into human population from farm animals particularly chicken but also results in increase in treatment failures of *C. jejuni* infections. So, the strategies alternative to antibiotics to decrease *C. jejuni* prevalence in chickens are urgently needed. It has been estimated that decreasing *C. jejuni* bacterial cell levels by $2\log_{10}$ could result in reduction of 90% campylobacteriosis cases (EFSA, 2020).

1.2.7 Transmission of *C. jejuni*

An overwhelming source of *C. jejuni* transmission in humans is chickens. Other animals such as cattle and pigs also contribute to a miniscule percentage of *C. jejuni* infections.

Transmission of *C. jejuni* from chickens to humans occurs primarily through consumption of chicken meat, cross-contamination of contaminated chicken meat in meat processing plants and of other food and water resources (Es-Soucratti et al., 2020). Chickens act as principle reservoir of *C. jejuni* because this bacterium is highly adapted to this animal. It has been experimentally shown that *C. jejuni* infection of only one bird is enough to make the whole chicken flock infected with *C. jejuni* (Horrocks et al., 2009). In the farm, *C. jejuni* primarily spreads through chicken feces when it contaminates water, feed, air, soil and litter. The bacterium gets access to each and every bird in the flock because all the birds share these resources. Aside from these sources, *C. jejuni* can also spread to chickens and to humans through farm workers, flies, rodents, and other animals that may be present in the farm (Ahmed et al., 2013; Newell et al., 2003). The factors that determine the spread of *C. jejuni* transmission among chickens include flock size, working activities, environmental water supplies, insects, staff, rodents, and population of other birds on the same farm are major contributors deciding the rate of transmission (Adkin et al., 2006; Horrocks et al., 2009). Moreover, warmer climates with low oxygen tension levels can also aid in *C. jejuni* transmission (Louis et al., 2005).

1.2.8 Campylobacterosis

Campylobacterosis is defined as an infection of the intestines that is caused by bacteria belonging to the genus *Campylobacter* particularly by *C. jejuni*. The infection in the gastrointestinal tract in some exceptional circumstances could also reach the bloodstream. Campylobacterosis is typically diagnosed as a moderate, self-limiting gastroenteritis that could be associated with 1–3 days of headaches, fever, and vomiting followed by abdominal pain with watery or in extreme cases, bloody diarrhea, lasting 3–7 days (Bolton, 2015). The antibody targeting *C. jejuni* surface lipooligosaccharides can auto-immune react with gangliosides in

the human nervous system, causing serious neurological disorders like Guillain-Barre and Miller Fisher syndrome (Man, 2011). For *C. jejuni* to infect and subsequently cause Campylobacterosis, CFU as low as 360 are sufficient (Hara-Kudo & Takatori, 2011). Studies have revealed that other bacterial pathogens are also directly linked to Campylobacterosis. In developing countries, *E. coli*, rotavirus, and other pathogenic infections were founded to make a person susceptible to Campylobacterosis, but no such evidence was reported in developed countries (Coker et al., 2002).

Campylobacterosis is one of the most common foodborne and gastrointestinal diseases around the world. It is usually perceived as a disease of the industrialized nations. In the United states, more people are affected by Campylobacterosis than any other gastrointestinal disease totaling around 1.5 million people each year (CDC, 2021; Hoffmann et al., 2021). In the European Union (EU), Campylobacterosis is also the most prevalent foodborne disease. Around 0.24 million people in the EU reportedly contracted Campylobacterosis in the year 2018 (EFSA, 2020). Aside from industrialized countries in the Americas and Western Europe, Campylobacterosis is also becoming more widespread in Africa, the Middle East, and Asia, especially among youngsters (Johnson et al., 2017). However, estimations of the burden of Campylobacterosis in developing countries is much harder due to the lack of surveillance programs and resources for executing them (Mughal, 2018). Some reports exist on the association between sex, age and living conditions and susceptibility to Campylobacterosis. A demographic study by Samuel et al. (2004) indicated that the *C. jejuni* infection rate is higher in males than in females of all ages. Green et al. (2006) collected data from Manitoba province, Canada and reported a high rate of Campylobacterosis in the 0-4 and 20-39 years' ages group in rural and urban areas of Manitoba, respectively, with the rate being higher in males. The difference was starker in the 0-4 year age group in which rural population showed seven times higher Campylobacterosis cases than urban

population for the same age group. The authors hypothesized that children living in rural conditions particularly near farms are more susceptible to *C. jejuni* infection than children living in cities. In Hesse, Germany, the rate was also found to be higher in individuals under five years and 5-14 years of age (Fitzenberger et al., 2010). In this study as well, living in rural areas was associated with significantly higher Campylobacterosis cases.

Human-to-human transmission of Campylobacterosis is sporadic. Usually, *Campylobacter* infections are resolved without any antibiotic therapy. In mild diarrheal symptoms, treatment supporting electrolyte and hydration balance is sufficient (Acheson & Allos, 2001). However, antibiotic therapy by macrolide antibiotic (erythromycin) and fluoroquinolones (ciprofloxacin) is needed in certain diarrheal cases (Balfour & Faulds, 1993). If the affected person is pregnant or already suffering from HIV, antibiotic therapy is also necessary (Acheson & Allos, 2001). Tetracyclines, Gentamicin (Shen et al., 2018), and Macrolides drugs are also effectively against *Campylobacter* infection but only for a limited period (Koningstein et al., 2011).

Antimicrobial resistant, mainly fluoroquinolone-resistant, *Campylobacter* strains are causing more infections in humans and animals in developing world where people use antibiotics more frequently than required. It has complicated the clinical treatment of multi-drug-resistant (MDR) *Campylobacter* bacteria (Fields & Swerdlow, 1999; Whelan et al., 2019). Moreover, resistant *C. jejuni* are more likely to cause serious campylobacterosis complications than non-resistant strains. This is evidenced by studies showing that extreme outcomes of campylobacterosis like Guillain-Barre Syndrome (GBS), intestinal hemorrhage, and toxic mega-colon are more common in people who are infected with the resistant *C. jejuni* strains (Chamovitz et al., 1983). A report presented by the Centers for Disease Control and Prevention (CDC) listed the drug-resistant *Campylobacter* strains as microorganisms that could pose serious level of threat to public health

care. The CDC also indicated that about 24% of *Campylobacter* strains are resistant to ciprofloxacin (fluoroquinolone) and macrolides. Furthermore, the report indicates that more than 300,000 *Campylobacter* infections are caused by antibiotic-resistant strains every year in United States (CDC, 2019; Shen et al., 2018).

1.2.9 Prevention of *C. jejuni* colonization

The colonization of *C. jejuni* in chickens can occur through various routes. Reducing the spread of this bacterium in chickens requires implementation of prevention and control measures (Nauta et al., 2009). Prevention and control are two different terms. The preventive measures involve the reduction of chances of *C. jejuni* infection in chickens. Control measures involve additional steps and strategies to minimize bacterial presence in poultry before slaughtering the chickens (Hermans et al., 2011). Efforts to reduce *Campylobacter* species colonization in chickens are more fruitful than the controlling environmental exposure due to the more difficulty faced in the latter case as these bacteria spread faster in the environment (Lin, 2009). The strategies include reducing *C. jejuni* colonization in chickens by increasing the resistance of chickens against *C. jejuni* infection and use of effective strategies alternative to use of antimicrobials. There have been many approaches used for this purpose in the past. These include vaccination (Hodgins et al., 2015), bacteriophage therapy (Kittler et al., 2014), probiotics and prebiotic usage (Arsi et al., 2015; Gálvez et al., 2007). However, after continuous efforts in this regard, no such efficient method has been reported to confer a complete barrier against *C. jejuni* infection in chickens. These measures have indeed shown some success in controlling *C. jejuni* spread in chicken flocks. However, they are ineffective against preventing and controlling complete *C. jejuni* spread. Due to the high compatibility of bacteria, it can multiply in nearly all conditions and develop resistance against these practical measures (Lin, 2009; Vandeputte et al., 2019). Emerging evidence suggests that

reconstituting the gut microbiota in chicken can confer resistance against *C. jejuni* colonization in chicken (Deng et al., 2020; Fu et al., 2021; Han et al., 2017). In the following section, we will discuss the role of microbiota in maintaining health of chicken as well as prevention of *C. jejuni* colonization and then we will discuss microbial modulation of chicken gut as a strategy to control *C. jejuni* colonization and transmission in chicken flocks.

1.3 Microbiota

The ecological communities of commensal as well as symbiotic and pathogenic microorganisms that can colonize the GIT and other parts of the animal body are called microbiota (Sender et al., 2016). Gut microbiota are the organisms that live in the digestive tract of living organisms and often provide beneficial effects to host body functioning. The microbes present in the gut region and their metabolites are collectively called microbiome (Sun, Jia, et al., 2018). It has been reported that the bacterial cells outnumber the host cells by nearly ten to one (Hooper & Gordon, 2001). Chicken at the first day of life shows microbiota presence in their gut, indicating that it acquires it at their embryonic stage either from the mother parent directly (Gantois et al., 2009) or through the pores of eggshells from the environment (Roto et al., 2016), which also constitute as an important source of microbiota transfer to chicken at the hatchery (Pedroso et al., 2005). Kizerwetter-Świda et al. (2008) reported the presence of microbiota in 18 and 20-day old embryos by showing their presence in the liver, yolk, and Ceca. Chicken microbiota is different from that of humans. Wei et al. (2013) reported the presence of 915 operational taxonomic units (OTUs) as broiler microflora. Among them, 13 phyla count for 90% of total microbiota, with *Firmicutes* (70%), *Bacteroidetes* (12.3%), and *Proteobacteria* (9.3%) being the highest in the count. The other phyla observed were *Actinobacteria*, *Cyanobacteria*, *Spirochaetes*, *Synergisteles*, *Fusobacteria*, *Tenericutes*, and *Verrucomicrobia* (Wei et al., 2013). A more recent study reported

a total of 117 genera, with *Ethanoligenes*, *Clostridium*, *Ruminococcus*, *Lactobacillus*, *Desulfohalobium*, and *Bifidobacterium* constituting the majority (Ballou et al., 2016). The microbial diversity is not same throughout the body and varies depending on the particular environment of the organ and also the age of the chickens (Deng et al., 2020; Hooper & Gordon, 2001), with majority of the microbiota residing in the GIT of animals (Guarner & Malagelada, 2003). Even within the GIT, variation in microbiota still exists. The cecum and ileum contain an abundance of Gram positive bacteria and are dominated by bacteria such as *Bacillus*, *Clostridia*, *Lactobacillus*, and *Streptococci* (Lu et al., 2003). However, differences in microbial taxonomic diversity exists between these organs (Awad et al., 2016; Feye et al., 2020). This difference becomes starker as the chicken mature. For example, the cecum in chickens is dominated by *Clostridiacae* while *Lactobacilli* are predominant in the ileum (Lu et al., 2003). Overall, the gut microbiota plays a crucial role in metabolism of nutrients as well as the formation of healthy immune response and present barrier against colonization by the foreign pathogens. So, gut microbial homeostasis is essential for all animals as it plays a vital role for the host's digestive, metabolic, and immune systems (Ballou et al., 2016).

The microbial antigens compartmentalized in the host by mucous epithelial cells layer stimulate the intestinal immune system regularly. The biochemical signals generated by microbiota play an essential role in maintaining the complex interaction of microbe-host symbiotic relations. The pattern recognition receptors recognize these microbial signals and help develop interaction between host and microbe (Mogensen, 2009). The role of epithelial cells in maintaining microbiota and host interaction is also significant. It maintains tolerance for commensal bacteria and offers a reduction in response to a pathogen (Ignacio et al., 2016). The intestinal homeostasis functions to control the penetration of bacteria through the epithelial barrier, and innate immune strategies

prevent bacteria from colonization in small intestine epithelial cells. The most commonly used natural processes for this function are antimicrobial peptide and mucus production (Huttenhower et al., 2012). Comparison of the gut microbiota in the conventionally raised chickens with germ free broiler chickens has also shown that gut microbiota is crucial for the development of chickens as SCFA increases enterocyte development in the chicken body (Chambers & Gong, 2011). Moreover, it is an essential source of crucial nutrients, including ammonium, vitamins, and some types of amino acids (Pan & Yu, 2014). The microbiota produces essential nutrients such as vitamins (B and K groups), organic acids like lactic acid, and short chain fatty acids (acetic, butyric, and propionic acid) as well as antimicrobial compounds like bacteriocins. So, microbiota is not only essential for maintaining immune homeostasis but also in providing nutrients necessary for growth (Shang et al., 2018).

1.3.1 Microbiota and pathogens

The association between gut microbiota and susceptibility to infection by pathogens was first shown by Adams and Prince (1959), who showed that mice free from gut microbes were more susceptible to the pathogen infection than mice with a strong microbiota community. It is widely accepted by scientists that early colonization of the gut by bacteria is important for the well-being and productivity of chickens, as it can modify the digestive tract shape and function as well as its influencing susceptibility of their hosts to various infectious diseases. The colonization of the GIT of birds is followed by the microbial population enrichment and increase in complexity and diversity of these bacterial communities. This is then followed by the maturation and stabilization of the microbial communities. The whole process normally takes around three weeks after hatching of the broiler chickens (Diaz Carrasco et al., 2019). The microbial communities or the microbiota that develops and matures in the GIT protects its host from various pathogens in

various ways. This includes adjusting pH values to create acidic environments or change oxygenation levels in the GIT to make it harder for pathogens that have certain pH and oxygen level requirements to colonize in the host's GIT (Kalliomäki & Walker, 2005; Marteyn et al., 2011). Moreover, as the microbiota is highly adaptable to its host's environment, the foreign pathogens that enter into a new niche in the host face difficulty in competing with the indigenous microbiota for nutrients and other resources (Lawley & Walker, 2013). Furthermore, the gut microbiota also produce secondary metabolites that inhibit the growth of foreign pathogens, which in combination with the stimulation the host innate and adaptive immune response by microbiota, serves as to clear the foreign pathogenic bacteria from the host's body (Han et al., 2017). The gut microbiota not only protects from infection in the GIT by bacteria, fungi, and viruses, but also impacts susceptibility to infections in various other organs such as brain, lungs, liver, and skin (Abaidullah et al., 2019). Because of these reasons, the role of chicken gut microbiota is important in maintaining health and protect the host from infections by pathogens. However, due to variability in chickens and their flocks, pinpointing the exact molecular mechanisms induced by the gut microbiota that has beneficial effect on chicken health and on protection against pathogenic bacteria has been difficult (Diaz Carrasco et al., 2019). Therefore, extensive research is required to understand the physiological roles and molecular dynamics of microbiota (Abaidullah et al., 2019).

