Understanding how barn sanitation practices impact the chicken gut microbiome and the significance of variations in early life gut microbial community structure on microbial functionality

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Animal Science

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ABSTRACT

Chemical disinfectants are widely used by Canadian broiler producers for barn sanitation, although little is known regarding their effects on chicken performance and the gut microbiome, which had been well recognized to have great importance to the host health. In this thesis, studies were conducted in commercial barns to evaluate the impacts of barn sanitation practices on chicken performance, the gut microbiome, and immune responses. The significance of variations in early-life gut microbial structure were also assessed with regards to their effects on the microbial population, microbial functional capacity, as well as indices of host immune response.

To determine the impact of barn sanitation practices on the chicken performance and the gut microbiome, barn cleaning with chemical disinfectants versus water-wash was conducted as a cross-over experiment. At the end of the production cycle, the flock mean body weight and mortality rate were comparable between the barn sanitation treatment groups. The barn water-wash resulted in a modest but significant effect on the structure of broiler cecal microbiota, with notable reductions in cecal *Campylobacter jejuni* occurrence and abundance. In addition, chickens from the barn water-wash group had increased level of cecal acetate, butyrate and total short-chain fatty acids that were negatively correlated with *C. jejuni* abundance.

To further assess the effects of the barn sanitation practices on chicken gut microbial functional capacity, particularly on microbial metabolism and antibiotic resistance, cecal content samples were subjected to shotgun metagenomic sequencing. At day 7, the gut microbiome of chickens from the chemically disinfected barns had decreased capacity of amino acid production with increased stringent response compared to the water-wash group. Similarly at day 30, the gut microbiome of chickens reared in chemically disinfected barns exhibited decreased abundance of the genetic pathways encoding amino acid and short-chain fatty acid biosynthesis due to

decreased cecal *Helicobacter pullorum* population. Our data suggested that the use of chemical disinfectants in barn cleaning were more effective in controlling persistent antibiotic resistance genes.

Finally, a study was conducted to investigate distinct cecal early-life microbial structures identified among commercial broiler chickens in gut microbial interaction, functionality, as well as host immune status. Week-old commercial broiler chickens were screened and chickens with distinct cecal *Bacteroides* composition were identified. Compared to the *Bacteroides*-under-representative group, the chickens with *Bacteroides*-over-representative cecal microbiota had increased microbial genetic potential of complex polysaccharide degradation and short-chain fatty acid production in the gut, which was supported by increased cecal short-chain fatty acid concentrations. In addition, chickens with high cecal *Bacteroides* had lower expression of interleukin-1β gene and higher expression interleukin-10 gene and tight-junction protein claudin-1 gene. The results indicated that elevated cecal *Bacteroides* may be beneficial to commercial broiler chickens in suppressing gut inflammation coincided with the increment of short-chain fatty acid production.

Collectively, this thesis provides insights into the role of barn sanitation practices during poultry production in the microbiome of commercial broiler chickens, and exhibits the significance of variations in early-life gut microbial community structure in microbial functionality and host immune status.

PREFACE

This thesis is an original work by Yi Fan, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Broiler barn sanitation", No. AUP00002377, 6/4/2018.

The study in Chapter 2 has been published as Yi Fan, Andrew J Forgie, Tingting Ju, Camila Marcolla, Tom Inglis, Lynn M McMullen, Benjamin P Willing, Douglas R Korver. The Use of Disinfectant in Barn Cleaning Alters Microbial Composition and Increases Carriage of *Campylobacter jejuni* in Broiler Chickens. Appl Environ Microbiol. 2022 May 24;88(10):e0029522. doi: 10.1128/aem.00295-22. Initial studies were designed by Douglas R Korver, Tom Inglis, and Lynn M McMullen. Douglas R Korver, Benjamin P Willing and Yi Fan designed the follow up experiments, Yi Fan conducted the experiments, collected, and analyzed the data, and wrote the manuscript. Andrew J Forgie, Tingting Ju, and Camila Marcolla helped with experiments and data analyses. All authors edited and approved for publication. Benjamin P Willing and Douglas R Korver supervised the study.

The study in Chapter 3 is expected to be submitted for publishing as Yi Fan, Tingting Ju, Tulika Bhardwaj, Douglas R. Korver, Benjamin P. Willing. The impact of barn disinfection and age on the cecal microbial functional capacity and resistome of broiler chickens. 2022. Yi Fan, Douglas R Korver, and Benjamin P Willing designed the study and experiments, Yi Fan conducted the experiments, collected and analyzed the data, and wrote the manuscript. Tingting Ju and Tulika Bhardwaj helped with data analyses. Benjamin P Willing and Douglas R Korver supervised the study.

The study in Chapter 4 was designed by Yi Fan and Benjamin P Willing. The manuscript was accepted by Microbiology Spectrum as Yi Fan, Tingting Ju, Tulika Bhardwaj, Douglas R. Korver, Benjamin P. Willing. Microbiology Spectrum. 2022. Week-old chicks with high *Bacteroides* abundance have increased short-chain fatty acids and reduced markers of gut inflammation. doi:10.1128/spectrum.03616-22. Yi Fan conducted experiments, collected and analyzed the data, and wrote the manuscript. Tingting Ju and Tulika Bhardwa helped with the data analyses. All authors edited and approved for publication. Benjamin P Willing and Douglas R Korver supervised the study.

Dedicated

To my wife and my best friend,

Dr. Justina Su Zhang

ACKNOWLEDGEMENT

I would like to express my sincerest gratitude to my supervisors Dr. Benjamin P. Willing and Dr. Douglas R. Korver, for their guidance and support during my PhD study. It was their warm encouragement that helped me get through tough times in this journey. Thank you for providing numerous opportunities to develop and expand my skills and knowledge as a scientist.

I would also like to thank Dr. Leluo Guan for serving on my supervisory committee and giving me invaluable advice and support throughout my research program. I would like to extend my gratitude to our collaboration partner Dr. Lynn M. McMullen and Dr. Tom Inglis for their advice and supports of the project. I am grateful for my examining committee members Dr. Xin Zhao and Dr. Simon Otto for their precious time to read my thesis.

I am appreciative to the Department of Agricultural, Food, and Nutritional Science, the Faculty of Graduate Studies and Research at the University of Alberta, the Alberta Chicken Producers, the Alberta Livestock and Meat Agency, the Canadian Poultry Research Council, the Results Driven Agriculture Research for the financial and academic support.

I want to thank all the members from the Willing lab, the Korver lab, the McMullen lab, and the Poultry Health Services. Special thanks to Dr. Tingting Ju, Dr. Andrew Forgie, Dr. Tulika Bhardwa, Taresa Chieng, and Patrick Ward for their contribution.

I also want to thank all my friends and family for their unconditional support. To my parents and my in-laws, thank you for your warmest encouragement when I was lost. To my wife, the mother of our two lovely children, thank you for your unconditional support and love. Thank you for being such a great mother for Mason and Tyler. Words cannot express my gratitude. I would never be able to get to this point without you.

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AGP: Antimicrobial growth promoter

AMR: Antimicrobial resistance

ANCOM: Analysis of composition of microbiome

ASV: Amplicon sequence variant

ARG: Antibiotic resistance gene

BAC: Benzalkonium chloride

BCFA: Branched-chain fatty acid

CARD: The comprehensive antibiotic resistance database

CE: Competitive exclusion

CFU: Colony-forming unit

CLDN1: Claudin-1 gene

CR: Conventionally raised

DAMP: Damage-associated molecular patterns

EDTA: Ethylenediaminetetraacetic acid

erm, 23S ribosomal RNA methyltransferase gene

FD: Full Disinfection with chemical disinfectants

FDR: False discovery rate

FFA: Free fatty acid receptor

GALT: Gut associated lymphoid tissue

GF: Germ free

GPR: G-protein coupled receptor

HB: high-*Bacteroides* group

hipO: hippurate hydrolase gene

IL: Interleukin

LB: low-*Bacteroides* group

LEfSe: Linear discriminant analysis effect size

LNU: Lincosamide nucleotidyltransferase

MGE: Mobile genetic element

MLS: Macrolide-lincosamide-streptogramin

NetB: necrotic enteritis B-like toxin gene

PAMP: Pathogen-associated molecular patterns

PCoA: Principal coordinate analysis

PCR: Polymerase chain reaction

PERMANOVA: Permutational Multivariate Analysis of Variance

QIIME2: Quantitative Insight into Microbial Ecology2

qPCR: Quantitative polymerase chain reaction

rpoB, gene encoding β-subunit of bacterial RNA polymerase

RPP: Ribosomal protection protein

RT-qPCR: Reverse-transcription quantitative polymerase chain reaction

SAT, Streptogramin A acetyltransferase genes

SCFA: Short-chain fatty acid

SEM: Standard error of the mean

vanR, vancomycin resistant gene R component

WW: Water-wash

Chapter 1: INTRODUCTION

As a major agriculture product, chicken is one of the most important high-quality protein sources for humans (1). Currently, the world's population is estimated to be 7.2 billion, and is expected to reach nearly 10 billion by 2050 (2). Modern agriculture has done well in providing food to nurture such a huge population. In fact the price of all meat products, relative to average income is currently the lowest in human history due to the success in mass production (3). The growth in global livestock production was largely credited to poultry industry, especially broiler chicken production (4). The United States Food and Drug Administration (USDA) forecasted that the broiler per capita consumption is expected to increase steadily, growing from 44.7 kg in 2023 to 46.1 kg by 2031 (5). With the continued expansion of the poultry industry, factors affecting chicken performance, zoonotic diseases, and food safety will continue to be an important concern to the general public, industry, and academia, especially in the context of eliminating preventive usage of antibiotics in livestock farming.

The gut microbiota, which can be beneficial to the host by maintaining gut homeostasis, has been identified as a potential tool to address these challenges. The gut microbiota refers to the totality of microorganisms associating with the host gastrointestinal tract (GIT) (6). The importance of the chicken gut microbiota to host health and performance had been well recognized. Emerging evidence has shown the importance of broiler commensal microbial community to nutrient metabolism, feed efficiency (7-9), competitively defending against pathogen infection and colonization (10, 11), and educating the host immune system (12, 13). Subsequently, various probiotic and prebiotic products have been developed and used in chicken production to help broiler chickens to establish symbiotic gut microbiota, and showed success in increasing chicken nutrient utilization (14), and prevent disease development (13, 15, 16).

Therefore, establishing healthy host-microbe interactions early in production may provide a possible alternative of antimicrobial growth promoters (AGPs) in production to help maintain, or even enhance broiler performance.

In production, broiler chickens are delivered to the production house shortly after hatch (normally within 24 h) and receive feed and water on farm. Notably, in the modern poultry industry, commercial broiler chickens are hatched and reared without the presence of hens. The absence of contact with the parent flock largely affects the early-life chick microbiota by limiting the transmission of chicken-derived commensals (17, 18).Thus, for broiler chickens, the barn is one of the early microbial exposures. Early life bacterial exposure is important to the microbiota assembly in the animal GIT and immune system development (19). It has been reported in human studies and various animal models that early microbial exposure persistently affects future host intestinal health and disease development (20-22). Previous studies have revealed that oral gavage of chicken-derived microbiota at hatch reduced pathogen colonization as well as increasing short chain fatty acid (SCFA) production in the chicken gut (23, 24). Therefore, establishing healthy host-microbe interactions early in production may provide a possible solution to help maintain broiler health in an environment free from antimicrobial growth promoters. Thus, improving the barn microbial environment, which might introduce beneficial early commensal-host interactions to the broiler chickens, may advance the development of a commensal microbiota in broiler chickens, and thus help improve chicken health in production.

1.1 The Chicken Gut Microbiota

The gut microbiota is considered a crucial organ that plays an integral role in maintaining the host health by modulating physiological functions including nutrition, metabolism, and immunity

(25). Complex interactions between various microbial molecules (e.g. DNA and lipopolysaccharide), metabolites (e.g. short chain fatty acids), and the host immune system strongly affect the host health (26). Although information on the avian microbiome is still limited compared to mammalian species, in this section, we will focus on some current knowledge of the chicken gut microbiome and its relationships to the host.

1.1.1 Chicken Intestinal Habitats and the Inhabitants

Previous studies on chicken microbiome revealed that more than 1000 species inhabit the chicken intestine (27, 28). Like other animal species, the chicken GIT is partitioned according to digestive function. The chicken GIT consists of the crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, and cloaca (29). The corresponding products of different physiological functions (e.g. host digestive enzymes and acid) segregate host digestive processes from most of the environmental microbes to ensure host priority of dietary substrate utilization (30). In addition, diverse environmental factors in the intestine, such as pH, digesta flow rate, dietary substrates, bile salts, and oxygen levels influence the assembly and development of specific bacterial communities. Generally, the complexity of the chicken gut microbiome increases along the chicken GIT from the proximal part (crop) to the distal part (cecum). The composition and complexity of the gut microbial communities largely increases in distal parts of the GIT, especially in the ceca.

In the proximal part of the chicken GIT, the beak collects food that is swallowed whole. Feed goes through the esophagus to the crop where it is moistened. Physiologically, the crop of chickens stores and softens the feed that can aid grinding and enzymatic digestion further down

the digestive tract (31). The chicken crop harbors $10^8 – 10^9$ CFU/g of bacteria (32). Compared to the distal part of the GIT, the crop microbiota has a simple composition, dominated by facultative anaerobes, predominantly lactobacilli (33, 34), which are believed to be responsible for decomposition of starch and the production of lactate and thus provides an acid barrier with pH of ~4.5. Diets may have an impact of the crop microbiota. Feed additives such as high amounts of phytic acid in plant-based diets promote the abundance of family *Aeromonadaceae* and *Flavobacteriaceae* while reducing the dominance of *Lactobacillus* in the crop (35). However, variations in microbiota among individual broiler chickens fed on similar diets have also been observed, indicating that there may also be other factors (e.g. initial microbial exposure, host genetics, etc.) impacting the crop microbiota (36).

The gizzard mechanically grinds feed particles. Similar to the crop, the most predominant bacteria in the gizzard are reported to be lactobacilli (37). However, due to the hydrochloric acid in the gastric juices provided by the proventriculus, the pH of the gizzard is kept relatively low (pH~2.6), resulting in lower bacterial abundance and less fermentation activity in the gizzard.

Degradable feed components are broken down to simple carbohydrates, amino acids, and fatty acids in the small intestine (duodenum, jejunum, and ileum) as well as at the brush border membrane along the small intestinal lining (38). In the small intestine, remainder of the digestion occurs at the duodenum, whereas the released nutrients are absorbed mainly in the jejunum and ileum. In the small intestine, the duodenum harbors the lowest bacterial density due to the short passage time and the presence of pancreatic juices and bile (37, 39). The duodenal microbiota is dominated by *Lactobacillus* species (40). Highly similar to the duodenal microbiota (41, 42), the jejunal microbial community is reported to nearly exclusively made up by *Lactobacillus* (7) but with increased abundance of members from the phylum Proteobacteria (e.g. *Brucella*,

Shigella/*Escherichia coli*) (41, 43). Compared to the duodenum and jejunum, the chicken ileum has increased pH and decreased oxygen level, making the microbial community of the ileum more diverse and higher in cell density (44), and it is also the most studied segment among the small intestine. Specifically, gram-positive bacteria from the phylum Firmicutes, mainly *Lactobacillus* which makes up more than 70% of the ileal microbiota, populated the ileum of chickens (45-47). A substantial part of the ileal digesta enters the ceca through reverse peristalsis for further microbial fermentation. The similar oxygen and nutrient availability between the two organs makes the ileal microbial communities sometimes overlap with the cecal microbiota (48). Thus, some bacteria often found in the chicken ceca such as members from the family *Enterococcaceae, Peptostreptococcaceae, Erysipelotrichaceae* and *Clostridiaceae* are also frequently detected in the chicken ileum (49, 50).

The abundance and complexity of the gut microbiota considerably increases in the chicken ceca due to the longest feed retention time (12 to 20 h) compared to other GIT segments (51). The chicken ceca can harbor as high as 10^{11} bacteria per gram of content (52). As mentioned above, although differing in bacterial load, the crop, gizzard, duodenum, jejunum, and ileum shared similar facultative anaerobic communities (predominantly *Lactobacillus*). Conversely, in the chicken ceca the low oxygen levels favor strict anaerobes. For example, the relative abundance of members from the order *Clostridiales* progressively increase from the crop (almost undetectable) to the ileum (10%) and are dominant in the ceca (over 60%) (52-54). The chicken cecal microbiota are dominated by the phyla Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria (55, 56). One major biological function of the chicken ceca is to offer a niche for the gut microbes to anaerobically recycle nitrogen and digest non-starch polysaccharides to produce SCFAs (57). The frequently reported genera from Firmicutes and

Bacteroidetes in the ceca are important amino acid utilizers and SCFA producers (e.g. *Bacteroides, Faecalibacterium*, *Blautia*, *Butyricimonas*, *Megamonas, Oscillibacter,* members from the *Ruminococcaceae* and *Lachnospiraceae* family, and members from the *Clostridiales* order) (28, 45, 54, 58, 59). Large variations have been reported in specific taxa. For example, *Lactobacillus* has been reported to range from relatively dominant (~8%) (45) to undetected in the chicken ceca (54). This variation may be due to the fact that since *Lactobacillus* are facultative anaerobes, the prevalence may be affected by the oxidative stress in the ceca. When the cecal environment is under oxidative stress (e.g. the presence of reactive oxygen species produced by the host during inflammation), the growth of obligate anaerobes may be suppressed, and thus lead to the expansion of facultative anaerobes (60). The variation in colonization pattern between studies might also be explained by the differences in chicken breed (host genetics), management, or diets, but age are most likely to play an important role as well and this will be discussed in the next section. Following Firmicutes and Bacteroidetes, in Proteobacteria, genus *Desulfohalobium* and *Shigella/Escherichia* were also frequently detected in the ceca (59, 61, 62).