1.3.2 Microbiota and *C. jejuni*

One of the most important factors in the colonization of chicken GIT by *C. jejuni* has been shown to be the intestinal microbiota of chickens (Han et al., 2017). A healthy balanced microbial community in chickens is dominated by beneficial gram-positive bacteria (at least 85 percent), while the remaining bacteria include *Clostridium* in younger birds, and

Campylobacter, *Escherichia coli*, and *Salmonella* in older birds in absence of any intestinal disturbance (Choct, 2009). It is unclear how much the microbiota in the chicken GIT influences the colonization by *C. jejuni*. The antibiotic treated and germ free chicken have been shown to be susceptible to *C. jejuni* colonization at significantly higher levels than chicken reared in more conventional farming systems. This highlights a role of natural chicken microbiota in conferring resistance against *C. jejuni* infection in chickens (Han et al., 2017). A study by Han et al. (2017) has shown that microbiota depletion in chicken due to the antibiotic treatment and germ-free rearing conditions increases the CFU of *C. jejuni* in chickens. The authors of the study speculated that lower intestinal diversity allows for the initial colonization of the chicken GIT by *C. jejuni*. Once initial colonization is complete, the resident *C. jejuni* reduce the intestinal integrity of chicken which paves a way for more *C. jejuni* to breach the intestinal barrier. This allows greater number of *C. jejuni* to colonize the GIT, which in absence of diverse and rich intestinal microbiota can spread to other organs as well due to compromised intestinal integrity. Still, not much is known about the specific gut microbes that help in competitive exclusion of *C. jejuni* or the bacteria that help *C. jejuni* to successfully colonize the chicken GIT (Deng et al., 2020). Experimental evidence exists for the association between high levels of *Clostridium perfringens* (*C. perfringens*) and increased susceptibility to *C. jejuni* colonization in chicken (Skånseng et al., 2006). On the other hand, high prevalence of *Actinobacteria* in chickens has been found to be associated with reduced *C. jejuni* colonization (Kaakoush et al., 2014). In mouse models, *Bifidobacterium, Clostridium cluster XI,* and *Lactobacilli* have been shown to have a protective role against *C. jejuni* colonization. Moreover, depletion of the gut microbes induced by antibiotics usage has been shown to contribute to *C. jejuni* colonization and development of enteritis in mice (O'Loughlin et al., 2015; Sun, Jia, et al., 2018).

1.3.3 Microbial metabolites and *C. jejuni*

The host-microbiota helps strengthen the host immune system and produces metabolites and nutrients that help improve the host growth rate. Generally, gut microbiota produces two types of metabolites: primary and secondary. Primary metabolites consisting of peptides, polysaccharides, and fatty acids are functional in every biological system. These metabolites are involved in production of SCFAs and vitamins by fermenting non-digestible components of the digestive system. Both the SCFAs and vitamins have been shown to aid in the growth of the host and also maintaining an effect on intestinal immune homeostasis (Bäckhed et al., 2005; Guarner & Malagelada, 2003). The secondary metabolites of gut microbiota are diverse compounds all of which are characterized by low molecular weight (i.e., around 3000 Daltons). They are functional only in specific biological processes in an organism. These secondary metabolites that are isolated from gut microbes that have antimicrobial properties are known as bacteriocins (Bérdy, 2005; Zacharof & Lovitt, 2012). The major difference between antibiotics and bacteriocins is that the former has broader spectrum activity while the activity of the latter is restricted mostly to strains belonging to same species (Zacharof & Lovitt, 2012). The production of bacteriocins is also crucial for containing *C. jejuni* population (Fu et al., 2021). Besides acting as antimicrobials, these metabolites have a crucial role in growth processes, cell replication, and other responding actions (Bérdy, 2005). These microbiota metabolites have also been demonstrated to suppress *C. jejuni* growth by indirect antagonism against these bacteria (Blaut & Clavel, 2007; Holmes et al., 2011).

One of the most important products of microbiota are bile acids. More than 95% of bile acids produced secreted into gut are successfully absorbed in the gut (Ridlon et al., 2006). In the hepatocytes, primary bile acids like chenodeoxycholic acid (CDCA) and cholic acid (CA) are produced from cholesterol, which are then conjugated with glycine or taurine (Chiang, 2004). Inside the intestine, bile salt hydrolase (BSH) are produced by bacteria which deconjugate

conjugated primary bile acids, which are then changed by microbiota to form secondary bile acids such as lithocholic acid (LCA), ursodeoxycholic acid (UDCA) and deoxycholic acid (DCA) (Archer et al., 1982; Gilliland & Speck, 1977; Ridlon et al., 2006). Bile acids have been linked to a number of chronic disorders, but current research has given indication of positive properties of secondary bile acids in promoting health and ameliorating diseases, such as enhancing gut motility. Anaerobic microbiota as well as their metabolite DCA protects chickens and mice from the *C. jejuni* colonization (Alrubaye et al., 2019) and intestinal inflammation, respectively (Bansal et al., 2020). Recent studies have revealed that DCA alters microbiota composition in chickens to prevent *C. jejuni* colonization (Alrubaye et al., 2019). It increases the population of phylum *Bacteroidetes* in chicken cecal microbiota and decreases *Firmicutes* population, which might be responsible for conferring protection against *C. jejuni* infection (Alrubaye et al., 2019). The particular commensal microbiota composition of the host gut determines vulnerability to *C. jejuni* infection. This has been demonstrated in the conventionally colonized wild type mice, which are immune to the colonization and infection by *C. jejuni* colonization even after high *C. jejuni* peroral infection doses (Heimesaat et al., 2019b). Recent findings suggest that some anaerobic bacteria and the metabolites (specifically DCA) protect *Il10*$^{-/-}$ mice from the intestinal inflammation caused by *C. jejuni*. These metabolites as well as the signaling cascades they induce are important events in controlling *C jejuni* infections and thus, may define new treatment targets (Sun et al., 2018).

1.3.4 The role of mouse microbiota against *C. jejuni*

Research on understanding the role of host microbiota in colonization resistance against *C. jejuni* and the biological processes that allow *C. jejuni* to colonize and causes disease symptoms in the hosts was hampered for long due to the lack of appropriate *in vivo* models. However, recently, a number of mice models have been developed that have shed light on this

topic (Giallourou et al., 2018; Heimesaat et al., 2019a, 2019b; Sun, Winglee, et al., 2018; Wang et al., 2019). Mice gut microbiota contain a total of 37 genera (Wang et al., 2019). Among all these bacteria, only eight bacteria namely, *Bifidobacterium*, *Butyricoccus*, *Clostridium XI*, *Coprobacillus*, *Hydrogenoanaerobacterium*, *Lactobacillus*, *Oscillibacter*, and *Roseburia*, are found to confer resistance to *C. jejuni* colonization in mice (Sun, Winglee, et al., 2018). On the other hand, high numbers of *E. coli* have been shown to facilitate *C. jejuni* colonization while enrichment of *Enterococcus spp.* and *Clostridium sensu stricto* in the mice microbiota has been shown to facilitate colitis induced by *C. jejuni* (Haag et al., 2012; Sun, Winglee, et al., 2018).

Specific pathogen free (SPF) mice have been shown to be naturally resistant to the colonization of *C. jejuni*. On the other hand, birds show one of the highest colonization rates of *C. jejuni* colonization albeit without development of any visible disease symptoms most of the time (Awad et al., 2018; Sun, Winglee, et al., 2018). Humans show one of the strongest responses to *C. jejuni* colonization due to the development of various disease symptoms which normally go away on their own in 1 to 2 weeks (Giallourou et al., 2018). The specific reason for this heterogeneity in *C. jejuni* colonization rates and responses by different hosts is unknown. One of the most probable reason for these different outcomes could be the difference in microbiota and specially gut microbiota between these organisms (Lawley & Walker, 2013). Mice gut microbiota acts as a protective shield against colonization of *C. jejuni* as shown by studies using conventionally raised mice and gnotobiotic and germ free (GF) mice. The former murine models successfully repel *C. jejuni* colonization while in the latter two groups, *C. jejuni* has been shown to easily colonize the mice (Bereswill et al., 2011; Smith & Tucker, 1978). Further evidence on the role of mice microbiota in protecting the host from *C. jejuni* colonization comes from antibiotic treated (AT) mice models. Administration of ampicillin in mice decreases bacterial diversity which has been

found to be correlated with increased susceptibility to *C. jejuni* colonization (O'Loughlin et al., 2015). Moreover, reconstitution of the mice microbiota with human microbiota has been shown to allow *C. jejuni* colonization in mice while the opposite has been found to be true when mice are reconstituted with mice microbiota (Bereswill et al., 2011). Furthermore, a recent study by Heimesaat et al. (2019b) has shown that mice containing high loads of *C. jejuni* due to depleted microbiota and *C. jejuni* infection could be successfully treated with FMT from healthy and conventionally raised mice. The authors showed that FMT treatment reduced the *C. jejuni* loads in mice by 7.5 folds within two weeks. Moreover, *C. jejuni* cells were even completely eliminated in 12.5% of mice that previously contained high numbers of *C. jejuni*. Besides, the FMT treatment also improved immune response and reversed the intestinal damage caused by the *C. jejuni* colonization in mice. In another study, Heimesaat et al. (2019a) also showed that FMT treatment could also treat mice model of human *C. jejuni* infection. This was demonstrated by two fold decrease in *C. jejuni* cells load and improvement in immune status in the human *C. jejuni* infection mice models within a week of FMT.

There is presently relatively little data on supporting the use of FMT to prevent *C. jejuni* colonization in chickens. However, in humans, the generation of bile acids by FMT has been shown to be highly efficient in treating reoccurring *Clostridium difficile* infections (Buffie et al., 2015; Silverman et al., 2010). However, there is lack of date that support the usage of mice microbiota to prevent *C. jejuni* chicken colonization. In the next chapter, we used SPF-mice microbiota to prevent *C. jejuni* chicken colonization.

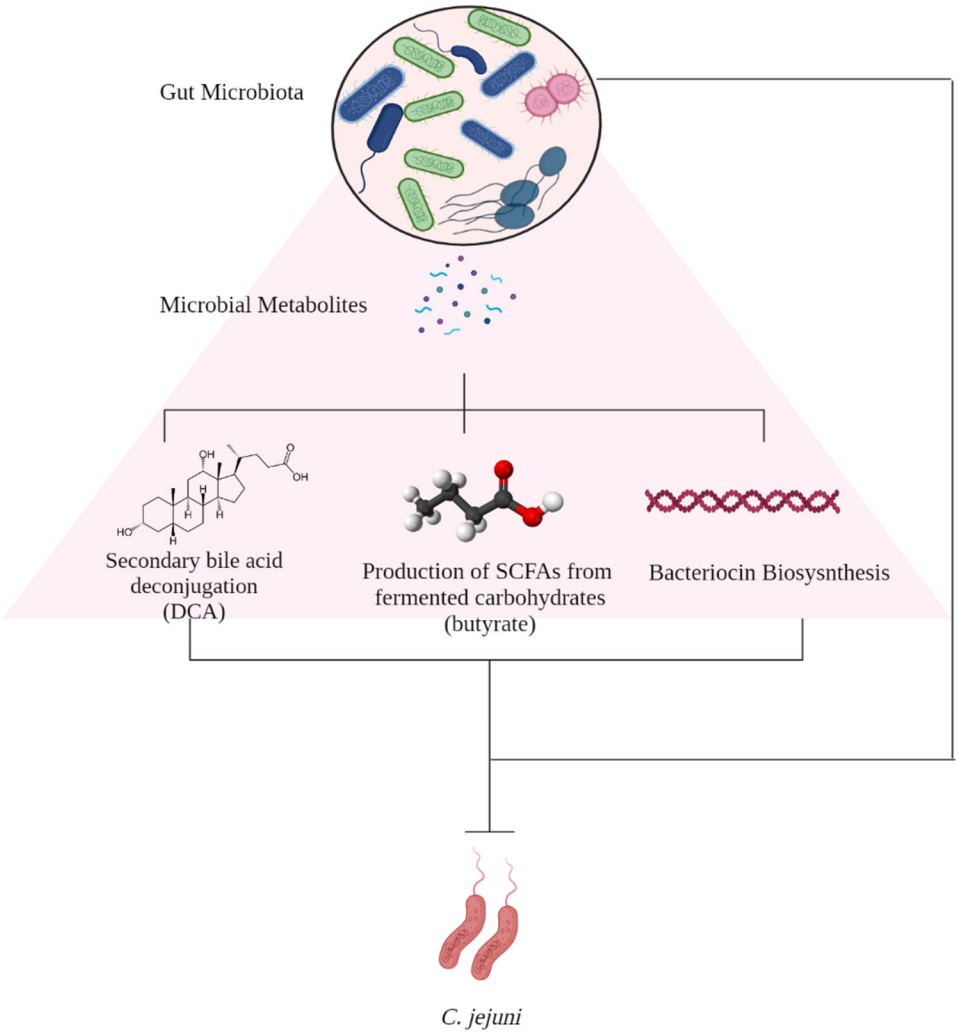

Figure 1.1 Microbiota and microbial metabolites illustration of inhibition *C. jejuni* in gut.

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CHAPTER II

Microbiota from Specific Pathogen-Free Mice Reduces *Campylobacter jejuni* Chicken Colonization

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2.1 Abstract

Campylobacter jejuni, a prevalent foodborne bacterial pathogen, is mainly transmitted from poultry with few effective prevention approaches. In this study, we aimed to investigate the role of microbiota on *C. jejuni* chicken colonization. Microbiota from specific pathogen-free (SPF) mouse stools were collected as SPF-Aerobe and SPF-Anaerobe. Birds were colonized with SPF-Aerobe or SPF-Anaerobe at day 0 and infected with *C. jejuni* AR101 at day 12. Notably, *C. jejuni* AR101colonized at 5.3 and 5.6 $\log_{10}$ *C. jejuni* CFU/g chicken cecal digesta at days 21 and 28, respectively, while both SPF-Aerobe and SPF-Anaerobe microbiota reduced pathogen colonization. Notably, SPF-Aerobe and SPF-Anaerobe increased cecal phylum *Bacteroidetes* and reduced phylum *Firmicutes* compared to those in the non-transplanted birds. Interestingly, microbiota from noninfected chickens, SPF-Aerobe, or SPF-Anaerobe inhibited AR101 in vitro growth, whereas microbiota from infected birds alone failed to reduce pathogen growth. The bacterium *Enterobacter*102 isolated from infected birds transplanted with SPF-Aerobe inhibited AR101 in vitro growth and reduced pathogen gut colonization in chickens. Together, SPF mouse microbiota was able to colonize chicken gut and reduce *C. jejuni* chicken colonization. The findings may help the development of effective strategies to reduce *C. jejuni* chicken contamination and campylobacteriosis.

Keywords: microbiota transplantation; foodborne pathogen; intestine; bacterial colonization; specific pathogen-free.