1.1.2 Successional Change of the Chicken Microbiota

The assembly of the chicken microbiome goes through successional stages. The age of the chickens is one of the most important drivers that influences GIT microbial community structure, cell density, and metabolic function. Previous studies revealed a series of temporal and successional microbial composition changes through broiler maturation (13, 45).

The chicken gut microbiota assembles as soon as 1 day of life (63). The colonization of the chick is believed to be initiated as it breaks the egg shell (64). As mentioned, in poultry

production, newly hatched chicks from hatcheries have no contact with adult birds, and thus the environment they are placed in will have a strong impact on their mostly naïve intestinal microbiome. At day 1, the cecal microbiota of the newly hatched chicks were dominated by *Enterobacteriaceae* and to a lesser extent *Enterococcus* (63). While the initial ceca colonization begins with facultative anaerobic bacteria, obligate anaerobic bacteria soon replaces the facultative anaerobes and dominate the ceca (45, 65). Some pathogens, such as *Clostridium perfringens* and *Campylobacter coli,* were detected in the GIT as early as 3 days of age (45). Over the first 3 days, the cecal microbial taxa diversity and richness progressively increases, and by day 7 the family *Ruminococcaceae* outnumbers *Enterobacteriaceae* leading the expansion of Firmicutes population, which dominates the ceca by day 14 (63, 65).

The broiler gut microbiome matures between 21 and 45 days post-hatch (13, 66-68). The alpha diversity of the chicken gut microbiome continues to change after 2 weeks post-hatch (13). Approximately 100 low abundance genera that were not detected at 3 weeks in broiler chicken became detectable after 6 weeks of age (13). More recently, Jurburg et al. reported three stages of successional microbiota changes by monitoring broiler chicken feces from day 1 to 35 after hatch (69). Briefly, they found that the first stage colonization was caused by vertical or environmental transmission leading to the colonization of *Streptococcus* (from order *Lactobacillales*) and *Escherichia/Shigella* (from order *Enterobacteriales*) in the gut. The second stage started at day 4, *Lactobacillales* and *Enterobacteriales* were displaced by a series of taxa including *Lachnospiraceae* and *Ruminococcus*-like species variants from order *Clostridiales*. In the third stage starting on day 10, the relative abundance of a diverse cluster of obligate anaerobic bacteria slowly increased, while the *Latobacillales*, *Enterobacteriales* and *Clostridiales* remain predominant (69). The maturation of the chicken microbiota coincides with

the maturation of the chicken intestine and plays an important part in maintaining intestinal homeostasis and the provision of nutrients (which will be further discussed in 1.2.5 and 1.2.6).

1.1.3 Early-life Bacterial Exposure

Early-life bacterial exposure is important to microbial assembly in the animal GIT and immune system development. It has been widely reported in a variety of animals that early microbial exposure significantly and persistently affects future host intestinal function and disease development by promoting epithelial cell turnover, increasing mucus production and epithelium integrity, and educating host immune response (20-22, 63, 70-74). It has been well reviewed that the development of human microbiome is significantly influenced by early age diet and environmental conditions of the individual host (75, 76). One major difference between birds and mammals is that mammals are delivered through a microbe-diverse and abundant vaginal tract (77), whereas birds are hatched from eggs that are considered to be internally sterile (35). In addition, mammals are fed by maternal milk, which provides bioactive molecules like immunoglobulin (78) and oligosaccharides (79, 80) to help their young develop their immune system and shape gut microbiota (81). In avian species, gut microbiota development relies on direct contact with other birds and the environment. For example, it has been shown that there is a high microbial similarity between the nest of the Pied Flycatchers (*Ficedula hypoleuca*) and their builders (82). Although this study is limited to cultured-based methods, which could only recover a subset of the overall microbial community, it indicates an opportunity for avian microbiota transmission in the wild. In addition, microbial transmission is also caused by maternal excreta consumption. Cooper et al. reported that ostrich chicks consume adult feces to help prime their digestion and performance by adding useful microbes (83). Kubasova et al.

revealed that hen contact at hatch helped young chickens establish more obligate anaerobes in the ceca, which is important for intestinal homeostasis (84). These studies indicated that microbial transmission from the parent flock is an important source of commensal microbiota for chickens.

Modern broilers are believed to be domesticated from the wild red jungle fowl (*Gallus gallus*) (85). Chickens are precocial birds and are relatively mature and mobile at hatch, which makes them economically suitable and labor-friendly for domestication. In modern broiler production, chicks are hatched in a clean hatchery, where eggs are hatched separately from the hens and fumigated to avoid bacteria contamination. This reduces the opportunity of the newly hatched chicks to be exposed to the parent-flock-derived microbes. Soon after, chicks are transferred to a clean barn covered by new bedding material on the floor with sufficient feed and clean water supply along with closely managed temperature, humidity, and ventilation. The production barns are often water-washed or chemically disinfected with the intent to reduce disease transmission and improve food safety. However, when trying to avoid pathogen transmission (i.e. *Salmonella* spp., *Campylobacter* spp., infectious bronchitis virus, avian influenza viruses, etc.), transmission of necessary chicken-derived commensals is also limited.

Recycled chicken litter is allowed in broiler production in many parts of the world, for example in the United States. Some previous studies found that exposure to used litter mimics the transmission from hen to chick and can impact early life broiler gut microbiota (20, 21), and thereby confer competitive exclusion against poultry pathogens (10, 86, 87). One study has shown that compared to fresh litter, used litter significantly decrease of the abundance of Proteobacteria in the gut microbiota of chicks over the first two weeks of life (88). More recently, it was reported that recycled litter increased the predominance of some potential

beneficial bacteria, such as *Faecalibacterium*, in the ceca of young chicks, and the effect remained as the chicken gut matured (89, 90). Research has also shown that the intestinal immune response of broilers reared on recycled litter is indicative of immune activation, which could negatively impact broiler production (91). Collectively, these studies highlight the importance of the initial environmental exposure to the chicken gut microbiome, which can offer useful information to the industry to improve management.

1.1.4 Host and Environmental Factors affecting the Chicken Gut Microbiota

As discussed above, chicken age and initial bacterial exposure are influential to the chicken gut microbiota. In the last decade, many studies have also recognized that host genetics (chicken type and breed) (92, 93), diet composition (94-97), antibiotic usage (53, 97, 98), production system (cage, intensive floor farming or extensive farming system) (99-101), flock densities (102), and litter management (will be further discussed in 1.2.2) could affect the gut microbial population of chickens.

Breeding programs have led to distinctive genetic variance between chicken types and breeds, in which the most pronounced difference are between laying hens (egg type chicken) and broiler chickens (meat type chicken). Such great differences in genetics contribute to the variations observed in intestinal immune functions (103). Simon et al. reported that intestinal immunoglobulin secretion was higher in broilers compared to laying hens (103), which may lead to variations of the gut microbiota between the two chicken types. Currently, studies comparing the gut microbiota between laying hens and broiler chickens are limited. The cecal microbiota of laying hens and broiler chickens differed significantly particularly when inoculated with

Campylobacter (92). The authors speculated that the differing microbial compositions of laying hens and broiler chickens was likely due to the intestinal immune responses caused by *Campylobacter* challenge (92). In addition, within broiler chickens, differences in the gut microbiota between breeds were also observed (104-106). For example, Cobb 500 and Ross 308 broilers harbored distinct ileal microbiota in which Bacteroidetes were absent in the Ross breed and Actinobacteria were absent in the Cobb breed (106, 107). However, Han et al. detected Bacteroidetes in the ileum of the Ross breed during a study examining *Campylobacter* colonization in different chicken breeds (92). Due to variations in experimental designs, animal management, initial microbial exposure, sequencing techniques in different studies, it is difficult to determine to which degree that the broiler chicken genetic backgrounds affect the gut microbiome.

Alterations in the chicken gut microbiome can be induced by changing the diets in as quickly as 1 day (45). The effects of diet on the chicken gut microbiome are well recognized and the focus of many studies. It has been shown that the type of diet (e.g. wheat and corn) (97), the nutritional composition of the diet (e.g. protein level) (94, 108), as well as the presence of feed additives can influence the chicken gut microbiome (109-112). For instance, reduced crude protein in broiler chicken diets resulted in elevated cecal *Lactobacillaceae* over time (94). The cecal microbiome of chickens fed the control diet and fishmeal-supplemented diet differed significantly, which in the latter, strongly reduced the abundance of some butyrate producers (e.g. *Butyrivibrio*) in the ceca (108). In addition, the supplementation of feed additives such as phytase (109), protease (110), β-glucan (111), and xylanase (112) had been shown to influence the chicken gut microbiome and intestinal homeostasis.

Antibiotic supplementation to the feed was also reported to have an impact on the chicken gut microbial population. Danzeisen et al. reported that subtherapeutic use of monensin depleted *Roseburia*, *Lactobacillus* and *Enterococcus* in the ceca, and enriched *Escherichia coli* when coupled with virginiamycin or tylosin (53). Very recently, Zou et al. studied the effects of AGP and diets on the chicken gut microbiome and suggested that although AGP had a relatively small effect on altering microbial taxonomic abundances, it disturbed interactions between microbes by affecting some key taxa of the microbial network, and thereby promoted the exclusion of other taxa (97).

Other environmental factors such as production system and management can also impact the broiler gut microbiome. For example, compared to indoor ground litter-raised broiler chickens, broilers raised in a cage system had decreased bacterial species richness and evenness in the ileum at day 13 (99). Access to an outdoor area in the extensive farming systems (free-range chickens) was demonstrated to increase the cecal microbial alpha diversity (101), and resulted in a higher cecal Bacteroidetes composition (100). In addition, increased flock density was associated with reduced crop microbiome richness (102).

1.1.5 Effects of Chicken Gut Microbiota on Nutrient Metabolism

Sharing a mutualistic relationship to the host, the gut microbiota contributes to host nutrient metabolism, particularly through the fermentation of undigestible nutrients. Commensals produce SCFAs (e.g. acetic acid, butyric acid, and propionic acid), organic acids (e.g. lactic acid), vitamins (e.g. vitamin K and vitamin B groups), and induce host basal immune responses with necessary metabolic costs (113-115).

SCFAs are products of the gut microbiota fermentation from partially- or non-digestible polysaccharides or proteins derived from the undigested portion of the host diet. Acetate, propionate, and butyrate were reported to be the most abundant SCFAs in the chicken gut (116). Some beneficial commensals can directly utilize the indigestible nutrients in the diet for SCFA production, or utilize metabolic intermediates from other bacteria to make SCFA through cross-feeding (117). SCFAs are rapidly absorbed by the intestinal epithelial cells and enhance intestinal integrity as direct energy sources to enterocytes (118). They also reduce the pH at the site of production. In addition, SCFAs are also able to bind to G protein-coupled receptors such as free fatty acid receptors (FFA2,3) expressed on various immune cells (e.g. monocytes and macrophages) and non-immune cells (e.g. intestinal epithelial cells, adipocytes and enteroendocrine cells) (119). By binding to these receptors, SCFAs regulate a series of host cell functions including gene expression, immune cell recruitment, differentiation and apoptosis (will be reviewed in section 1.1.6) (120). Feed intake was also found to be regulated through the interactions between SCFAs and FFA2/FFA3 expressed by the host enterocytes (121).

In addition, the intestinal microbiota is an important source of vitamin production. Some commensals can synthesize vitamin K and B vitamins, including biotin, cobalamin, folacin, niacin, pyridoxine, riboflavin, and thiamine (114, 122, 123).

In turn, the available nutrients in the GIT niches also select microbial taxa that can colonize the host gut. The variation in the chemical structures of undigested fibers influences utilization by the GIT inhabitants, as specific enzymes are required for specific fiber types. The gut microbiota has a wide-ranging ability to digest and utilize these complex molecules. It was shown that the total genome of the gut microbiota harbors different functional gene families including 130 glycoside hydrolases, 22 polysaccharide lyases, and 16 carbohydrate esterases

(124). It makes the microbiome highly adapted to different energy sources of undigested fibers. Successful colonization is often based on the ability to produce a specific set of enzymes that have a high binding efficiency and rate of reaction towards available nutrient sources, coupled with the ability to obtain and produce products. This is supported by the potential to colonize around fiber particles with greater attachment and utilization functional capacities than potential competitors (125).

1.1.6 Effects of Chicken Gut Microbiota on Host Intestinal Health and Immunological Modulation

The importance of the gut microbiota to chicken intestine development as well as in maintaining gut homeostasis has been well recognized for decades. Some early research revealed that the intestinal–bacterial interactions were established shortly after hatch or feeding (39). The establishment of the intestinal microbiota was essential for early intestinal development (126). For example, at the villus area of the small intestine significantly increased in conventional raised (CR) chickens compared to germ-free (GF) at 5 d post hatch (127). The absence of microbiota in GF chickens resulted in poorly developed lymphoid follicles and decreased T and B lymphocytes compared to the CR birds (128). Compared to chicks at hatch, increased pro-inflammatory cytokines and chemokines, such as interleukin (IL)-1, IL-8, and K203, were observed in the gut of 2-day-old chickens after initial contact to feed and bacteria (129). The gut-associated lymphoid tissue (GALT) of chickens consists of the cecal tonsils, the Meckel's diverticulum, and the bursa of Fabricius. Bar-Shira et al. reported that the T and B cell maturation in the GALT is closely related to the development of the chicken gut microbiota (129). The initial innate immune response consists of a heterophil response in combination with

maternal antibodies followed by a secondary mucosal response after feeding and the presence of microbiota in the gut (130). Furthermore, the changes of intestinal T-cell receptors over time have been shown to be dependent upon the gut microbiota by comparing GF and CR chickens (131). Goblet cell mucin production was also impacted by the microbiota. Forder et al. compared the small intestinal mucin profile between CR chicken and chickens with limited microbiota, and found that the intestinal mucin profile of the CR birds was indicative of mature goblet cells, suggesting that increased microbial exposure leads to mature goblet cell formation (132).

Correlations have been made between the relative abundance of different bacteria and a series of cytokine expression levels in the chicken gut. For example, *Escherichia/Shigella*, *Parasutterella*, and *Vampirovibrio* have been associated with the expression of the pro-inflammatory cytokine gene *IL-6* in the cecal tonsils (13), whereas *Ruminococcus, Clostridium,* and *Lactobacillus* were reported to be negatively correlated to the expression of the pro-inflammatory cytokine gene interferon gamma (IFN-γ) and *IL-6* in the cecal tonsils and small intestine (133, 134). These studies indicated that the gut microbiome composition is connected to the host intestinal immune status.

One mechanism by which the microbiota promotes host health is through the production of SCFAs, particularly in modulating the host immune status. Complex interactions between SCFAs, gut microbes, and the host immune system have been reviewed by van der Hee & Wells (135). Briefly, SCFAs are imported into enterocytes and tissues via transporters and paracellular transport. SCFA receptors expressed on enterocytes and immune cells in the lamina propria and mucosal lymphoid tissue can activate signaling pathways to regulate host immune response according to the SCFA concentration to maintain intestinal homeostasis (135). For example, butyrate exhibited anti-inflammatory properties and also could be used as a source of energy for

enterocytes (136). Butyrate signaling through G-protein coupled receptors (e.g. FFA2, FFA3, and GPR109a) can confer anti-inflammatory properties in the GIT by down-regulating the expression of cytokines and chemokines (137). Intestinal macrophages and dendritic cells respond to the presence of butyrate through the niacin receptor, and thereby lead to increased production of the anti-inflammatory cytokines (e.g. *IL-10*), enhancing Treg cell differentiation to maintain gut homeostasis (118). Thus, SCFA producers in the gut are of great importance to the host health from an immunologic viewpoint. In fact, it has been shown that butyrate producers in the chicken gut, such as members in *Lachnospiraceae* family, were shown to exhibit anti-inflammatory effects by significantly increase peripheral Treg cells and *IL-10* monocytes (138).

On the other hand, disruption of the intestinal microbial homeostasis, for example, pathogen or opportunistic pathogen over-growth, can result in dysbiosis and lead to altered gut immunological profile and even expression of diseases. With an increase in pathogens in the microbiota, the pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) stimulates the pattern recognition receptors such as membrane-bound Toll-like receptors in the gut epithelium, and therefore activate myeloid differentiation factor 88 (MyD88). MyD88 activation will cause the nuclear translocation of nuclear factor kappa-B, and initiate the onset of inflammation, which decreases gut integrity for the infiltration of immune cells coming from the lamina propria or mucosal lymphoid tissue (139). Decreased gut integrity also allows pathogens, PAMP, and DAMP into the circulation, reaching other organs such as liver and spleen, and triggering systemic inflammation. When pathogens, PAMP, or DAMP reach the liver, similar to the intestine, they will trigger the onset of inflammation recruiting immune cells. In addition, the gut microbiota exhibits the ability to modulate bile acids and thereby modifies the signaling properties and subsequent actions of host bile acid receptors

(140, 141). It was shown that the gut microbiota influenced bile acid profile and farnesoid-X receptors signaling (140), which has been associated with inhibition of inflammation and improved gut integrity (142). In turn, bile acids can also influence the intestinal microbial composition (143, 144). Moreover, the host can also influence the intestinal microbes via IgA antibody production and secretion into the intestine through the bile duct (145), making it more effective in regulating the gut microbial populations.

1.1.7 Effects of Chicken Gut Microbiota on Pathogen Defense

Both poultry and zoonotic pathogens have long been a major concern to the poultry industry. The pathogens harbored by chickens are capable of causing severe illness in both humans and poultry. One prevalent pathogen is *Salmonella*, which makes up a small portion of the chicken intestinal microbial community (18). *Salmonella* can cause diarrhea, fever, abdominal pain, and can be fatal in the immunocompromised populations (e.g. infants, pregnant women, senior citizens, or immunosuppressed patients) (146).