2.2 Background

Campylobacter jejuni colonizes asymptomatically in the intestinal tract of poultry and causes a prevalent foodborne campylobacteriosis around the world [1,2]. *C. jejuni* resistant to macrolides, fluoroquinolones, aminoglycosides, or carbapenems has been detected in samples from children and adults worldwide [1–7]. More than 20 cases of campylobacteriosis per 100,000 population were reported in the USA in 2019 [8], and more than 220,000 people were affected in Europe in 2019 [9]. The case number was more than the total incidences induced by eight other bacterial pathogens [10]. More than 14.35 cases per 0.1 million people were caused by the pathogen in 2020 [11]. Moreover, *C. jejuni* often causes severe post-infectious complications, such as arthritis [12], the neurodegenerative disorder Guillain–Barr syndrome [13], irritable bowel syndrome [14], and inflammatory bowel diseases (IBD) [15,16].To reduce campylobacteriosis, different measures have been implemented to reduce enteritis by reducing *C. jejuni* contamination in animal food, particularly pre- and post-harvest poultry. The intervention methods include strict biosecurity on farms [17], vaccines [18], probiotics [19], phages [20], decontamination of poultry carcasses in the post-slaughter process [21], facility design and management, reducing contamination in feed, transportation, and other sources, and other strategies [2]. It is estimated that decreasing Campylobacter count on chicken carcasses by 100 times decreases human campylobacteriosis 30-fold [22]. Although those reduction measurements reduce some *C. jejuni* contamination, improved and alternative strategies are much needed, as reflected in the consistent high level of campylobacteriosis incidence reported in the Morbidity and Mortality Weekly Report from the Infectious Disease Database at CDC between 1996 and 2017 [23].

The gastrointestinal tract of humans and animals is inhabited by trillions of microbes, collectively called the microbiota [24,25]. The gut microbiota modulates essential host physiology and various host functions such as the intestinal barrier, nutrition, and immune homeostasis [25–29]. Specific

pathogen-free (SPF) Il10$^{-/-}$ mice are naturally resistant to *C. jejuni* 81–176-induced colitis, while the mice become susceptible to campylobacteriosis after being treated with the broad-spectrum antibiotic clindamycin [30]. Sequencing and bioinformatic analysis of 16S rDNA revealed that microbiota-mediated bile acid metabolism was essential for preventing *C. jejuni*-induced colitis. Increasing evidence is emerging on gut microbiota preventing *C. jejuni* colonization in poultry [31–33]. Apart from naturally transmitting microbiota from wild hens to turkey chicks, the turkey microbiota transmission is disrupted in modern industrialized poultry production, partly because eggs are hatched by a hatchery instead of hens [34]. Poultry chicks obtain their microbiota from the environment and/or farms, where most of the microbes are not natural inhabitants of the bird gut [35]. The application of antibiotics as growth promoters further drives the dysbiosis of birds in commercial poultry production [36]. In our previous studies, we found that transplanting bile acid deoxycholic acid-modulated microbiota to hatched chicks reduced the colonization of *C. jejuni* human clinical isolate 81–176 and chicken isolate AR101 in pre-harvest chickens [37].

Because SPF mice are naturally resistant against a *C. jejuni* infection [30,38], in this study, we hypothesized that SPF mouse microbiota would be able to colonize chickens and reduce *C. jejuni* chicken colonization. Our data indicate that the mouse SPF-Aerobe and SPF-Anaerobe microbiota shaped the chicken intestinal microbiota. Furthermore, the SPF-Aerobe and SPF-Anaerobe indeed reduced *C. jejuni* AR101 in vitro growth and chicken colonization. These findings will help the development of effective strategies against *C. jejuni* chicken colonization.

2.3 Materials and methods

2.3.1 Mouse microbiota preparation and chicken experiments of microbiota transplantation and *C. jejuni* infection

The performed animal experiments were in accordance with the Animal Research: Reporting of In Vivo Experiments (https://www.nc3rs.org.uk/arrive-guidelines accessed on 22

August 2019) and approved by the Institutional Animal Care and Use Committee of the University of Arkansas (protocols No. 20009 for mice and 20011 for chickens). For the bird experiment with SPF microbiota, a total of 135 zero-day-old Cobb 500 broiler chicks were randomly allocated into cohorts of 15–30 birds per group, as detailed in Supplementary Table S1. The birds obtained from Cobb-Vantress (Siloam Springs, AR, USA) 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 days 0–10 and a grower diet during days 11–28. The basal diet was formulated as described earlier [37,52]. Stool from eight-week-old SPF BL6 *Il10*$^{-/-}$ mice fed a chew diet was freshly collected and immediately suspended in 30% glycerol PBS stock and stored at −80 °C. The stool samples were cultured on brain heart infusion (BHI, BD Biosciences, Franklin Lakes, NJ, USA) agar plates at 42 °C for 48 h under aerobic or anaerobic conditions using the GasPak system (BD Biosciences, Franklin Lakes, NJ, USA) and collected as SPF-Aerobe and SPF-Anaerobe microbiota. The microbiota was added glycerol at final 30% and stored at −80 °C. Before the chicken colonization experiment, the SPF-Aerobe and SPF-Anaerobe microbiota were cultured on a BHI plate for 48 h, collected in PBS, and enumerated by OD_{600} and plating. OD_{600} of 1 was estimated at about 10^8 CFU/mL. At chicken experiments, chicks at day 0 were orally gavaged once with PBS or 10^8 CFU/bird SPF-Aerobe or 10^8 CFU/bird SPF-Anaerobe. For the chicken experiment of *Enterobacter*102, a total of 90 zero-day-old Cobb 500 broiler chicks were randomly allocated into cohorts of 30 birds per group. The birds were fed and raised similarly to those in the SPF microbiota experiment. The chickens were orally gavaged once with PBS or 10^8 CFU/bird of *Enterobacter*102 in 0.5 mL/bird on day 0. Two days before infection, frozen stock of *C. jejuni* AR101 (isolated at Dr. Billy Hargis's lab at University of Arkansas at Fayetteville) were cultured microaerobically at 42 °C for 48 h on *C. jejuni*-selective blood plates. The motility of *C.*

jejuni was ensured under a microscope as described before [53] and routinely examined on semisolid MH (0.4% agar) plates. *C. jejuni* AR101 in PBS was estimated as that OD_{600} of 0.468 was 10^{10} CFU/mL. The bacterium was also serially diluted, cultured on the *Campylobacter*-selective plates, and enumerated 48 h later. The *Campylobacter*-selective plate was prepared in-house and it consisted of Bolton's Campylobacter Enrichment (CE) Broth (Neogen Food Safety, Lasing, MI, USA), 1.5% agar (VWR, USA, OH), 5% lysed horse blood (VWR, Radner Township, PA, USA), five antibiotics (20 mg/L cefoperazone, 50 mg/L cycloheximide, 20 gm/L trimethoprim, 20 mg/L vancomycin, and 0.35 mg/L polymyxin B), 500 mg/L ferrous sulfate, and 200 mg/L triphenyl-tetrazolium chloride (TTC) (all from Sigma-Aldrich, St. Louis, MO, USA). The ferrous sulfate and TTC were used to make *C. jejuni* colonies dark red. The birds were gavaged with 1 mL PBS or 10^9 CFU/bird *C. jejuni* AR101 at day 12 [37]. Chicken body weight was measured at days 0 and 28. Because of the pen size constrains, the birds were randomly euthanized at days 21 and 28 to collect cecal samples for enumerating *C. jejuni*, and the exact bird numbers are listed in figure legends.

This experiment was conducted until 28 days of age because of the pen size constrain. Cecal digesta samples of all the groups were collected for DNA isolation. Another set of cecal digesta were serially diluted ten-fold with sterile PBS and cultured on the *Campylobacter*-selective plates at 42 °C for 48 h under a microaerophilic atmosphere. Emerged colonies were positively determined as *C. jejuni* only when they were dark red and shining, round, and with a smooth surface. The colonies were also examined under a microscope for size and motility evaluation [53]. The CFU per gram digesta was then calculated.

2.3.2 Estimation of microbiota composition at phylum level

Cecal digesta samples were collected, and DNA was extracted using bead beater disruption and phenol: chloroform separation method as described before [54]. Briefly, 0.1 g of fecal sample

suspended in 500 μL PBS was transferred to a 2 mL screw cap tube containing 85 μL of 10% SDS solution, 500 μL of phenol/chloroform (25:24), and 0.3 g sterile 0.1 mm zirconia beads (BioSpec, Bartlesville, OK, USA). The samples were homogenized on a Fisher brand Bead Mill 24 Homogenizer (Fisher Scientific, Pittsburg, PA, USA) for 3 × 30 s at high speed with a 10 s pause for each run. After centrifugation, the supernatant was further extracted twice with 500 μL of chloroform (25:24), and the top aqueous layer was collected and mixed with 1/10 Vol (~50 μL) 3M sodium acetate (pH 5.2) and 2.5 Vol (~1.25 mL) ethanol overnight at –20 °C. After centrifugation, the DNA pellet was washed once with 70% ethanol and resuspended in 100 μL DNase/RNase-free H_2O. The abundance levels of five phyla of gut bacteria were determined by real-time PCR according to the manufacturer's recommendation. Briefly, each PCR reaction mixture comprised 4 μL of BioRad iTaq Universal SYBER Green Super mix (BioRad, Hercules, CA, USA), 1.6 μL of template DNA (~4 ng), 0.6 μL of 5 μM primer mix, and 1.8 μL of DNase/RNase H_2O. The amplification reaction was performed in a BioRad 384 Real-Time PCR machine (BioRad, Hercules, CA, USA) using the following program: 1 min at 95 °C, followed by 30 cycles of 30 s each at 95 °C, 60 s at 60 °C. The gene primers [37] used included universal 16S rRNA: 16S357F 5′-CTCCTACGGGAGGCAGCAA-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*: 798cfbF 5′-CRAACAGGATTAGATACCCT-3′,cfb967R 5′-GGTAAGGTTCCTCGCGTAT-3′; *Firmicutes*: 928FirmF 5′-TGAAACTYAAAGGAATTGACG-3′, 1040FirmR 5′-ACCATGCACCACCTGTC-3′; *Actinobacteria*: Act920F3 5′-TACGGCCGCAAGGCTA-3′, Act1200R 5′-TCRTCCCCACCTTCCTCCG-3′. The relative percentage of each phylum was

calculated following the relative PCR quantification method [55] similar to that in this paper [55]. Briefly, the $2^{-\Delta\Delta CT}$ value of each phylum gene expression Ct in one sample was calculated using the universal 16S rRNA gene expression Ct. The percentage of each phylum was then calculated by the phylum $2^{-\Delta\Delta CT}$ value in one sample divided by the sum of all phylum $2^{-\Delta\Delta CT}$ values in the same sample and multiplied by 100.

2.3.3 Isolation of *Enterobacter*102

When chicken cecal digesta were cultured on *C. jejuni* selective plates, pink colonies were grown on the plate, compared to dark red *C. jejuni* colonies. The pink colony was named *Enterobacter*102. Under a light microscope, *Enterobacter*102 was rod-shaped and larger than *C. jejuni*. *Enterobacter*102 was able to grow aerobically, stained Gram-negative, and showed pink colonies on a MacConkey plate.

2.3.4 Identification of bacterial species using 16s DNA and sanger sequencing

Either *C. jejuni* AR101 or *Enterobacter*102 was derived from a single colony. To isolate DNA for Sanger sequencing, the bacteria were spread on the respective agar plates. The bacteria were collected, and DNA was extracted. Genomic DNA from *C. jejuni* AR101 or *Enterobacter*102 was amplified by PCR of the 16S rDNA gene region with universal primers (27Fw1: 5′-AGAGTTTGATCMTGGCTCAG-3′, 1492R: 5′- CGGTTACCTTGTTACGACTT-3′) following the instructions in this webpage https://chmi-sops.github.io/mydoc_16S_Sanger.html (accessed on 20 July 2020). The PCR products were gel-purified and Sanger-sequenced at Eurofins Scientific using primers of 27Fw1, 1492R, and universal primer 515Fw2: 5′-GTGCCAGCMGCCGCGGTAA-3′. The sequences were assembled and aligned using the NCBI genome database. The bacteria were given species names with >98.0% and 95.0% of 16S rDNA sequence homology for *Campylobacter jejuni* and *Enterobacter* sp., respectively. The 16s rDNA sequences were uploaded at NCBI with submission numbers of SUB10285129 and SUB10285090.

2.3.5 *In Vitro* co-culture of *C. jejuni* with various microbiota

The impact of various microbiota on *C. jejuni* growth was evaluated. Briefly, *C. jejuni* AR101 from frozen stocks was cultured and grown on the *Campylobacter*-selective plates in a microaerophilic atmosphere for 48 h using the GasPak system (BD Biosciences, Franklin Lakes, NJ, USA). *C. jejuni* at 6.3×10^8 CFU was co-cultured with noninfected microbiota at 2.0×10^8 CFU, SPF-Aerobe at 1.8×10^8 CFU, or SPF-Anaerobe at 8.4×10^8 CFU in 1 mL of CE broth. *C. jejuni* at 7.7×10^8 CFU was co-cultured with Cj-MB at 6.7×10^7 CFU, Cj-SPF-Aerobe at 1.6×10^9 CFU, or Cj-SPF-Anaerobe at 1.5×10^9 CFU in 1 mL of CE broth. *C. jejuni* at 1.8×10^8 CFU and 4.4×10^7 CFU *Enterobacter*102 were co-cultured in 1 mL of CE broth. The experiments were carried out in triplicate. Because *C. jejuni* growth would be reduced within 24 h in anaerobic conditions [54], the co-culture bacteria were incubated for 24 h at 42 °C under anaerobic conditions using the GasPak system (BD Biosciences, Franklin Lakes, NJ, USA) to mimic cecal air conditions. *C. jejuni* growth was measured by serial dilution and plating on the *Campylobacter*-selective plates for enumeration. When co-culture with *C. jejuni*, *Enterobacter*102 was counted with pink colonies compared to dark red colonies of *C. jejuni*. Cj-MB, Cj-SPF-Aerobe, and Cj-SPF-Anaerobe themselves could not grow a single colony on the *Campylobacter*-selective plates, suggesting that *C. jejuni* lost culturability after storing with microbiota.

2.3.6 Statistical analysis

All 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 using GraphPad Prism 7.0 software. *C. jejuni* CFU was transformed with a formula of $\log_{10}$ (CFU + 1). The results were considered statistically significant if p-values were <0.05.

2.4 Results

2.4.1 Mouse microbiota reduced *C. jejuni* AR101 chicken colonization

Mouse SPF-Aerobe and SPF-Anaerobe microbiota was prepared from SPF mouse stools and transplanted to zero-day-old chicks. DNA from *C. jejuni* chicken isolate AR101 was isolated, and 16S rDNA was PCR-amplified, Sanger-sequenced, and confirmed to be in 99.0% alignment with *C. jejuni*. The birds were infected with AR101 at day 12. Consistently with our previous reports [37], *C. jejuni* was not detected in noninfected birds, suggesting clean housing at our poultry facility. Notably, mouse SPF-Aerobe and SPF-Anaerobe microbiota reduced *C. jejuni* AR101 cecal colonization by more than 1-log compared to that of only infected birds (Cj AR101) at day 21 (3.8 ± 0.2 and 4.1 ± 0.0 vs. 5.3 ± 0.4 $\log_{10}$ *C. jejuni* CFU/g cecal digesta, respectively) (Figure 1A). The SPF-Aerobe and SPF-Anaerobe continued to reduce *C. jejuni* chicken colonization compared to that of the infected control birds at day 28 (4.0 ± 0.6 and 4.9 ± 0.1 vs. 5.6 ± 0.2 $\log_{10}$ *C. jejuni* CFU/g cecal digesta, respectively) (Figure 1B). Notably, the SPF-Anaerobe microbiota with or without *C. jejuni* AR101 infection increased the accumulative body weight compared to that of noninfected birds from day 0 to day 28 (1606 ± 17.7 and 1683 ± 43.1 vs. 1463 ± 47.6 g/bird, respectively) (Figure S1), while SPF-Aerobe microbiota did not increase the bird weight gain. These results suggest that SPF-Aerobe and SPF-Anaerobe effectively reduce *C. jejuni* AR 101 colonization in the chicken.