Salmonella pathogenicity in chickens were found to be age-dependent. Young chickens are the most susceptible to *Salmonella* infection, especially in first few days post-hatch, and pathogen susceptibility is decreased in mature chickens when their gut microbiota are more diverse and stable (10). In fact, the concept of commensal competitive exclusion (CE) was first proposed in poultry studies regarding excluding *Salmonella* colonization in the chicken gut (10). Nurmi and Rantala found that commensal members of the chicken gut microbiota inhibited *Salmonella enterica* colonization by competitive exclusion (10, 147). Subsequent studies reported that introducing adult chicken cecal content or the bacteria derived from the mature

chicken gut to young chicks could significantly reduce *Salmonella* infection (23, 148). These studies solidified that the early development of the gut microbiota in chicken plays an important role in pathogen defense.

Research on the application of CE advanced from studying *Salmonella* exclusion, to other poultry-related pathogens, for example *Campylobacter*. *Campylobacter* is one of the most important zoonotic pathogens that is highly associated with poultry (149). Colonizing the lower intestinal tract of the chickens, some species of *Campylobacter,* namely *C. jejuni* and *C. coli,* are a contributor to human illness resulting in campylobacteriosis. For a long time, *C. jejuni* has been considered as a commensal of the chicken microbiota as the colonization of *C. jejuni* in the chicken gut did not seem to cause obvious clinical signs (150). However, recent studies reported that *C. jejuni* colonization could increase pro-inflammatory cytokine gene expression in the chicken gut (151), and impact the gut epithelium, which led to pathogen translocation (152, 153). Attempts to exclude *Campylobacter via* CE have been made. Colonizing young broiler chicks with a diverse set of adult chicken commensal bacterial isolates succeeded in reducing *Campylobacter jejuni* colonization (154, 155).

In addition to the potential impact on human health by zoonotic diseases, the economic impact of poultry diseases is a major concern of chicken producers. Bacterial pathogens such as *Clostridium perfringens* can cause necrotic enteritis, resulting in reduced growth and feed efficiency, and in severe cases, increased mortality (156). The annual economic losses caused by necrotic enteritis was estimated to be over US$6 billion globally (157). To date, in-feed antimicrobial additives are used in broiler farming to control necrotic enteritis. Concerns over the spread of antimicrobial resistance from agriculture has led to legislation and consumer pressure to reduce antibiotic use in animal production globally (158). Efforts have been made to find

effective alternatives of antimicrobial additives to control necrotic enteritis. Previous research has demonstrated that using a cocktail of probiotics reduced the level of cecal *C. perfringens* and associated intestinal lesions, which showed the potential to be an alternative to antibiotics (159). Overall, these studies have demonstrated the importance of the microbiota in pathogen defense in the chicken gut, which have proven to have potential in preventing and controlling pathogens in chickens.

1.2 The Potential Effects of the Broiler Production Environments on the Commercial Broiler Chicken Microbiota

1.2.1 Current Chicken Barn Sanitation Practices in the Canadian Poultry Industry

As discussed above, the relatively high hygiene levels of hatcheries and the absence of the hens at hatch have adverse effects on the development of broiler healthy microbiota (160). In practice, newly hatched chicks are delivered to the broiler production house shortly after hatch and receive feed and water on arrival. Therefore, broiler production houses are important in the initial assembly and development of the broiler gut microbiota. According to the current on-farm-food safety assurance program by the Chicken Farmers of Canada for broiler production, all production barns must be sanitized with chemical disinfectants at least once annually, and the reuse of chicken litter from a previous flock is not allowed (161). Within the year, producers have the option to clean the barns by blower/sweep clean, water wash or using chemical disinfectants. Limited by the lack of scientific information comparing the effects of these cleaning practices on productivity, chicken health, and chicken gut microbiology, many producers continue to use chemical disinfectants after each flock assuming “the cleaner, the

better". Currently, not much information is available to review how frequently chicken producers apply chemical disinfectants to sanitize the broiler barns in Canada. Course et al. surveyed 36 broiler chicken producers in Ontario on barn cleaning procedures for a period of 10 months and reported that for the 696 barn cleaning observation, chemical disinfection was the most frequently used practices (48.3%) (162).

Using chemical disinfectants may be efficient in removing microorganisms (163), but may also reduce the transmission of beneficial microbes derived from one flock to the next. As environmental chicken-derived bacteria were proven to be important in the early life gut microbiota assembly and development (84), the absence of these commensals may lead to decreased ability of the chicken gut microbiota to competitively exclude pathogens . Moreover, this may also result in selection of disinfectant-resistant pathogens that further increases the risk of pathogen contamination of chicken meat (164). To date, very limited information is available regarding how chemical disinfected barns affect chicken gut microbiota and intestinal health. Alternatively, to study the impact of the rearing environments on the assembly and development of the chicken microbiota, studies have been performed using recycled chicken litters.

1.2.2 The Interactions between Recycled Litter, the Chicken Gut Microbiota, and the Host Responses

Recycled chicken litter mainly consists of bedding material, chicken fecal waste, feed, water, and feathers (165). In most commercial poultry operations outside of Canada, the microbial communities in the recycled litter are carried over from one flock to the next (166). It was reported that recycled chicken litter contained bacteria ranging from 10^7 – 10^{10} CFU/g of dry

material (167, 168). In a production barn, recycled litter sampled from different locations also varied in microbial composition due to different temperatures, moistures, and litter depth (169). In addition, previous studies also reported different microbial compositions in recycled litters. Generally, members from phyla Firmicutes and Actinobacteria were reported to dominate the microbial communities of recycled litter. In addition, *Bacillales, Lactobacillales,* and *Enterococcus* were also often reported to be detected in the recycled chicken litter (89, 168, 169). Compared to the recycled litter, fresh litter was high in *Acinetobacter*, *Pseudomonas*, and *Enterobacteria*, whereas the used chicken litter contained more intestinal-type bacteria (89). Wang et al. compared the microbiota of fresh and recycled litter, and reported an increase of halotolerant/alkaliphilic bacteria in recycled litter indicating increased urea utilization (90).

As discussed, exposure to recycled chicken litter had an impact on the chicken gut microbiota and increased pathogen defense (10, 86, 87). Particularly, the recycled litter increased the microbial densities in the broiler gut microbiota of chicks over the first two weeks of life, resulting in an increased predominance of *Clostridiales* and *Lactobacillus* spp. in the chicken gut in later life (89). More recently, recycled litter was reported to increase the predominance of some potentially beneficial bacteria, such as *Faecalibacterium*, a SCFA producer whose increased abundance in young broiler ceca continued as the chicken matured (90). As mentioned previously, recycled chicken litter could effectively suppress pathogen colonization in the chicken gut. Early studies reported significant reduction of zoonotic pathogen *Salmonella* Typhimurium and *Salmonella* Enteritidis in the intestine of the chickens raised on recycled litter comparing to sterilized recycled litter group, which suggested that the pathogen exclusion effects were exerted by the viable bacteria presented in the recycled litter (23, 170). Very recently, Valeris-Chacin et al. used 16S rRNA sequencing to study the microbiome of the chicken litter.

They reported that isolation frequencies of *Campylobacter* from the chicken litter was negatively associated with *Bifidobacterium*, *Anaerosporobacter*, and *Stenotrophomonas* relative abundance in the recycled litter (171).

The chicken immune system was also found to be affected by recycled litter. Lee et al. compared the immune responses from chickens reared on clean litter, recycled litter from a previously healthy flock and recycled litter from a farm with history of a gangrenous dermatitis (GD) outbreak. Their results indicated that the GD litter stimulated the chicken humoral immune responses. Particularly, higher serum antibodies against *Eimeria* or *C. perfringens* were observed in chickens raised on GD litter compared with those on the fresh or used litter. In addition, recycled litter also decreased the expression of cecal tonsil pro-inflammatory cytokines (165). However, the study done by Shanmugasundaram et al. reported that compared with broiler chickens raised on fresh litter, those raised on used litter had increased expression of pro-inflammatory cytokine *IL-1β* and *IL-4* with a decrease expression of anti-inflammatory cytokine *IL-10* in cecal tonsils (91). Reasons behind these distinct results were not further explored. It may be caused by the differences of the chicken age, litter microbiome, and the gut microbiota between these studies, or variations of environmental factors such as diets and litter contaminants. However, to date, there is still no consensus on whether using recycled litter confers beneficial or harmful effects on the chicken immune response.

1.2.3 Current Knowledge of Barn Disinfections and its Effects on the Barn Environmental Microbiology, the Chicken Gut Microbiota, and Host Health

Currently, limited information is known regarding the effects of barn disinfections on the barn environmental microbiology, chicken gut microbial communities and the chicken performance. Recently, one study by Course et al. examined chicken barn sanitation procedures (i.e. dry cleaning, water cleaning, and chemical disinfection), and evaluated the effects on the pathogen detection odds in the barn environments (162). By comparing these cleaning methods on wood-floor and cement-floor broiler production barns, they found that the odds of detecting *S. enterica* were higher on water cleaning wooden floors compared to dry cleaned concrete floors; whereas *E. coli* concentration was lower in chemically disinfected barns compared to dry cleaned barns (162). Interestingly, they also found that the odds of having quaternary ammonium compound-resistant *E. coli* isolates were lower in full disinfected barns compared to the dry cleaned barns.