2.4.2 SPF-Aerobe and SPF-Anaerobe modulated the chicken microbiota

We reasoned that the colonization reduction of AR101 in chickens might come from chicken microbiota alteration by the mouse microbiota transplantation. To assess this hypothesis, we used phylum-specific primers to analyze the microbiota composition change. Notably, SPF-Aerobe and SPF-Anaerobe reduced the relative abundance of the phylum *Firmicutes* compared to that of uninfected birds (61.3 and 52.9 vs. 97.5%) and infected birds (51.9 and 50.9 vs. 86.7%,

respectively), while the relative abundance of *Bacteroidetes* was increased compared to that of uninfected birds (38.4 and 44.7 vs. 2.3%) and infected birds (42.0 and 47.6 vs. 12.4%, respectively) (Figure 2). Interestingly, *C. jejuni* colonization modulated chicken cecal microbiota of the phyla *Bacteroidetes* and *Firmicutes*. Importantly, most of the relative abundance was significant (Table 1). These results indicate that SPF-Aerobe, SPF-Anaerobe, and *C. jejuni* were able to colonize and change the microbiota in the chicken gut.

2.4.3 Chicken noninfected microbiota and mouse SPF microbiota reduced *C. jejuni* growth

Upon validation of transplanted mouse SPF microbiota reducing *C. jejuni* chicken colonization, we reasoned that the mouse SPF microbiota would directly inhibit *C. jejuni* AR101 growth, while the chicken microbiota would not. To examine this hypothesis, *C. jejuni* AR101 inoculum was co-cultured with microbiota from noninfected, SPF-Aerobe, and SPF-Anaerobe chickens in the Campylobacter Enrichment (CE) Broth at 42 °C for 24 h under anaerobic conditions. Notably, both SPF-Aerobe and SPF-Anaerobe reduced *C. jejuni* AR101 by more than 1-log compared to the *C. jejuni* AR101 culture-alone group (6.8 ± 0.2 and 6.3 ± 0.1 vs. 8.6 ± 0.3 $\log_{10}$ *C. jejuni* CFU/mL, respectively) (Figure 3). Interestingly, the microbiota from noninfected chickens also reduced *C. jejuni* AR101 growth by more than 2-log compared to that in the *C. jejuni* AR101 culture alone (6.4 ± 0.4 vs. 8.6 ± 0.3 $\log_{10}$ *C. jejuni* CFU/mL, respectively). Notably, each microbiota at 24 h increased the number of CFU compared to that at 0 h (Figure S2). Because of the unexpected result of noninfected chicken microbiota reducing *C. jejuni* AR 101 in vitro growth, we then modulated our hypothesis that *C. jejuni* possibly modulated chicken microbiota for its growth and colonization. To address this reasoning, we co-cultured *C. jejuni* AR101 with chicken microbiota from only infected birds (Cj-MB), transplanted with SPF-Aerobe and infected birds (Cj-SPF-Aerobe), and transplanted with SPF-Anaerobe and infected birds (Cj-SPF-Anaerobe). Interestingly, the three-chicken microbiota themselves could not grow a single colony

on the *Campylobacter*-selective plates (Figure 4), suggesting that *C. jejuni* lost culturability after storing with microbiota. Notably, Cj-MB did not reduce *C. jejuni* AR101 in vitro growth compared to that in positive control of Cj AR101 culture alone. Consistently, Cj-SPF-Aerobe and Cj-SPF-Anaerobe reduced *C. jejuni* AR101 in vitro growth by more than 3-log compared to that in the Cj AR101 culture-alone group (3.7 ± 0.6 and 0.8 ± 0.5 vs. 7.3 ± 0.1 $\log_{10}$ *C. jejuni* CFU/mL, respectively). Consistently, each microbiota at 24 h increased number of CFU compared to that at 0 h (Figure S3). These results suggest that *C. jejuni* modulated the chicken microbiota for its growth and colonization, while the transplanted mouse SPF microbiota resisted against pathogen growth.

2.4.4 An Aerobic bacterial isolate reduced *C. jejuni* AR101 *In Vitro*

Next, we wanted to identify and isolate the individual bacteria from the protective SPF microbiota. About 100 bacterial colonies were isolated using BHI plates at 42 °C under anaerobic conditions for 48 h. The colonies were individually co-cultured with *C. jejuni* for 24 h, and then *C. jejuni* was enumerated on the *Campylobacter*-selective plates prepared in-house. Unfortunately, none of the bacteria were able to inhibit *C. jejuni* growth using the co-culture method. By accident, during one chicken trial, a bacterial colony from birds gavaged with mouse SPF-Aerobe was able to grow with pink color on the *Campylobacter*-selective plate compared to the dark red color of *C. jejuni*. The bacterial colony was selected and later named *Enterobacter*102. We reasoned that this bacterium might resist *C. jejuni* infection. *Enterobacter*102 was rod-shaped, stained Gram-negative, and had the same size as *E. coli*. *Enterobacter*102 also grew in pink colonies on a MacConkey agar plate. The DNA from *Enterobacter*102 was isolated, and 16S rDNA was PCR-amplified, Sanger-sequenced, and confirmed to be in 95% alignment with *Enterobacter* sp. To functionally dissect the interaction between *Enterobacter*102 and *C. jejuni* AR101, in vitro co-culture was performed. Interestingly, *Enterobacter*102 showed the ability to reduce *C.*

jejuni AR101 colonization by more that 2-log compared to that in the Cj AR101 culture-alone group (4.6 ± 0.1 vs. 7.3 ± 0.1 $\log_{10}$ *C. jejuni* CFU/mL) (Figure 5). Not surprisingly, the number of *Enterobacter*102 increased at 24 h compared to that at 0 h (Figure S4). Although the reduced *C. jejuni* could result from depleted nutrients in the presence of a microbiota (SPF microbiota or *Enterobacter*102), the comparable growth between Cj AR101 and Cj-MB AR101 in Figure 4 suggested that the microbiota was an important factor influencing *C. jejuni* growth. These results suggested that *Enterobacter*102 has potential to reduce *C. jejuni* AR101 chicken colonization.

2.4.5 *Enterobacter*102 reduced *C. jejuni* AR101 chicken colonization

Encouraged by the result of *Enterobacter*102 reducing *C. jejuni* AR101 in vitro growth, we then performed chicken experiments. The birds were colonized with 10^8 CFU/chick of *Enterobacter*102 at day 0, infected with *C. jejuni* at day 12, and euthanized at days 21 and 28. Consistently with the in vitro experiments, *Enterobacter*102 reduced *C. jejuni* AR101 chicken colonization by more than 1-log at day 21 in comparison to that in only infected birds of the Cj AR101 group (4.0 ± 0.0 vs. 5.3 ± 0.4 $\log_{10}$ *C. jejuni* CFU/g cecal digesta) (Figure 6A). Notably, *Enterobacter*102 continued to reduce *C. jejuni* AR101 chicken colonization by more than 2-log at day 28 (2.4 ± 0.9 vs. 5.7 ± 0.2 $\log_{10}$ *C. jejuni* CFU/g cecal digesta) (Figure 6B). These results suggest that the bacterial isolate of *Enterobacter*102 inhibited *C. jejuni* AR101 growth and reduced the pathogen's chicken colonization.

2.5 Discussion

Poultry is the main reservoir of the prevalent foodborne bacterial pathogen *C. jejuni* which asymptomatically colonizes the birds [39]. However, the pathogen fails to colonize SPF or conventionally raised mice [30,38]. We then hypothesized that the microbiota from SPF mice might resist against *C. jejuni* infection, while the chicken microbiota might be susceptible to the pathogen. Here we report that mouse SPF-Aerobe and SPF-Anaero microbiota reduced *C.*

jejuni chicken colonization at days 21 and 28. Notably, SPF-Aerobe and SPF-Anaerobe increased chicken cecal phylum *Bacteroidetes* and reduced phylum *Firmicutes* compared to those in the infected-alone birds. Interestingly, the uninfected chicken microbiota, SPF-Aerobe, or SPF-Anaerobe inhibited AR101 in vitro growth. Microbiota from birds transplanted with SPF-Aerobe or SPF-Anaerobe and infected inhibited AR101 in vitro growth, whereas microbiota from *C. jejuni*-infected-alone birds did not. *Enterobacter*102 isolated from infected birds transplanted with SPF-Aerobe reduced AR101 in vitro growth and chicken colonization. Altogether, these findings revealed that mouse SPF microbiota is able to colonize the chicken gut and resists against *C. jejuni* colonization in chickens, suggesting a potential strategy to reduce *C. jejuni* chicken contamination.

A notable observation from this study is that the mouse microbiota was able to be successfully transplanted into chickens and to reduce *C. jejuni* chicken colonization. It is a well-known medical practice to transplant a healthy donor's microbiota to treat a human *Clostridium perfringens* infection [40]. The microbiota compositions of human recipients are comparable to those of the human donor's, and the *C. difficile* infection is reduced. Consistently, microbiota composition in recipient piglets is similar to that of human donors in an inter-mammalian microbiota transplantation [41], suggesting that it is feasible to transplant microbiota between animals within the class level of Mammalia. In the current study, we successfully transplanted mouse (class Mammalia) microbiota to chickens (class Aves), suggesting it is possible to transplant microbiota between animals within the phylum level of Chordata. Apparently, the difference of body temperature (42 °C in chickens and 37 °C in mice) and intestinal anatomy between the animals did not reduce the donor mouse microbiota colonization in the recipient chickens. A meta-data analysis study showed that chicken microbiota at the phylum level is mainly

comprised of 13 phyla, including *Firmicutes* (70.0%), *Bacteroidetes* (12.3%), *Proteobacteria* (9.3%), and other small proportions of *Actinobacteria*, *Cyanobacteria*, *Spirochaetes*, *Synergisteles*, *Fusobacteria*, *Tenericute*, and *Verrucomicrobia* [42]. Consistent with this finding, we found that birds without a mouse microbiota transplantation had the phylum *Firmicutes* majority, while microbiota-transplanted birds dramatically reduced *Firmicutes* and increased *Bacteroidetes*, independently of *C. jejuni* infection. Interestingly, the microbiota in mice is composed of the phyla *Firmicutes* at 54% and *Bacteroidetes* at 30% [43], which is close to the composition of our transplanted chicken microbiota. A field survey study reported that birds from the farms with the highest *Campylobacter* counts show the highest percentage of *Firmicutes* and the lowest percentage of *Bacteroidetes* in their microbiota, although microbiota composition is highly variable between or within farms [44]. In addition, the significant reduction of *C. jejuni* colonization by SPF-Aerobe and SPF-Anaerobe microbiota in both days 21 and 28 suggested that the microbiota may continue to reduce pathogen colonization for a longer period of time. This experiment was cut short because of the pen size constrain. It would be interesting in the future to conduct follow-up experiments to reduce *C. jejuni* colonization by SPF microbiota for birds at the market age of days 35–45. Together, these data showed that mouse SPF microbiota is transplantable to reduce *C. jejuni* chicken colonization.

After the evaluation of the protective effect of the mouse SPF microbiota, it is imperative to isolate and identify individual bacteria in the microbiota against *C. jejuni* chicken colonization for further functional evaluation. In a human longevity study, Sato and colleagues have plate-cultured, isolated, and evaluated a group of 68 bile acid metabolizing bacteria [45]. They found that *Parabacteroides merdae* and *Odoribacteraceae* strains produced isoalloLCA and reduced

Gram-positive multidrug-resistant pathogens, such as *C. difficile* and vancomycin-resistant *Enterococcus faecium* [45]. A microbiota with higher level of genera *Clostridium XI*, *Bifidobacterium*, and *Lactobacillus* is associated with resistance to *C. jejuni*-induced colitis in mice [30]. Interestingly, probiotics *Bifidobacterium longum* PCB133 and a xylo-oligosaccharide do not decrease *C. jejuni* chicken colonization [46]. We have co-cultured *C. jejuni* with various ATCC or lab-isolated bacteria, such as *Bifidobacterium longum* and *Clostridium scindens*, and we did not find the bacteria to reduce *C. jejuni* in vitro growth (data not shown). During our search for individual microbiota against *C. jejuni*, we found that the *Enterobacter*102 from microbiota of SPF-Aerobe grew as pink colonies on the *Campylobacter*-selective plates. Later, we found that *Enterobacter*102 reduced *C. jejuni* in vitro growth and chicken colonization. Probiotic application of *Enterobacter* sp. improves both Mediterranean fruit fly (medfly) pupal and adult productivity and reduces rearing duration [47]. Most other reports showed that *Enterobacter* sp. is a pathogen and induces intestinal inflammation [48,49]. Future research on how *Enterobacter*102 reduces *C. jejuni* growth and chicken colonization is much needed. We are working on identifying *Enterobacter*102 and other bacterial candidates by culture-isolation and 16S rDNA Sanger sequencing. Together, these data suggest that individual bacteria in the SPF microbiota might be able to be isolated and used to reduce *C. jejuni* growth and chicken colonization.

Another interesting finding from the current study is that the microbiota from noninfected birds at day 28 was able to reduce *C. jejuni* in vitro growth, while microbiota from infected-alone birds failed to reduce pathogen growth. The results suggest that *C. jejuni* might have modulated the chicken microbiota for facilitating pathogen colonizing and thriving in the gut. It is a consensus that intestinal microbiota influences *C. jejuni* colonization and induction of enteritis [24,50], as also discussed in the paragraphs above. However, few reports showed that *C. jejuni* modulates the

microbiota to benefit its own colonization. *Salmonella* Enteritidis infection reduces the overall diversity of the chicken microbiota population with an expansion of the *Enterobacteriaceae* family for promoting pathogen colonization [51]. In the current study, we found that a *C. jejuni* infection increased the phylum *Bacteroidetes* compared to that in noninfected birds. Future research is needed to identify which specific bacteria are increased to facilitate *C. jejuni* colonization.

In conclusion, the mouse SPF microbiota was able to colonize chicken ceca and reduced *C. jejuni* chicken colonization. The reduction of *C. jejuni* chicken colonization might come from reduced bacteria in the phylum *Firmicutes* and/or increased bacteria in the phylum *Bacteroidetes*. Notably, *Enterobacter*102 reduced *C. jejuni* in vitro growth and chicken colonization. Altogether, these findings provide a feasible strategy to reduce *C. jejuni* chicken contamination and human campylobacteriosis.

Author Contributions

A.A., Y.F., T.A. and X.S. designed the experiments and wrote the manuscript with input from co-authors M.B., B.A. and H.W. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The animal protocols No. 20009 and 20011 were approved by the Institutional Animal Care and Use Committee of the University of Arkansas at Fayetteville.