To investigate the effects of rearing environment cleaning in pathogen incidences on the chicken gut, de Castro Burbarelli et al. examined the effects of chicken pen cleaning using neutral detergent versus a protocol using acidic and alkaline detergents with chemical disinfectants and found that the protocol resulted in increased chicken body weight at 42 days of age with little impact on the *Campylobacter* incidence at the same age (163). However, this study only focused on the absence/presence of *Campylobacter* on research-pen level without examining the gut microbiota or the gut nutrient metabolites, making it difficult to explain the increased chicken performance observed in the protocol.

1.3 The Effects of Distinct Microbial Communities on the Chicken Host

In microbiome research, studying variations in microbial structure and composition can offer insight into complex host-microbe-metabolite interactions. Arumugam et al. first described three clusters in the human gut microbiota, and suggested that population-level analysis of the gut microbiome variation could help understand host–microbial symbiotic states, which might respond differently to diet and drug intake (172). Human studies revealed that the distinct microbial communities were correlated with long-term dietary patterns but independent of host phenotypes such as gender, age or body mass index (173). Compared to other animal models, such as mice, limited information is known regarding distinct chicken gut microbial communities and their effects on the host. Previously, some distinct microbial communities in the chicken gut microbiome had been identified (174, 175). However, in chicken studies the causes of these distinct microbial populations have yet to be determined.

Kaakoush et al. reported that the chicken fecal microbiomes could be separated into 4 robust enterotypes, namely the 1) Firmicutes-dominant, 2) the Firmicutes- and Proteobacteria-dominant group, 3) the Firmicutes- and Actinobacteria-dominant group, and 4) the Firmicutes- and Bacteroidetes-dominant group (174). Particularlly, the dominant Firmicutes were *Lactobacillus* and *Peptostreptococcaceae* were the first 3 groups; whereas the Firmicutes from group 4 were dominated by *Ruminococcaceae.* Sharing dominance with Firmicutes, the dominant Proteobacteria were *Escherichia-Shigella* and *Enterobacter*; the dominant Actinobacteria were *Corynebacterium* and *Brevibacterium*; and the dominant *Bacteroidetes* were *Alistipes* and *Bacteroides* (174). Kaakoush and colleagues speculated one possible factor that led these distinct microbial communities was the rearing environment (the conventional indoor farm vs. free range farm). Chickens reared on the conventional indoor farm had more gram-negative

bacteria (e.g. Proteobacteria) in their gut, as compared to free-range chickens, highlighting that the rearing environments greatly influence the chicken gut microbial composition (174). However, the authors did not explore changes in metabolite profile or host responses, leaving the question of how different microbial composisions affect host health unexplained (174).

A subsequent study of duodenum digesta using 16S rRNA sequencing and metabolomic techniques reported 3 distinct duodenal microbial communities, namely 1) the *Bacteroides* and *Escherichia/Shigella*-dominant group, 2) the *Ochrobactrum*- and *Rhodococcus*- dominant group, and 3) the *Bacillus* and *Akkermansia* group (175). In this study, associations between duodenal metabolites and the distinct microbial communities were drawn. Cellobiose, α -D-glucose, D-mannose, and D-allose were positively correlated to the group 2 microbial communities with β-hydroxybutyric acid being negatively correlated (175). Furthermore, the authors examined how these distinct microbial communities affect the host. Their results revealed that chickens harboring the group 2 duodenal microbiota had higher serum triglyceride level and fat deposition compared to the chickens with group 1 microbiota at 77 days of age (175).

Recently, studies trying to link distinct microbial communities to specific host diseases or phenotypes were criticized by some researchers. They have suggested that grouping the microbiota of individual subjects based on the dominance of certain genera may have oversimplified a complex situation. However, studies on distinct microbial communities are very informative if they include assays measuring biomarker signatures (e.g. antibiotic resistance genes, functional genes, and metabolites) and robust bioinformatics to establish possible relationships.

1.4 Gut Microbiota and Antibiotic Resistance

In 1951, the United States Food and Drug Administration approved the use of antimicrobial agents without veterinary prescription. However, emerging diseases caused by antibiotic-resistant bacteria had caused continuously expending concern of antibiotic usage in livestock production. Annual global deaths linked to antimicrobial resistance (AMR) was projected to reach 10 million by 2025 with cumulative medical expenses and economic loss reaching $100 trillion dollars (176). Livestock farming accounts for over 50% of all antibiotic usage globally (177). Concerns over the spread of antimicrobial resistance from agriculture has led to legislation and consumer pressure to reduce antibiotic use in animal production globally. In 2003, the European Union gradually banned all in-feed administration of non-therapeutic concentration of antibiotic agents in the livestock industry, whilst the World Health Organization called for the eliminating the use of livestock growth-promoting antibiotics in the near future (178). In Canada, the Chicken Farmers of Canada has put great effort to progressively phase out prophylactic antimicrobial use in chicken farming. According to the Chicken Farmers of Canada, by the end of 2018 preventive usage of antibiotics that are of very high (category I) and high (category II) medical importance (e.g. Aminoglycosides, Carbapenems, Cephalosporins, Fluoroquinolones, Fusidic acid, Glycopeptides, Polymyxins, Lincosamides, Macrolides, Penicillins, Quinolones, Streptogramins, sulfamethoxazole) were banned in chicken production (179). However, subsequent data following the antibiotics ban in the EU showed significantly increased broiler mortality rate correlating with decreased disease resistance (180). Therefore, effective alternatives of prophylactic antibiotics are highly warranted to reduce antibiotic usage whilst maintaining livestock productivity.

1.4.1 Usage of Antibiotics and Antimicrobial Resistance in Poultry Farming

As mentioned, livestock farming was reported to account for more than 50% of the global usage of antibiotics, which was projected to exceed 200,000 tones by 2030 (177). AGPs were defined as antibiotics added as feed additives at low subtherapeutic levels for the purpose of improving growth and feed efficiency (181). As a consequence, AGPs apply selective pressure to the microbial communities in the livestock GIT, which stimulates the mutation and transmission of antibiotic resistance genes (ARGs) among the microbes and thus foster the increase in the relative abundance of resistant populations (182). Microbes carrying ARGs can be excreted together with animal feces to the environments causing contaminations (183). Once ARGs disseminate into environments such as soil and water, it increases the likelihood of human exposure (183). This limits the number of antibiotics that are effective for therapeutic treatments conducting higher incidence of disease. In this sense, livestock waste can put pressure to the public health system (184).

Generally, the most frequently detected ARG classes in livestock waste include resistance to β-lactams (e.g. *bla*), macrolide-lincosamide-streptogramin (MLS) B (e.g. *erm*), FCA (fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol) (e.g. *fca*), sulfonamides (e.g. *sul*), and tetracyclines (e.g. *tet*); whereas aminoglycosides (e.g. *aac*), vancomycin (e.g. *van*) and multidrug (e.g. *mdr*) have also been detected (185-187).

In intensive broiler chicken production, antibiotics such as bacitracin, bambermycin, salinomycin, tetracycline, tylosin, and virginiamycin are often used (188, 189). Likely due to the high stocking density during production and short production cycle, the chicken gut microbiome were reported to have higher densities of ARGs compared to other livestock species (186). One important ARG reservoir in chicken production is the chicken litter. It accumulates ARG inputs

from the excreta and other residues like spilled feed containing ARGs during production. Previously, it was reported that poultry litter harbored high densities of AMR bacteria, mobile genetic elements (MGEs) and antibiotic residuals (190-192), and AMR bacteria were even still detectable in broiler production houses after removal of the used litter and after disinfection treatment (162).

The commercial chicken gut microbial communities were also known to be a reservoir of ARGs and MGEs (the complete collection of ARGs and MGEs in a given microbial community were also termed as "resistome") (193, 194). Studies profiling human, swine and poultry resistomes showed that the human gut shared the highest similarities in mobile ARGs with the chicken gut, indicating high-possibilities of horizontal ARG transfer between humans and poultry (195, 196). In particular, the poultry gut microbiota was reported to carry more than 500 ARGs that can be classified into over 200 types, being the highest in ARG numbers and abundance compared to humans and swine (195).

Currently, compared to the swine and ruminant resistome, information on the chicken gut resistome is still limited (197). Previously, a study across 9 European countries focusing on the chicken, swine and human fecal resistome revealed that the majority of ARGs found in chicken feces were tetracycline-, aminoglycoside-, and MLS- resistant genes (198). One recent study studied the fecal resistome of chickens at farms and the ones at live poultry markets in China (195). Generally, AMR against aminoglycoside, tetracycline, MLS, and β-lactam were more abundant than those associated with other classes (195). However, due to variations in chicken feed composition and antibiotic management between countries, it was hard to determine the factors affecting the chicken fecal resistome. Some previous studies have explored the effects of different classes of antibiotic administration on the chicken fecal resistome (199, 200). It was

reported that ampicillin was positively associated with the abundance of most β-lactam and bacitracin ARGs, and negatively correlated to the abundance of tetracycline ARGs in chicken feces (199). Xiong et al. reported that increased abundance of tetracycline ARGs were detected in chicken fecal resistome when therapeutic dosages of chlortetracyclines were added in the feed. Interestingly, the increased abundance of tetracycline ARGs coincided with decreased abundance of multi-drug resistant genes, indicating, not surprisingly, the classes and dosage of antibiotics involved in poultry production influence the chicken microbial resistome (200).