Data Availability Statement

The data are presented in this paper.

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Conflicts of Interest

The authors declare no conflict of interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Table 2.1 Significant p-values of relative abundance at the phylum level between groups

Group A	**Compared to group B**	**Phylum**	***p*-value**
Noninfected	SPF-Aerobe	*Bacteroidetes*	< 0.001
		Firmicutes	< 0.001
	SPF-Anaerobe	*Bacteroidetes*	< 0.001
		Firmicutes	< 0.001
	Cj AR101	*Bacteroidetes*	0.02
		Firmicutes	0.04
Cj AR101	SPF-Aerobe+Cj AR101	*Bacteroidetes*	< 0.001
		Firmicutes	< 0.001
	SPF-Anaerobe+Cj AR101	*Bacteroidetes*	< 0.001
		Firmicutes	< 0.001

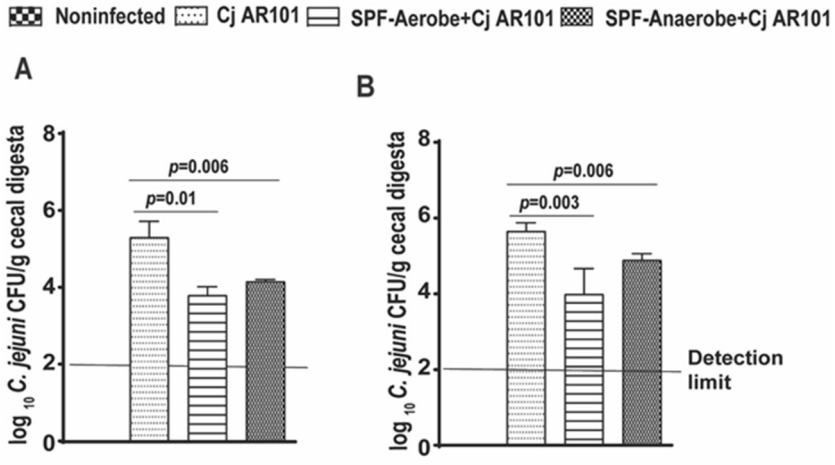

Figure 2.1 Murine microbiota reduced *C. jejuni* AR101 chicken colonization

Zero-day-old broiler chickens precolonized with SPF-Aerobe and SPF-Anaerobe were infected with *C. jejuni* AR101 at 12 days of age. The birds were euthanized at days 21 and 28. The bird cecal digesta was collected, serially diluted, and cultured on Campylobacter-selective agar plates prepared in-house at 42 °C under microaerobic atmosphere. (A) *C. jejuni* chicken colonization in the ceca of the birds at day 21. The bird number for each group was: noninfected (n = 10), Cj AR101 (n = 10), SPF-Aerobe (n = 5), and SPF-Anaerobe (n = 10). (B) *C. jejuni* chicken colonization in the ceca of the birds at day 28. The bird number for each group was: noninfected (n = 20), Cj AR101 (n = 20), SPF-Aerobe (n = 10), and SPF-Anaerobe (n = 20). All graphs depict the mean + SEM. Significant if $p < 0.05$. The results are representative of three independent experiments.

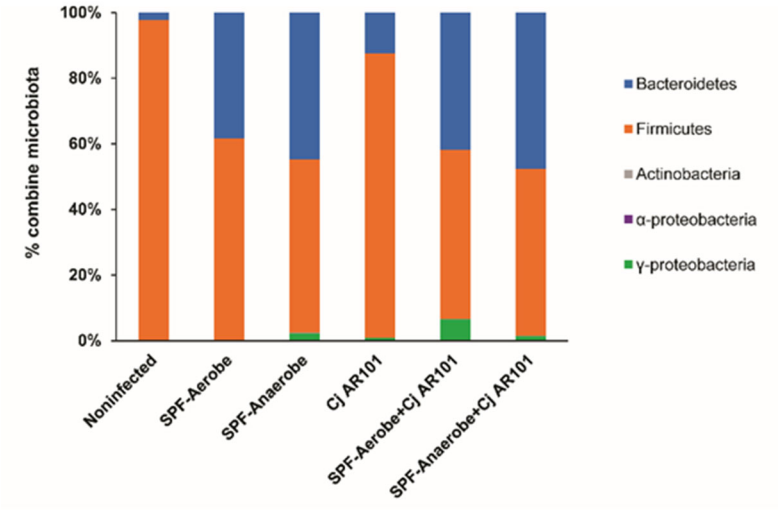

Figure 2.2 SPF microbiota modified the chicken microbiota at day 28

The birds were colonized with microbiota and infected with *C. jejuni* AR101 at day 12 as in Figure 1. Cecal digesta was collected at day 28, and DNA was extracted. Real-time PCR was per-formed to calculate bacterial composition at the phylum level. The detailed p-values were listed in Table 2.1. The results are representative of three independent experiments.

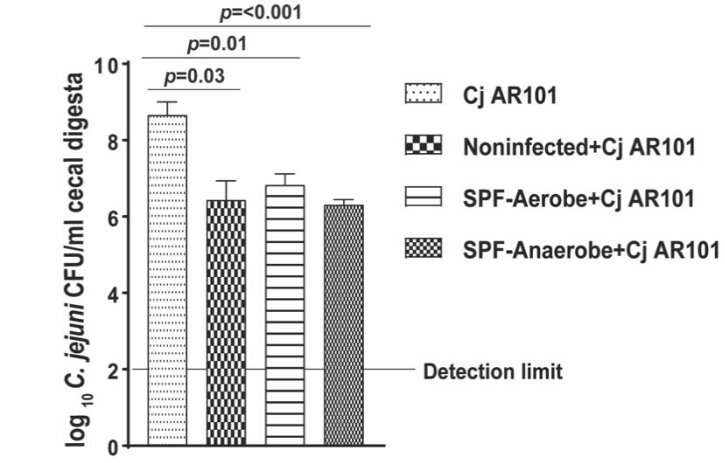

Figure 2.3 *In vitro* co-culture of noninfected chicken microbiota and *C. jejuni* AR101

AR101 was co-cultured for 24 h with microbiota from noninfected, SPF-Aerobe, or SPF-Anaerobe birds in vitro. AR101 growth was quantified by serially diluting and plating on the *Campylobacter* selective agar plates. All graphs depict the mean + SEM. Significant if $p < 0.05$. The results are representative of three independent experiments.

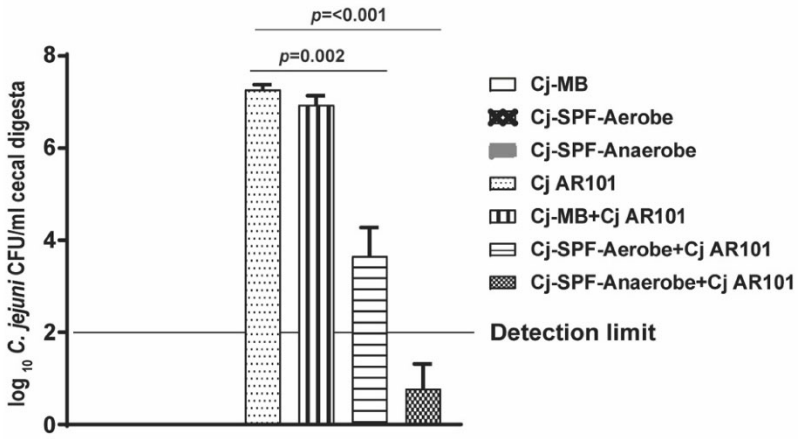

Figure 2.4 *In vitro* co-culture of *C. jejuni*-modulated microbiota and *C. jejuni* AR101

C. jejuni AR101 was co-cultured with microbiota from infected-alone birds (Cj-MB), transplanted with SPF-Aerobe and infected birds (Cj-SPF-Aerobe), and transplanted with SPF-Anaerobe and infected birds (Cj-SPF-Anaerobe). AR101 growth was quantified by serially diluting and plating on the *Campylobacter*-selective agar plates. All graphs depict the mean + SEM. Significant if $p < 0.05$. The results are representative of three independent experiments.

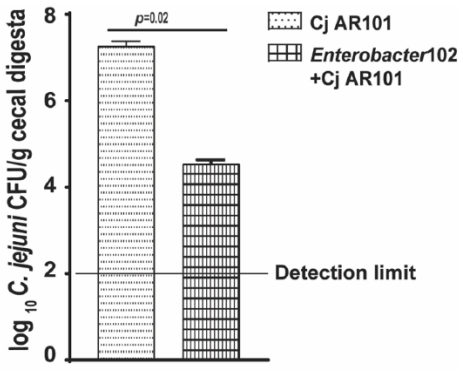

Figure 2.5 *In vitro* co-culture of Enterobacter102 and *C. jejuni* AR101

C. jejuni AR101 was co-cultured with *Enterobacter*102. AR101 growth was quantified by serially diluting and plating on *Campylobacter*-selective agar plates. All graphs depict the mean + SEM. Significant if $p < 0.05$. The results are representative of three independent experiments.

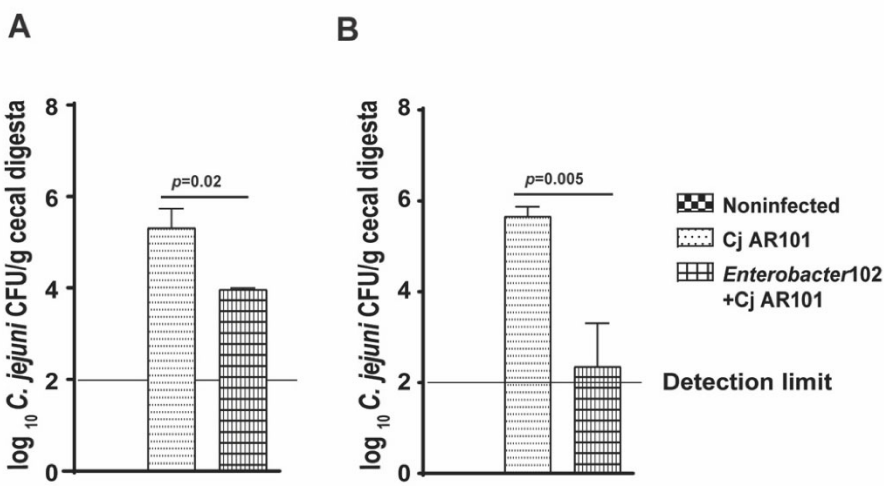

Figure 2.6 *Enterobacter*102-modulated *C. jejuni* AR101 chicken colonization

Zero-day-old broiler chicks were precolonized with *Enterobacter*102 and infected with *C. jejuni* AR101 at day 12. The birds were euthanized at days 21 and 28. The bird cecal digesta were collected, serially diluted, and cultured on *Campylobacter*-selective agar plates under a microaerobic atmosphere at 42 °C. (A) *C. jejuni* chicken colonization in the ceca of the birds at day 21. The bird number for each group was: noninfected (n = 10), Cj AR101 (n = 10), *Enterobacter*102 + Cj AR101 (n = 10). (B) *C. jejuni* chicken colonization in the ceca of the birds at day 28. The bird number for each group was: noninfected (n = 20), Cj AR101 (n = 20), *Enterobacter*102 + Cj AR101 (n = 20). All graphs depict the mean + SEM. Significant if $p < 0.05$. The results are representative of three independent experiments.

Table S2.1 Number of birds in each group of SPF microbiota experiments

Group	**Pens/group**	**Number of birds/group**
Noninfected	2	30
SPF-Anaerobe	2	30
Cj AR101	2	30
SPF-Aerobe+ Cj AR101	1	15
SPF-Anaerobe+ Cj AR102	2	30

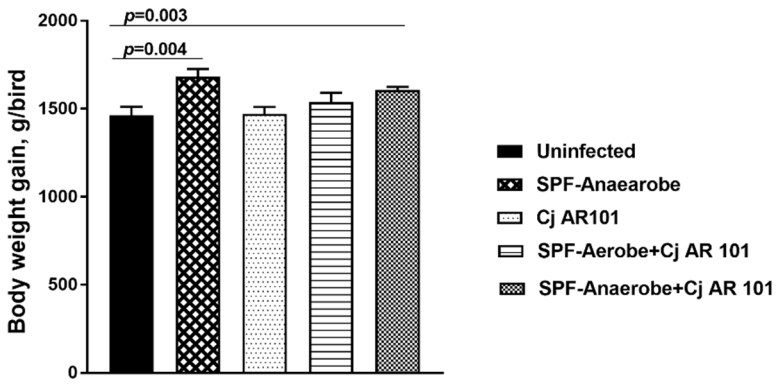

Figure S2.1 Accumulative body weight gain during d 0-28

Cohorts of chicks were colonized with mouse SPF microbiota and infected as in Figure 1. The bird weight was measured at d 0 and 28. The bird number was same as d 28 in Figure 1. Showed were accumulative body weight gain during d 0-28. All graphs depict mean + SEM. Significant if $p < 0.05$. Results are representative of 3 independent experiments.

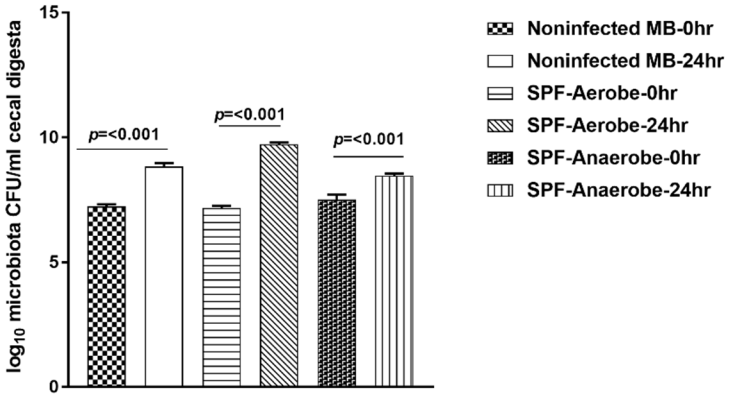

Figure S2.2 SPF-Microbiota growth co-cultured with *C. jejuni* for 24 hr

SPF microbiota was co-cultured with *C. jejuni* for 24 hr as described in Figure 3. Microbiota growth was quantified by serially diluting and plating on BHI plates. All graphs depict mean + SEM. Significant if $p < 0.05$. Results are representative of 3 independent experiments

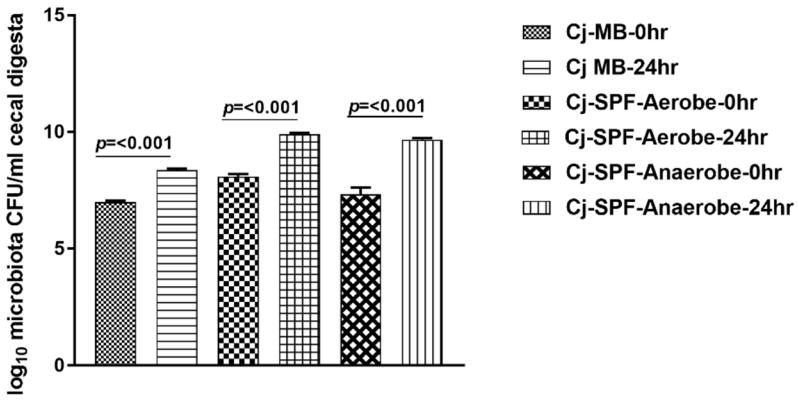

Figure S2.3 Cj-SPF-Microbiota growth co-cultured with *C. jejuni* for 24 hr

Cj-SPF microbiota was co-cultured with *C. jejuni* for 24 hr as described in Figure 4. Microbiota growth was quantified by serially diluting and plating on BHI plates. All graphs depict mean + SEM. Significant if $p < 0.05$. Results are representative of 3 independent experiments

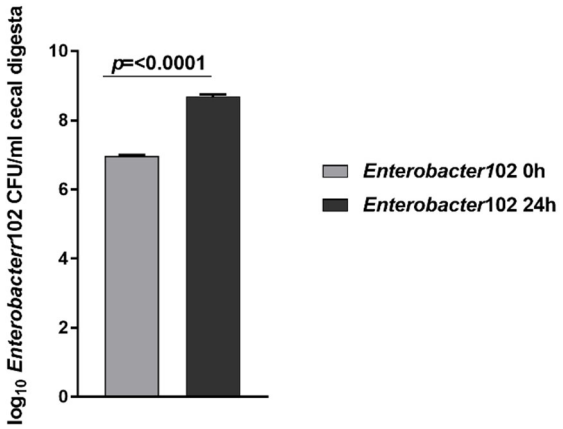

Figure S2.4 *Enterobacter*102 growth co-cultured with *C. jejuni* for 24 hr.

*Enterobacter*102 was co-cultured with *C. jejuni* AR101 for 24 hr. *Enterobacter*102 growth was quantified by serially diluting and plating on BHI plates. All graphs depict mean + SEM. Significant if $p < 0.05$. Results are representative of 3 independent experiments.

CHAPTER III

Transplantation of specific pathogen free mouse microbiota increases broiler chicken productivity

3.1 Abstract

Antimicrobial growth promoters have been used to sustain the efficient industrialized animal production, driving antimicrobial overuse and resistance. It is urgent to develop antimicrobial free alternatives as growth promoters in poultry production, but few effective antimicrobial alternatives are currently available. The objective of this study was to investigate the effect of transplanting microbiota on the bird growth performance. Mouse specific pathogen free (SPF) stool was cultured on Brain Heart Infusion (BHI) agar under anaerobic or aerobic condition and collected as SPF-Aerobe and SPF-Anaerobe microbiota. Day-old birds were tagged, weighed, and randomly assigned to 8 pens with 15 birds/pen. The birds were orally gavaged with PBS (3 pens), 10^8 CFU/bird SPF-Aerobe (2 pens) or SPF-Anaerobe (3 pens). The feed intake and individual bird weight were measured at d 0, 14, 21 and 28. The broiler chickens were euthanized at d 28. Intestinal digesta was collected to measure nutrient and bacteria levels. Notably, SPF-Aerobe and SPF-Anaerobe significantly increased body weight gain by 18% and 12% during d 0 to d 14, respectively, compared to the negative control. No significant difference of feed intake was observed among the groups. SPF-Aerobe significantly reduced periodic feed conversion ratio compared to the negative control by 20% during d 21 to 28. SPF-Aerobe and SPF-Anaerobe significantly reduced accumulative feed conversion ratio compared to the negative control by 18% and 14%, respectively, during d 0 to 28. SPF-Anaerobe at d 28 significantly increased the levels of macro-nutrients of gross energy, protein and fat in the digesta of the small intestine compared to the negative control. SPF-Anaerobe transplantation at d 28 increased phylum *Bacteroidetes* but reduced *Firmicutes* in the digesta of small intestine and ceca compared to the negative control. In

conclusion, microbiota was able to improve feed efficiency and early bird body weight gain, and microbiota reconstitution could be used an effective antibiotic alternative to improve poultry productivity.

3.2 Introduction

Poultry meat is one of the most consumed animal protein around the world and it accounted for 50.4% of the animal meat consumption in 2021 in USA [1]. The big market share of poultry meat has been benefited from the efficiently industrialized poultry production, leading to the inexpensive and high-quality poultry meat. The efficiency of poultry production has been achieved because of numerous technology and operation advancements, such as genetic selection, veterinary medicine, antimicrobial growth promoters (AGP), specialization, and vertical integrated industrialization [2]. Broiler chickens through commercial quantitative genetic selection from 1957 to 2005 increased growth by more than 400% at d 42, while reducing feed conversion ratio by 50% [3]. To sustain the fast broiler growth, various husbandry and veterinary innovations have been developed such as vaccination, AGP, and ecologically controlled housing. To promote economic efficacy, poultry production has been gradually specialized on individual production segments including broiler breeder farms, hatchery, broiler grow-out farms, feed mill, processing plants, and allied industries (e.g. feed additive, vaccine, medicine) [2]. Vertical integration has further improved the poultry production by owning and controlling multiple stages of the production as well as the transportation and marketing. The improved poultry production is evident by reduced wholesale broiler meat price. For example, $ 0.299/lb meat in 1960 [4] was inflation-adjusted to $1.47/lb in 2021 [5], a 42% reduction compared to the real price of $0.854/lb in 2021 [4].

The animal intestine harbors a complex community of trillions of microbes including bacteria, archaea, virus, and eukarya. These microbes called microbiota, and their metabolic activities and products are collectively defined as the microbiome [6]. Modern birds, including chickens, evolved from theropod dinosaurs around 150 million years ago [7]. Before industrialization era or in certain countryside, chicken hens have laid and hatched eggs and raised their chicks, just like their ancestor dinosaurs and wild birds. In the process, the chicks have acquired microbiota from their parents and living environment. Interestingly, the successful practice of specialization in modern poultry production [2] has changed many aspects of chicken life, including microbiota transmission. In the industrialized poultry production, eggs from breeder hens have been collected, cleaned, and hatched in hatchery and the chicks were raised on fresh or used bedding or on wire mesh, where the microbiota of the chicks have been acquired from the living environment and different from their parents'. The microbiota composition of broiler breeder hen feces are composed of 76% *Firmicutes*, 13 *Actinobacteria*, and 4.3% *Bacteroidetes* phyla [8]. The small intestinal microbiota of broilers at d 28 is predominant with 97% phylum *Firmicutes* [9]. The cecal microbiota of broilers at d 37 has 86% *Firmicutes* and 13% *Proteobacteria* phyla [10]. The separation of breeders and chicks might contribute to the microbiota composition difference.

Although the disruption of microbiota transmission from parents to their offspring chicks is apparent, the consequence hasn't being realized until the recent emergency of antimicrobial resistance, which is partly caused by AGP usage in poultry production for decades [11]. Increasing pressure from consumers and government regulations is mounting for taking actions to restrict AGP, resulting in a variety of challenges in poultry production [12], such as reduced growth performance and the re-emerging enteric disease of *Clostridium perfringens*-induced necrotic

enteritis [13]. Many alternatives have been investigated with various success rates on growth performance, such as direct feed microbes (probiotics) [14], short chain fatty acids [15], and bile acids [16, 17]. Recently, we found that specific pathogen free (SPF) mouse microbiota prevents *Campylobacter jejuni* chicken colonization [18]. Moreover, microbial metabolite secondary bile acid deoxycholic acid improves broiler chicken body weight gain [16] and prevents *Eimeria maxima* and *C. perfringens*-induced acute necrotic enteritis [19]. In this study, we hypothesized that SPF microbiota would improve chicken growth performance. We reconstituted broiler chicken intestinal microbial community using the SPF mouse microbiota and the growth performance was measured. We found that SPF Anaerobe microbiota improved chicken feed conversion ratio and early body weight gain. The results from this study may help developing new antimicrobial free alternatives for poultry production.

3.3 Materials and methods

3.3.1 Mouse microbiota preparation and transplantation

Animal experiments were performed in accordance with the Animal Research: Reporting of In Vivo Experiments (https://www.nc3rs.org.uk/arrive-guidelines) and approved by the Institutional Animal Care and Use Committee of the University of Arkansas (protocol No. 20009 for mice and 20011 for chickens). For this study, SPF BL6 *Il10*$^{-/-}$ mice were maintained in bio-secure settings and fed with ad libitum water and chewing diet cages with Alpha Dry bedding. Fresh stools were collected from male and female mice between 8 and 10 weeks of age. The stool samples were cultured on brain heart infusion (BHI, BD Biosciences, NJ) agar plates at 42 °C for 48 hr under aerobic or anaerobic conditions using the GasPak system (BD Biosciences, NJ) and collected as SPF-Aerobe and SPF-Anaerobe microbiota. The microbiota was added glycerol to final 30% and stored at -80 °C. Before chicken colonization experiment, the SPF-Aerobe and SPF-

Anaerobe microbiota were cultured on BHI plate for 48 hr, collected in PBS, and enumerated by OD_{600} and plating. OD_{600} of 1 was estimated at about 10^8 CFU/ml. Chicks at d 0 were orally gavaged once with 0.5 ml PBS or 10^8 CFU/bird SPF-Aerobe, or 10^8 CFU/bird SPF-Anaerobe.

3.3.2 Management and performance recording of broilers chicken

A total of 120 zero-day-old Cobb 500 broiler chicks (Cobb-Vantress, Siloam Springs, AR) were randomly allocated into 15 birds per pen. Chicks were transported to an ecologically controlled housing and were neck-tagged and randomly placed to floor pens. Groups of negative control (PBS), SPF-Aerobe, and SPF-Anaerobe had 3, 2 and 3 pens per group, respectively. Feed and water were supplied ad libitum and temperature was maintained according to their age. The broiler chicks were fed a corn-soybean meal-based starter diet for the first d 0 to 10 and a grower diet during d 11 to 28. The diets were based on corn and soybean meal and formulated to meet or exceed published nutrient recommendations as described before [20] and in Table 1. The feed consumption per pen and body weight per bird were measured at d 0, 14, 21 and 28. The birds were euthanized at d 14, 21 and 28 for sample collection and pen density reduction. Bird numbers of negative control, SPF-Aerobe, and SPF-Anaerobe were (62, 48, 22), (28, 20, 20), (45, 33, 28) during d 0-14, 14-21, and 21-28, respectively. The digesta in ceca and middle small intestine around diverticulum of birds at d 28 were collected and stored at -80 °C.

3.3.3 Laboratory analysis of feed and digesta macro-nutrients

Frozen small intestine digesta and feed samples were frozen-dried and ground using an electric grinder to ensure an evenly ground sample. Dry matter, gross energy, ash, nitrogen, and ether extract (fat) were evaluated in the digesta and feed samples. A bomb calorimeter was used to determine the gross energy (Parr 6200 bomb calorimeter, Parr Instruments Co., Moline, IL). The amount of dry matter was calculated based on AOAC [21] method 934.02. Nitrogen was

measured by combustion method standardized with EDTA (method 990.03 [21]). The fat was determined using AOAC [21] method 920.39. The nutrient levels of grower feed were 3887 cal/gm, 88%, 3.4%, 9.1 and 3.3 for calories, dry matter, ash, fat and nitrogen.

3.3.4 Estimation of microbiota composition at phylum level

DNA from the eight pooled digesta of small intestine or ceca per group was extracted using bead beater disruption and phenol: chloroform separation method as described before [18, 22]. The abundance of five phyla of gut bacteria were determined using SYBR Green PCR Master mix on a Bio-Rad 384-well Real-Time PCR System (BioRad, Hercules, CA, USA). The qPCR reactions were performed according to the manufacturer's recommendation. The primers were used as described before [16], including universal *16S rRNA*: 16S357F 5′-CTCCTACGGGAGGCAGCAA-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*: 798cfbF 5′-CRAACAGGATTAGATACCCT-3′,cfb967R 5′- GGTAAGGTTCCTCGCGTAT-3′; *Firmicutes*: 928FirmF 5′-TGAAACTYAAAGGAATTGACG-3′, 1040FirmR 5′-ACCATGCACCACCTGTC-3′; *Actinobacteria*: Act920F3 5′-TACGGCCGCAAGGCTA-3′, Act1200R 5′-TCRTCCCCACCTTCCTCCG-3′. The relative fold change of each phylum in one sample were normalized against universal *16S rRNA*. The percentage of each phylum was then calculated as the phylum relative folds divided by total folds of all five phyla.

3.3.5 Bacterial enumeration small intestine digesta

Eight pooled small intestine digesta per groups was weighed at around 0.1 g and suspended in 500 µl PBS in a 2 ml screw cap tube containing 0.3 g sterile 0.1mm zirconia beads (BioSpec,

Bartlesville, OK, USA). The samples were briefly homogenized on a Fisher brand Bead Mill 24 Homogenizer (Fisher Scientific, Pittsburg, PA, USA). The samples were then 10x serially diluted and plated on Brain Heart Infusion (BHI) agar. The plates were incubated on either aerobic or anaerobic conditions at 42 ºC for 48 h using the GasPak system (BD Biosciences, NJ).

3.3.6 Statistical analysis

All values are shown as mean ± standard error of the mean as indicated. Data were analyzed by One-way ANOVA followed by a Fisher LSD multiple comparison test using Prism 7.0 software. The microbiota counts were transformed with a formula of $\log_{10}$ (CFU + 1). Data were considered statistically significant if P values were < 0.05.

3.4 Results

3.4.1 The effect of SPF microbiota on periodic and accumulative bodyweight gain

As shown in Table 2 of periodic body weight gain per pen, the SPF-Aerobe and SPF-Anaerobe microbiota significantly increased periodic body weight gain by 18 % and 12%, respectively, compared to negative control during d 0 to 14. There was no significance of periodic body weight gain during d 14 to 21 and d 21 to 28. Similarly, no significant accumulative body weight gain was observed between groups during d 0 to 21 and d 0 to 28 (Table 3).

Because birds were individually weighed, we also calculated statistics using body weight gain per bird. The SPF-Aerobe and SPF-Anaerobe microbiota significantly increased periodic body weight gain d 0 to 14 (0.39 and 0.37 vs. 0.33 kg/bird, respectively, $P = 0.0005$) and d 14 to 21 (0.35 and 0.40 vs. 0.33 kg/bird, respectively, $P = 0.005$) (Supplemental Table S1). Consistently, the SPF-Aerobe and SPF-Anaerobe microbiota significantly increased accumulative body weight gain d 0 to 21 (0.77 and 0.82 vs. 0.68 kg/bird, respectively, $P < 0.0001$) and d 0 to 28 (1.35 and 1.42 vs. 1.32 kg/bird, respectively, $P = 0.04$) (Supplemental Table S2).

3.4.2 The effect of SPF microbiota on periodic and accumulative feed intake and feed conversion ratio

Interestingly, there was no significant difference of periodic or accumulative feed intake between the groups of SPF-Aerobe, SPF-Anaerobe, and negative control, as shown in Tables 4 and 5. During the early days (d 0-14 and 14-21) of SPF-mouse microbiota transplantation, there was no significant difference of periodic feed convention ratio between the groups (Table 6). During d 21 to 28, SPF-Aerobe significantly reduced periodic feed conversion ratio by 20% compared to negative control. Notably, SPF-Aerobe and SPF-Anaerobe significantly reduced accumulative feed conversion ratio compared to the negative control by 18% and 14%, respectively, during d 0 to 28 (Table 7).