1.4.2 Mutation and Transmission of ARGs and Antibiotic Resistant Mechanisms

As an ancient survival strategy, microorganisms had developed antibiotic resistance to overcome environmental threats. The emerge of resistance to antimicrobial agents reflects an aspect of microbial evolution, which can be acquired by genetic mutations or horizontal gene transfer (e.g. transformation, conjugation and transduction) (201). Under the stress of antimicrobial agents (e.g. adverse growth conditions), genetic errors can occur to bacterial genes that lead to reduced or loss of antibiotic affinity to their targets, and therefore confer survival advantage to the microorganisms (202). Surviving bacteria then pass these ARGs to their progeny cells causing the spread of antibiotic resistance. In addition, horizontal gene transfer (interspecies and/or intraspecies) is also another important route for bacteria to acquire ARGs. Bacteria are known to transmit genes including ARGs via the acquisition of mobile genetic elements (e.g. plasmid, transposons, integrons, bacteriophage, and retroviruses, etc.) naturally or under environmental pressure (203, 204). In this sense, misuse of antibiotics may accelerate the emerge of antibiotic resistance through mutation and transmission through vertical and horizontal gene transfer, which consequently leads to the spread of ARGs.

The molecular basis of how different types of antibiotics inhibit the growth of bacteria were well studied and reviewed (205). Antibiotics can inhibit 1) bacterial cell wall synthesis (e.g. β-lactams and vancomycin), 2) protein synthesis by targeting bacterial ribosome (e.g. aminoglycosides, tetracyclines, and MLS), 3) DNA replication by targeting DNA gyrase (e.g. quinolones), 4) RNA translation by targeting RNA polymerase (rifamycin), or 4) inhibit folic acid metabolism (e.g. sulfonamides) (205). To survive, bacteria have developed a series of resistance mechanisms. The resistance mechanism details are as follows.

To prevent accumulation of antibiotic in the bacterial cell, some bacteria alter membrane permeability (i.e. increasing efflux and decreasing uptake). Bacterial efflux pumps actively transport antibiotics out of the bacterial cells. Efflux pumps widely contribute to antibiotic resistance among both Gram-positive and Gram-negative bacteria. A variety of genes encode wide-range efflux pumps (e.g. the resistant nodulation division family and the major facilitator superfamily) and substrate-specific efflux pumps (e.g. tetracycline efflux pumps) (206-209). One other mechanism to alter the bacterial cell membrane permeability is through decreasing uptake. For example, *Pseudomonas* can decrease the membrane permeability by down-regulating the expression of outer membrane porin protein or by replacing porin protein with more selective channels to limit the diffusion of hydrophilic antibiotics (210).

ARGs can also confer resistance through modification (protect, alter, or replace) of the antibiotic target sites. For example, antibiotic target protection proteins can bind to the functional protein, such as the bacterial ribosomal proteins, thereby help bacteria to gain resistance against antibiotics (211, 212). Ribosomal protection proteins (RPPs) are good examples for this resistance mechanism. RPPs are a group of proteins that can competitively bind to the bacterial ribosome subunits resulting in the protection of the targets of specific antibiotics (e.g.

tetracyclines, macrolides, lincosamides and streptogramin) (211). Notably, genes encoding RPPs were often located on mobile genetic elements (213, 214) indicating a high capability of being horizontally transferred. In recent years, RPP gene families encoding tetracycline-resistance RPPs and the erythromycin ribosome methylases (*erm,* MLS-resistant) were frequently identified in a great variety of bacteria in livestock farming-related niches (215-222), urging a re-evaluation of the current guideline of antibiotic usage in agriculture.

Inactivation of antibiotics is another major mechanism of antibiotic resistance. A large group of ARGs can encode enzymes that can inactivate antibiotics by chemically them (223) or by destroying them (224). For instance, tetracycline destructases can destroy the chemical structure of tetracycline by oxidizing the covalent bonds of the antibiotic molecule (224). Unlike the alteration of permeability or change of the antibiotic targets, the ability to eliminates the intracellular and/or extracellular antibiotic challenge permanently by inactivation enzymes and destructases were considered to have a huge clinical impact (224). In fact, some recently developed antibiotics (e.g. tigecycline and omadacycline) had overcome resistant conferred by efflux pumps (225) and RPPs (226), yet currently limited measures were available in controlling drug resistance by antibiotic inactivation enzymes.

1.4.3 Resistance to Antibiotics Related to Poultry Production

Aminoglycosides were used for treating enteric infection in poultry (227), and are of high importance in treating serious human infections (category II antibiotics) (179). Bacterial resistance against aminoglycosides is mainly driven by modification of target of aminoglycosides (the bacterial ribosome). Genes encoding either acetyltransferases (*AAC(2),*

AAC(3), and *AAC(6)*), nucleotidyl transferases (*ANT(2), ANT(3), ANT(4), ANT(6),* and *ANT(9)*), and phosphotransferases (*APH(2), APH(3), APH(3), APH(4), APH(6), APH(7),* and *APH(9)*) were often detected in poultry farming.

β-lactams are one of the most important antibiotic classes in human bacterial infection treatments (category I). As an important member of β-lactams, penicillin was once approved as feed additives in Canada (189). Currently, it is highly restricted in Canadian poultry farming (179). Multiple resistant mechanisms against β-lactams have been documented. The major bacterial resistance against β-lactam, which has been well reviewed by Paterson & Bonomo, is exerted via the production of β-lactamases (e.g. *blaTEM* gene encoding extended-spectrum β-lactamases) (228). In addition, some gram-positive bacteria showed resistance by the acquisition of mutated penicillin binding proteins (229). The mechanism through decreasing outer membrane permeability and increasing expression of efflux pumps were widely reported (230, 231).

The macrolide, lincosamide and streptogramin antibiotics are category II antibiotics (179), which are often used to treat infections caused by Gram-positive bacteria. Interestingly, although macrolide, lincosamide and streptogramin are completely different in molecular structures, they are often studied as a group (termed as "MLS") due to the similarity in the inhibition of bacterial protein synthesis targeting the bacterial ribosome (232). Historically, some antibiotics in this group (e.g. lincomycin and virginamycin) were used as growth promoters in broiler chicken production (189, 233), and thus MLS-resistant genes were frequently detected in samples related to poultry production. The most frequently found ARGs conferring MLS-resistance in poultry farming belongs to the *erm* gene family (e.g. *ermB, ermC, ermG, ermF,*

ermX), which encode rRNA methylases that modifies the bacterial ribosome environments (220-222).

Tetracycline antibiotics were categorized as medium importance to human use in Canada (category III) (179), and they were also once used as feed additives by Canadian poultry producers (189). Likely due to the extensive use of tetracycline for decades, high frequency and diversity of tetracycline resistant genes were detected in poultry (192, 198, 234, 235). Tetracycline antibiotics target the bacterial ribosome and inhibit protein synthesis (236). The mechanisms of resistance against tetracycline include antibiotic target alteration by RPPs (e.g. *tet(W), tet(M), tet(O), tet(32),* etc.), tetracycline efflux pumps (e.g. *tet(40), tet(42), tet(K),* etc.), and tetracycline inactivation enzymes (e.g. *tet(X)*).

1.4.4 The Use of Chemical Disinfectants and its Effects on ARGs

Biocidal agents such as benzalkonium chloride (BAC), hydrogen peroxide, glutaraldehyde, ethanol, sodium hypochlorite, were widely used in agriculture for disinfecting purposes (237). On one hand, chemical disinfectants demobilize antibiotic-resistant bacteria and/or destruct ARGs by applying oxidation stresses (e.g. chorine, ozone). Conversely, they stress bacteria to adapt; a behavior that can promote antibiotic resistance through co-selection (238). Currently there is no consensus on whether chemical disinfectants induce ARG proliferation, or if they are beneficial in controlling antibiotic resistance. Some studies indicated that chemical disinfectants could stimulate microbial ARG proliferation and transfer in the environment as a stressor (239-241). Benzalkonium chloride, a widely used quaternary ammonium compound for controlling bacteria, fungi and viruses, was found to increase the

resistance to ampicillin, cetotaxime, and sulfamethoxazole among a series of food-related bacterial isolates (239, 242), and co-selected ARGs (243). Sodium hypochlorite (also referred as liquid bleach) is widely used in hospital, agriculture, and other industries as a chemical disinfectant. It was shown that adapted to sodium hypochlorite, some *Salmonella* species also developed associated resistance to some antibiotics (e.g. gentamicin, chloramphenicol, and tetracycline, etc.) (244). Notably, some bacteria such as *E. coli* can enter the viable but non-culturable state to persist from sodium hypochlorite and potentially various antibiotics (245).