3.4.3 The effect of microbiota transplant on nutrients of small intestine

We reasoned that the improved growth performance by microbiota transplantation was resulted from elevated nutrient metabolism. We then measured macro-nutrient levels in the digesta of the middle of the small intestine. Notably, SPF-Anaerobe significantly increased gross energy in the digesta by 11% compared to the negative control (Figure 1A). SPF-Anaerobe also significantly increased digesta fat and protein by 63% and 54%, respectively, compared to the negative control (Figure 1B).

3.4.4 SPF microbiota colonized in the chicken gut

The improved growth performance and increased macro-nutrient levels indicate a successful microbiota transplantation. To further reaffirm whether the chicken gut microbiota was reconstituted with mouse microbiota, we used five phyla-specific primers to analyze changes in microbiota composition in the small intestine and ceca. In small intestine, SPF-Aerobe and SPF-Anaerobe significantly reduced the relative abundance of the phylum *Firmicutes* compared to the

negative control (59 and 56 vs. 79%, respectively), while increased the relative abundance of the phylum *Bacteroidetes* compared to the negative control (40 and 43 vs. 21%, respectively) (Figure 2A). Consistently, in ceca, SPF-Aerobe and SPF-Anaerobe significantly reduced the relative abundance of the phylum *Firmicutes* compared to the negative control (63 and 59 vs. 92%, respectively,), while increased the relative abundance of the phylum *Bacteroidetes* compared to the negative control (37 and 41 vs. 7%, respectively) (Figure 2B). These results indicate that SPF-Aerobe and SPF-Anaerobe were able to reconstitute chicken gut microbiota.

3.4.5 SPF-Mouse microbiota growth in small intestine digesta of broilers chicken

To further investigate the impact of SPF microbiota transplantation, we also cultured the bacteria under aerobic or anaerobic condition using the digesta in the bird small intestine. Notably, SPF-Aerobe and SPF-Anaerobe transplantation significantly increased aerobes by more than one log compared to the negative control (6.2 and 6.7 vs. 5.3 $\log_{10}$ CFU/g digesta, respectively) (Figure. 3A). Consistently, SPF-Aerobe and SPF-Anaerobe transplantation increased anaerobes by around 2 logs compared to the negative control (7.3 and 7.5 vs. 5.7 $\log_{10}$ CFU/g digesta, respectively) (Figure. 3B). These results illustrate that SPF-Aerobe and SPF-Anaerobe transplantation have increased bacterial load in chicken small intestine.

3.5 Discussion

Although the industrialization of poultry production and the use of antimicrobial growth promoters have greatly improved the production efficiency, the associated issues, such as antimicrobial resistance [23] and animal welfare [24], have been becoming urgent to be addressed. Among various problems, the disruption of microbiota transmission from parent to offspring has been largely overlooked. In this study, we reasoned that broiler chicks from commercial hatchery would be colonized with environmental (e.g. hatchery, transportation vehicles, and grower farms)

microbiota. The environmental microbiota would not be optimal for chicken growth. To examine this hypothesis, we reconstituted broiler chicken microbial community by transplanting mouse SPF microbiota. The choose of mouse microbiota was based on our previous observations that SPF mice are resistant to foodborne pathogen *C. jejuni* colonization [18]. We found that SPF-Aerobe and SPF-Anaerobe increased chicken feed conversion ratio and early body weight gain compared to negative control. The nutrient levels of gross energy, protein, and lipid in small intestine of SPF-Anaerobe birds were higher compared to those of negative control birds. Microbiota composition in small intestine and ceca of SPF-Anaerobe and SPF-Aerobe birds showed difference to the negative control, indicating the success of the microbiota transplantation. Altogether, these results suggest that it is possible to transplant microbiota for improving chicken growth performance.

Accumulating evidence is pointing to the influence of microbiota on body weight gain. Excessive body weight gain in humans is becoming epidemic issues around the world. Microbiota has been found to be one of the key factors influencing body weight gain in humans and mice. Germ-free (GF) C57BL/6 mice colonized with microbiota from the cecum of conventionally raised mice induces a 60% increase in body fat content and insulin resistance within 14 days despite food intake reduction [25]. Uncultured or cultured fecal microbiota from adult female twin pairs discordant for obesity is transmissible to increase total body and fat mass in GF mice which are fed low-fat chew or high-fat diet [26]. Furthermore, germ-free mice colonized with an 'obese microbiota' results in a significantly higher total body fat increase than 'lean microbiota' [27]. Although the donor microbiota in our study was obtained from adult SPF mice with normal body weight, the transplantation of the mouse microbiota was able to increase recipient chicken feed conversion ratio and early body weight gain. The results suggest that chicken microbiota naturally

acquired from our farm environment was not optimal for chicken growth and it was necessary to establish different microbiota for increasing the bird growth performance. It would be reasonable to argue that the chicken farms with productivity underperformance would increase productivity by transplantation of "high performance" microbiota. Furthermore, it would be interesting to see whether transplanting microbiota from fast growing birds may increase recipient chicken growth better compared to microbiota from birds with same genetic background but slow growing.

Based on the current knowledge, we reasoned that the increased growth performance by microbiota transplantation came from enhanced efficiency on nutrient digestibility, absorption, and metabolism. A core group of eighty-nine carbohydrate active/digestive enzyme families are present across 85% of the human gut microbiota, while ten of the enzyme families are positively correlate with higher body mass and encoded in phylum *Firmicutes* bacteria [28]. *Bacteroides thetaiotaomicron* have two hundred sixty glycoside hydrolases in its genome [29]. The order *Lactobacillales and families Lachnospiraceae*, and *Streptococcaceae* are positively associated with fecal protease activity, whilst the family *Ruminococcaceae* and an unclassified family *Coriobacteriales* are negatively associated with fecal protease activity [30]. Transplantation of microbiome from obese mice increases the mouse capacity to harvest energy from the diet [27]. Studies using GF and conventionalized mice show that the microbiota colonization increases absorption of monosaccharides from the gut lumen, resulting in de novo hepatic lipogenesis [25]. In our study, SPF-Anaerobe microbiota transplantation increased the macro-nutrient levels of energy, protein, and fat in the middle of small intestine compared to the negative control. Those increased macro-nutrients might be absorbed and utilized by chickens, resulting in improved growth performance. Interestingly, SPF-Aerobe microbiota transplantation didn't significantly increased digesta protein and fat, while the microbiota transplantation increased feed conversion

ratio. These data suggest that more comprehensive nutrient metabolism analysis in the chicken microbiota transplantation experiments is needed to understand how much the microbiota impacts nutrient digestion, absorption and metabolism.

It is a consensus that some of microbiota members are not culturable, but it is inconclusive whether the culturable microbiota recapitulates the function of the whole microbiota. In medical practice, a healthy donor's feces is transplanted without culture to treat a human *Clostridium perfringens* infection [31]. The microbiota compositions of the recipients are comparable to those of the human donor's, and the *C. difficile* infection is reduced. Microbiota composition of recipient piglets is comparable to that of human donors' in an inter-mammalian fecal microbiota transplantation [32]. Interestingly, either uncultured or cultured fecal microbiota from human twin pairs shows different effects on increasing total body and fat mass in GF mice and the recipient mouse microbiota is consistent with the human donors' [26]. These results show that it is feasible to transplant microbiota within class Mammalia. A notable observation from our study is that the culturable mouse microbiota was able to successfully colonize chickens and to increase chicken growth performance, suggesting the possibility that culturable microbiota between animals of class Mammalia (mouse) and class Aves (chickens) in phylum Chordata could transplantable and work functionally. Interestingly, the difference of intestinal anatomy and body temperature (42 °C in chickens and 37 °C in mice) did not negatively impact the microbiota transplantation. Chicken microbiota at the phylum level is mainly consisted of 13 phyla of *Firmicutes* (70.0%), *Bacteroidetes* (12.3%), *Proteobacteria* (9.3%), and other small proportions of *Actinobacteria*, *Cyanobacteria*, *Spirochaetes*, *Synergisteles*, *Fusobacteria*, *Tenericutes*, and *Verrucomicrobia* [33]. The chicken microbiota composition is often variable from farms to farms. The small intestinal microbiota of broilers at d 28 is predominant with 97% phylum *Firmicutes* [9]. The cecal

microbiota of broilers at d 37 has 86% *Firmicutes* and 13% *Proteobacteria* phyla [10]. Consistent with these findings, we found that phylum *Firmicutes* was the dominant in birds without a mouse microbiota transplantation, while microbiota-transplanted birds reduced *Firmicutes* and increased *Bacteroidetes*. Interestingly, the microbiota composition of mice is 54% phyla *Firmicutes* and 30% *Bacteroidetes* [34], which was close to the composition of our transplanted chicken microbiota. How the microbiota composition is correlated to growth performance remain largely elusive. The relative abundance of genera *Bacteroides* and *Lactobacillus* is higher in the slow-growing breed birds compared to the fast-growing breed birds, while that of genera *Cloacibacillus* and *Megasphaera* is the opposite [35]. Further research on the relationship between microbiota members and growth performance will be helpful.

In conclusion, the mouse SPF microbiota was able to colonize chicken small intestine and ceca and to increase chicken growth performance. The improvement of chicken growth performance might come from increased nutrient availability. The change of growth might be related to the reduced bacteria in the phylum *Firmicutes* and/or increased bacteria in the phylum *Bacteroidetes*. Altogether, these findings provide a feasible antimicrobial alternative on increasing poultry productivity.

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Table 3.1 The composition of the experimental diets fed to broilers between d 0 and 28 post-hatched

Ingredient, % as-fed	Starter (0–11 d)	Grower (12–28 d)
Corn	58.17	61.62
Soybean meal (46.8%)	32.9	27.08
DDGS[a]	4	6
Soybean oil	1.34	2
Limestone	1.25	1.22
Dicalcium phosphate	0.9	0.74
Salt	0.45	0.42
DL-methionine	0.31	0.26
L-lysine HCl	0.24	0.24
L-threonine	0.09	0.08
Trace mineral premix[b]	0.1	0.1
Vitamin premix[c]	0.1	0.1
Sepremix[d] **(0.06%)**	0.02	0.02
Choline chloride (60%)	0.05	0.04
Santoquin	0.02	0.02
Phytase[e]	0.01	0.01
Inert filler[f]	0.05	0.05
Calculated composition, % unless noted otherwise		
AME_n, kcal/kg	3,015	3,098
CP	22.01	20
Digestible lysine	1.18	1.05
Digestible TSAA	0.89	0.8
Digestible threonine	0.77	0.69
Calcium	0.9	0.84
Available P	0.45	0.42

[a]DDGS, distillers dried grains with solubles
[b]Supplied the following per kg of diet: manganese, 100mg; zinc, 100mg; copper, 10.0mg; iodine, 1.0mg; iron, 50mg; magnesium, 27 mg.
[c]Supplied the following per kg of diet: vitamin A, 30,863 IU; vitamin D3, 22,045 ICU; vitamin E, 220 IU; vitamin B12, 0.05mg; menadione, 6.0mg; riboflavin, 26mg; dpantothenic acid, 40mg; thiamine, 6.2mg; niacin, 154mg; pyridoxine, 11mg; folic acid,
3.5mg; biotin, 0.33 mg.
[d]Supplied 0.12mg of selenium per kg of diet.
[e]Optiphos®, (Huvepharma Inc., Peachtree City, GA.) provided 250 FTU/kg of diet.
[f]Clinacox®, (Huvepharma Inc., Peachtree City, GA), provided 1 mg/kg diclazuril to the diet at the expense of the inert filler.
AMEn, nitrogen-corrected apparent metabolizable energy.

Table 3.2 Effect of SPF microbiota on periodic body weight (kg) of broilers

	Age (d)			
Diet	0 to 14	14 to 21	21 to 28	N (pens)
Negative control[1]	0.33±0.013[b]	0.34±0.046	0.59±0.035	3
SPF-Aerobe[1,2]	0.39±0.013[a]	0.35±0.010	0.62±0.041	2
SPF-Anaerobe[1,3]	0.37±0.006[a]	0.40±0.060	0.59±0.050	3
P value	0.03	0.74	0.87	

[a,b] Means within a column, without common superscript are significantly different (P <0.05).
[1]Basal diet (no growth promoter or coccidiostat).
[2]Bird gavaged with 10^8 CFU/bird SPF aerobic microbiota at d 0.
[3]Bird gavaged with 10^8 CFU/bird SPF anaerobic microbiota at d 0.

Table 3.3 Effect of SPF microbiota on accumulative body weight (kg) of broilers

	Age (d)			
Diet	0 to 14	0 to 21	0 to 28	N (pens)
Negative control[1]	0.33±0.013[b]	0.68±0.050	1.31±0.059	3
SPF-Aerobe[1,2]	0.39±0.013[a]	0.77±0.020	1.35±0.021	2
SPF-Anaerobe[1,3]	0.37±0.006[a]	0.80±0.067	1.42±0.135	3
P value	0.03	0.34	0.73	

[a,b] Means within a column, without common superscript are significantly different (P <0.05).
[1]Basal diet (no growth promoter or coccidiostat).
[2]Bird gavaged with 10^8 CFU/bird SPF aerobic microbiota at d 0.
[3]Bird gavaged with 10^8 CFU/bird SPF anaerobic microbiota at d 0.

Table 3.4 Effect of SPF microbiota on periodic feed intake (kg) of broilers

	Age (d)			
Diet	0 to 14	14 to 21	21 to 28	N (pens)
Negative control[1]	0.39±0.023	0.56±0.120	1.10±0.081	3
SPF-Aerobe[1,2]	0.40±0.046	0.50±0.006	0.93±0.059	2
SPF-Anaerobe[1,3]	0.37±0.004	0.58±0.083	0.99±0.048	3
P value	0.79	0.86	0.30	

[a,b] Means within a column, without common superscript are significantly different (P <0.05).
[1]Basal diet (no growth promoter or coccidiostat).
[2]Bird gavaged with 10^8 CFU/bird SPF aerobic microbiota at d 0.
[3]Bird gavaged with 10^8 CFU/bird SPF anaerobic microbiota at d 0.

Table 3.5 Effect of SPF microbiota on accumulative feed intake (kg) of broilers

	Age (d)			
Diet	0 to 14	0 to 21	0 to 28	N (pens)
Negative control[1]	0.39±0.023	0.95±0.141	2.04±0.217	3
SPF-Aerobe[1,2]	0.40±0.046	0.89±0.052	1.83±0.007	2
SPF-Anaerobe[1,3]	0.37±0.004	0.95±0.086	1.94±0.130	3
P value	0.79	0.94	0.70	

[a,b] Means within a column, without common superscript are significantly different (P <0.05).
[1]Basal diet (no growth promoter or coccidiostat).
[2]Bird gavaged with 10^8 CFU/bird SPF aerobic microbiota at d 0.
[3]Bird gavaged with 10^8 CFU/bird SPF anaerobic microbiota at d 0.

Table 3.6 Effect of SPF microbiota on periodic feed conversion rates of broilers

	Age (d)			
Diet	0 to 14	14 to 21	21 to 28	N (pens)
Negative control[1]	1.18±0.102	1.59±0.154	1.88±0.067[a]	3
SPF-Aerobe[1,2]	1.02±0.152	1.41±0.059	1.51±0.004[b]	2
SPF-Anaerobe[1,3]	1.00±0.0120	1.47±0.025	1.67±0.063[ab]	3
P value	0.35	0.53	0.03	

[a,b] Means within a column, without common superscript are significantly different (P <0.05).
[1]Basal diet (no growth promoter or coccidiostat).
[2]Bird gavaged with 10^8 CFU/bird SPF aerobic microbiota at d 0.
[3]Bird gavaged with 10^8 CFU/bird SPF anaerobic microbiota at d 0.

Table 3.7 Effect of SPF microbiota on accumulative feed conversion rates of broilers

	Age (d)			
Diet	0 to 14	0 to 21	0 to 28	N (pens)
Negative control[1]	1.18±0.102	1.36±0.134	1.60±0.078[a]	3
SPF-Aerobe[1,2]	1.02±0.152	1.17±0.120	1.31±0.065[b]	2
SPF-Anaerobe[1,3]	1.00±0.0120	1.20±0.012	1.37±0.025[b]	3
P value	0.35	0.40	0.04	

[a,b] Means within a column, without common superscript are significantly different (P <0.05).
[1]Basal diet (no growth promoter or coccidiostat).
[2]Bird gavaged with 10^8 CFU/bird SPF aerobic microbiota at d 0.
[3]Bird gavaged with 10^8 CFU/bird SPF anaerobic microbiota at d 0.

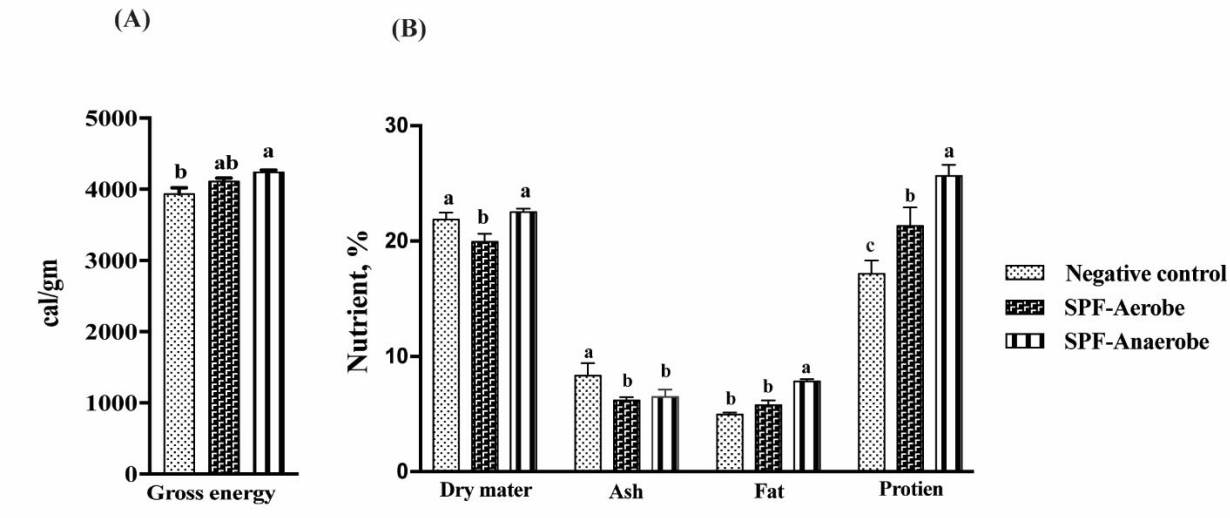

Figure 3.1 The effect of microbiota transplant on gross energy and nutrients of small intestine.

Frozen small intestine digesta from 8 pooled samples per group was dried and grounded to evaluate the gross energy (**A**) and the percentage of macro-nutrient including dry mater, ash, fat and protein (**B**). All graphs depict mean ± SEM. Different letters means significant $p<0.05$.

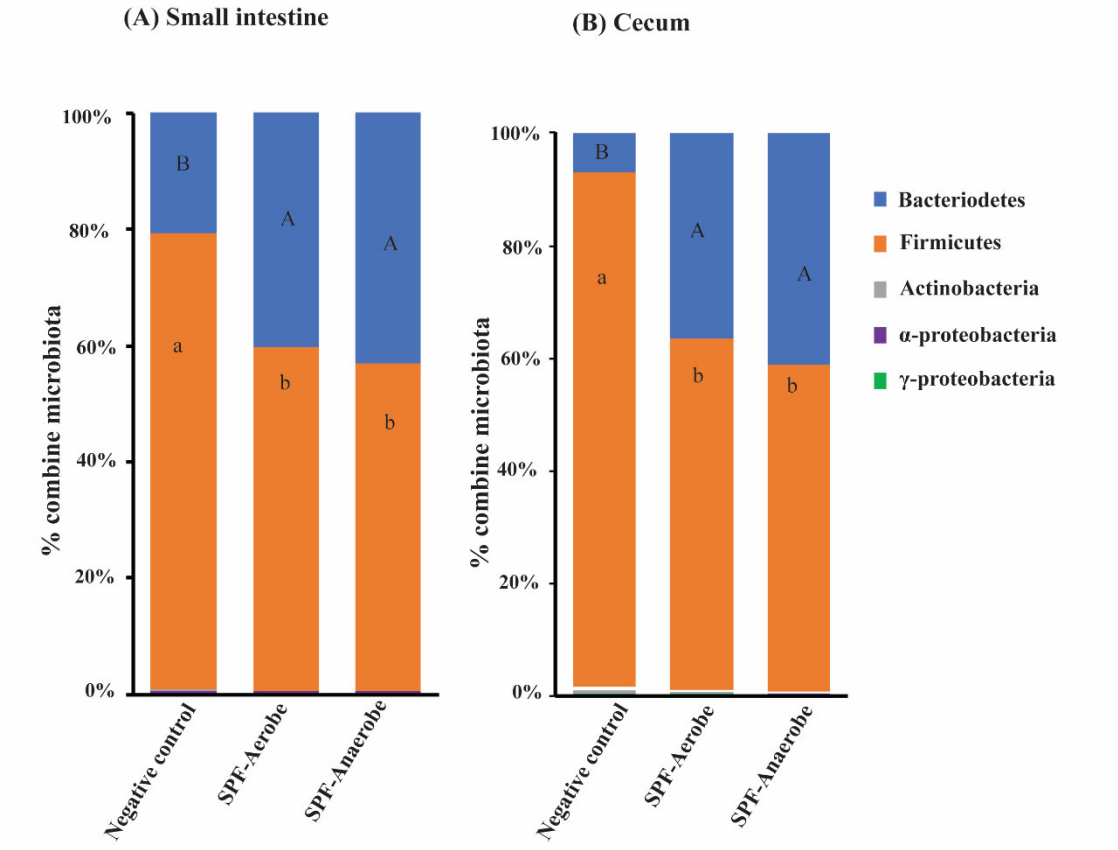

Figure 3.2 SPF microbiota modulated the chicken microbiota in small intestine and ceca.

Zero-day-old broiler chickens were colonized with SPF-mouse microbiota. The birds were euthanized at d 28. Small intestine digesta (A) and cecal digesta (B) from 8 pooled samples per group was collected and DNA was extracted. Real-time PCR was performed to calculate bacterial composition at the phylum level. The significance of the phylum *Bacteroidetes* is represented by A-B, whereas the significance of the phylum *Firmicutes* is represented by a-b, $p<0.05$.

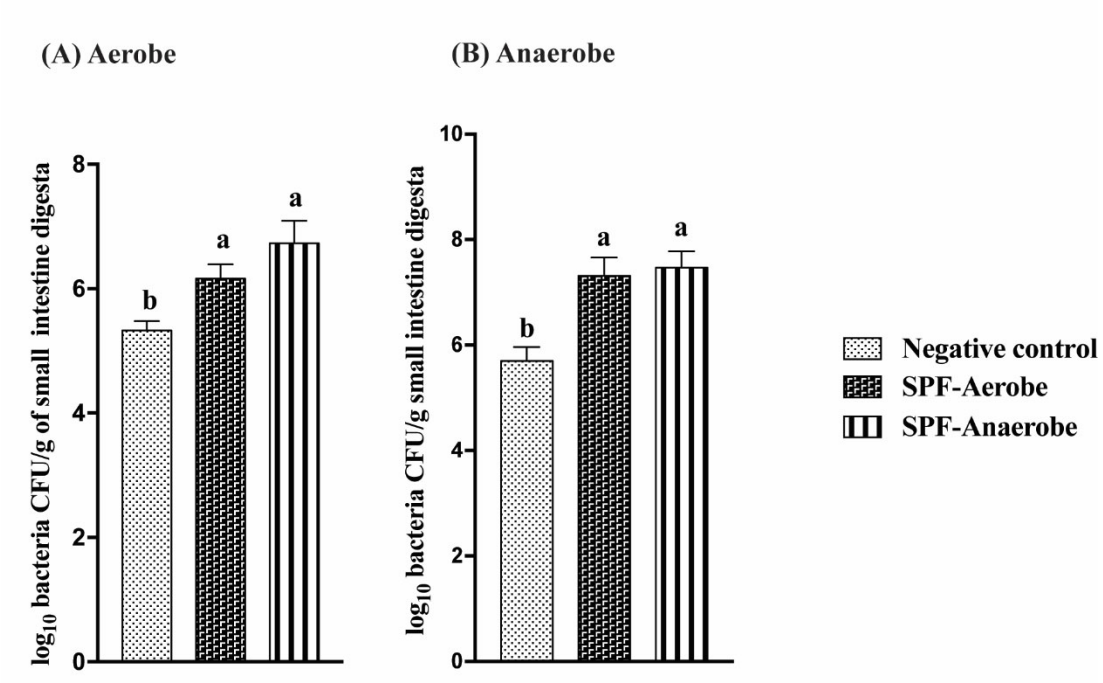

Figure 3.3 Microbiota enumeration in small intestine digesta.

Small intestine digesta from 8 pooled samples per group was collected from bird at d28 and serially diluted to enumerate microbiota in both aerobic (**A**) and anaerobic condition (**B**). All graphs depict mean ± SEM. Different letters means significant $p<0.05$.

Table S3.1 Effect of SPF microbiota on periodic body weight (kg) of broilers per bird

	Age (d)		
Diet	0 to 14	14 to 21	21 to 28
Negative control	0.33±0.009[b]	0.33±0.014[b]	0.58±0.018
SPF-Aerobe	0.39±0.011[a]	0.35±0.007[ab]	0.62±0.035
SPF-Anaerobe	0.37±0.012[a]	0.40±0.018[a]	0.59±0.021
P value	0.0005	0.005	0.61

[a,b] Means within a column, without common superscript are significantly different (P <0.05).
[1]Basal diet (no growth promoter or coccidiostat). Bird number N = 62, 48, 22 during d 0-14, 14-21, and 21-28, respectively.
[2]Bird gavaged with 10^8 CFU/bird SPF- mouse aerobic microbiota, bird number N = 28, 20, 20 during d 0-14, 14-21, and 21-28, respectively.
[3]Bird gavaged with 10^8 CFU/bird SPF- mouse anaerobic microbiota, bird number N = 45, 33, 28 during d 0-14, 14-21, and 21-28, respectively.

Table S3.2 Effect of SPF microbiota on periodic body weight (kg) of broilers per bird

	Age (d)		
Diet	0 to 14	0 to 21	0 to 28
Negative control	0.33±0.009[b]	0.68±0.019[b]	1.32±0.041[b]
SPF-Aerobe	0.39±0.011[a]	0.77±0.015[a]	1.35±0.026[ab]
SPF-Anaerobe	0.37±0.012[a]	0.82±0.024[a]	1.42±0.042[a]
P value	0.0005	<0.0001	0.04

[a,b] Means within a column without common superscript are significantly different (P <0.05).
[1]Basal diet (no growth promoter or coccidiostat). Bird number was the same as in Supplemental Table S1.
[2]Bird gavaged with 10^8 CFU/bird SPF- mouse aerobic microbiota
[3]Bird gavaged with 10^8 CFU/bird SPF- mouse anaerobic microbiota

CHAPTER IV

4.1 Conclusion

This study evaluated the effect of SPF-mouse microbiota on *C. jejuni* chicken colonization. SPF-mouse microbiota was cultured in aerobic and anaerobic conditions and isolated as SPF-Aerobe and SPF-Anaerobe. The study indicated that both SPF-Aerobe and SPF-Anaerobe mouse microbiota successfully colonized in chicken ceca and inhibited *C. jejuni* colonization in chickens. Reduced bacteria in the phylum *Firmicutes* and/or increased bacteria in the phylum *Bacteroidetes* might explain the decrease in *C. jejuni* chicken colonization. Consistently, SPF-mouse microbiota inhibited *C. jejuni* growth in *vitro.* Moreover, we were able to isolate bacterium from the SPF-anaerobe microbiota and identified as *Enterobacter*102. *Enterobacter*102 was able to inhibit *C. jejuni* growth in *vitro* and chicken colonization. Moreover, we studied the effect of SPF-mouse microbiota on broilers chicken growth performance. The study showed that SPF-Aerobe and SPF-Anaerobe significantly increased body weight growth by 18% and 12%, respectively, during day 0 to day 14. There were no significant differences in feed intake between the groups. During day 21 to 28, SPF-Aerobe substantially decreased periodic feed conversion ratio by 20% compared to the negative control. SPF-Aerobe and SPF-Anaerobe microbiota reduced accumulative feed conversion ratio compared to negative control by 18% and 14% respectively, during d 0-28. In addition, SPF microbiota increased the amounts of gross energy and macro-nutrients including, protein and fat in small intestine digesta compared to the negative control. Lastly, SPF-mouse microbiota transplantation increased phylum *Bacteroidetes* and reduced *Firmicutes* in small intestine and cecal digesta.

Following those discoveries, several future directions could be explored. It will be important to identify specific bacterial members in the SPF microbiota using 16S rDNA

sequencing and individual bacterial culturing. The identified bacteria could be individually and combinedly assessed for their capacity to reduce *C. jejuni* growth *in vitro* and colonization in chickens. The bacteria could also be individually and combinedly examined for improve chicken productivity. Secondly, the microbial metabolites produced by the microbiota could be the contributing factors and be identified using targeted and untargeted metabolomics. The discovery of the metabolites could be coupled with the identification of specific microbiota members. The identified microbiota metabolites could be used to prevent *C. jejuni* infections in chickens and humans. Thirdly, host immune responses modulated by the microbiota could be contributing factors. Although no host immune responses have been investigated in these projects, it was possible that the interaction of host immune response, microbiota, and metabolites occurred. The anti-inflammatory response by microbiota is implicated in gnotobiotic experiments. Altogether, these findings and future research may help the development of effective strategies to reduce *C. jejuni* chicken contamination and campylobacteriosis in humans and increase poultry productivity.