Some studies have also indicated that the use of chemical disinfectants could control antibiotic resistance by decreasing ARG abundance. It has been showed that quaternary ammonium compounds and sodium hypochlorite treatment of swine manure significantly decreased the abundance of selected ARGs (i.e. *erm(B), erm(C), erm(F), intI1, tet(Q),* and *tet(X)*) (246). It was also reported that oxidants such as chlorine and hydroxyl radical exhibited the potential to destroy ARG located both in *E. coli* cells and on plasmids (247). However currently, limited information is available regarding chemical disinfectant-treated rearing environments and their effects on the animal gut resistomes.

1.6 Research Objectives and Hypotheses

The production barn acts as an important microbial source for the chickens. Cleaning/ disinfection practices impact the microbial abundance and diversity of the rearing environment, which may influence the assembly and development of the chicken gut microbiome.

Objectives: This thesis was designed to evaluate the effects of barn cleaning practices at the production level to provide scientific evidence for the Canadian poultry industry to make rational decisions regarding the most appropriate barn cleaning method.

Specifically our aims were to:

1) assess how barn cleaning practices influence the chicken productivity, the chicken gut microbiota and pathogen load, as well as cecal SCFA concentrations

2) evaluate the gut microbial functional consequences that result from the different barn cleaning practices, particularly with respect to the genetic potential of microbial metabolism and the ARG incidence of the gut microbiota; and

3) explore how early-life distinct cecal microbial populations affect microbe-microbe interactions, the gut microbial functionality and the subsequent effects on host immune activation.

Hypotheses:

1) Compared to barn water-wash, the application of chemical disinfectants during barn cleaning will alter the composition of the chicken gut microbiota resulting in increased pathogen carriage.

2) The use of disinfectants in barn cleaning will impact the cecal microbial functional capacities of nutrient metabolism and increased incidence of ARGs in the chicken gut.

3) Distinct cecal *Bacteroides* composition in week-old broiler chickens results in different microbial functionalities that altered the cecal SCFA production and host immune status.

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Chapter 2: THE USE OF DISINFECTANT IN BARN CLEANING ALTERS MICROBIAL COMPOSITION AND INCREASES CARRIAGE OF *CAMPYLOBACTER JEJUNI* IN BROILER CHICKENS[*]

2.1 Introduction

In broiler chicken production, biosecurity measures are important to maintain flock health and food safety. Regulations of the current on-farm-food safety assurance program require Canadian broiler chicken producers to clean barns with disinfectants at least annually (1). Barn cleanouts within the year using water wash (**WW**) can be done without disinfectants; however, in practice many producers continue to perform full disinfection (**FD**) using chemical disinfectants after each flock. Using chemical disinfectants removes a high proportion of microbes (2), but may also reduce the transmission of beneficial microbes between flocks. This could lead to a potential loss in the microbes that can out-compete pathogens in the environment. It may also result in selection of disinfectant-resistant pathogens that further increases the risk of pathogen contamination of animal products (3). To date, little information is available regarding how these cleaning measures affect chicken health and zoonotic pathogen colonization. Furthermore, emerging evidence has shown the importance of broiler commensal microbial community to nutrient metabolism, feed efficiency (4-6), host resistance to pathogens (7), and immune system development (8, 9). In chicken production, the establishment of a symbiotic microbiota has been shown to increase nutrient utilization (10), and prevent disease development (11). Therefore, establishing healthy host-microbe interactions early in production may provide a possible solution

[*] This chapter has been published as Yi Fan, Andrew J Forgie, Tingting Ju, Camila Marcolla, Tom Inglis, Lynn M McMullen, Benjamin P Willing, Douglas R Korver. The use of disinfectant in barn cleaning alters microbial composition and increases carriage of *Campylobacter jejuni* in broiler chickens. Applied and Environmental Microbiology. 2022 May 24;88(10):e0029522. doi: 10.1128/aem.00295-22.

to help maintain, or even enhance broiler performance in an environment free from antimicrobial growth promoters. Broiler gut microbiota assembly and development is significantly influenced by the initial environment to which they are exposed (12). Thus, placing newly hatched broilers in an environment with microbiota from a previous healthy flock may advance the development of a commensal microbiota in broiler chickens.

Previous work has shown that exposure to reused broiler litter altered early-life gut microbiota and increased infection resistance to pathogens in broiler chickens (13-15). Reused litter induced changes in the broiler gut microbiota of chicks over the first two weeks of life, resulting in an increased predominance of *Clostridiales* in the gut (16). More recently, the application of reused litter was reported to increase the predominance of some potentially beneficial bacteria, such as *Faecalibacterium*, a short-chain fatty acid (**SCFA**) producer whose increased abundance in young broiler ceca continued as the chicken matured (17). Commensal microbes and SCFAs are important in maintaining gut homeostasis. For example, butyrate increases intestinal epithelial oxygen consumption, which helps to maintain an anaerobic environment (18). SCFAs also modulate host immune responses by suppressing pro-inflammatory cytokine expression to achieve homeostasis (19).

Both poultry and zoonotic pathogens have long been a major concern to the poultry industry. The poultry gut microbiota plays an important role in pathogen exclusion. For example, commensal members of the chicken gut microbiota inhibited *Salmonella enterica* colonization by competitive exclusion (15, 20). Colonizing broiler chicks with a diverse set of adult chicken commensal bacterial isolates reduced *Campylobacter jejuni* colonization (21). In addition to the potential impact on human health by zoonotic diseases, the economic impact of poultry diseases is a major concern of chicken producers. Bacterial pathogens such as *Clostridium perfringens* can

cause necrotic enteritis, resulting in reduced growth and feed efficiency, and in severe cases, increased mortality (22). To date, in-feed antimicrobial additives are used in broiler farming to control necrotic enteritis. Efforts have been made to find effective alternatives to control necrotic enteritis. Concerns over the spread of antimicrobial resistance from agriculture has led to legislation and consumer pressure to reduce antibiotic use in animal production globally. Previous research has demonstrated that using a cocktail of probiotics reduced the level of cecal *C. perfringens* and associated intestinal lesions, which showed the potential to be an alternative to antibiotics (23). However, antibiotics remain the most effective and widely used approach to keep the prevalence of *C. perfringens* infections low in the poultry industry (24).

To date, limited information is available on how WW and FD affect pathogen prevalence, gut microbial communities, nutrient metabolism, and host responses in chickens. In this study, a cross-over experiment was designed using seven similar commercial chicken barns to compare WW and FD over the course of four production cycles at two locations. We evaluated the effects of barn cleaning method on the commercial broiler intestinal microbiota, occurrence of *Campylobacter* and *Salmonella*, abundance of *C. jejuni* and *C. perfringens*, as well as SCFA profile in 30-day-old broiler ceca.

2.2 Materials and methods

2.2.1 Broiler production house and barn cleaning

A commercial broiler company in Alberta, Canada provided all the chickens and facilities including a total of seven similarly engineered single-storey, cement-floored production houses at two locations for this study. The broiler facilities were environmentally-controlled metal houses

with solid sidewall ventilation. Four alternating water and feed lines ran the entire length of each house.

For FD, chicken manure, used litter, and organic matter were completely removed from the chicken house after depopulation followed by a two-step disinfection: 1) All surfaces were thoroughly covered with foam containing 7% sodium hydroxide, 7% 2-(2-2-butoxyethoxy) ethanol, 6% sodium laureth sulfate, 5% sodium N-lauroyl sarcosinate, and 5% tetrasodium ethylenediaminetetraacetic acid for 60 mins, and subsequently rinsed with high-pressure followed by low-pressure water-wash with the water temperature set at 35℃; 2) after the broiler house was air-dried, all surfaces were covered with foam containing 10% glutaraldehyde, 10% benzalkonium chloride, and 5% formic acid for 60 mins followed by high-pressure water rinse. After the two-step disinfection, broiler houses were left to air-dry overnight followed by placement of fresh wood shavings (~10 to 15 cm deep). For WW, manure and used litter were removed, followed by low-pressure water rinse with the water temperature set at 35℃ of the facility surfaces, air dried, and placement of fresh wood shavings (~10 to 15 cm deep). The current study was performed on 28 production flocks, and the FD and WW treatments were each applied on 14 production flocks. For each chicken barn, two flocks of each treatment were assigned according to the schedule shown in Table 2.1.

2.2.2 Chicken management and sample collection

Animal use for this experiment was approved by the Animal Care and Use Committee: Livestock of the University of Alberta following the Canadian Council on Animal Care guidelines (25). For each flock, 14,000 Ross 308 broiler chicks were placed at 1 day of age and confined to

half of the house, then allowed access to the entire house at 7 days of age. All chickens were fed *ad libitum* and reared from 1 day of age through processing at about 32-35 days of age when the average target live weight of 1.8 kg is reached. Each flock had a placement based on a maximum stocking density of 30 kg/m^2. Overall mortality rate and body weight of broilers at day 32 were recorded for each barn.

To investigate if *Campylobacter* and *Salmonella* were present after barn cleaning, environmental samples including litter samples, feeding pan and drinkers, as well as shoe-cover samples were collected 3-6 hours before chicken placement of each flock. For litter samples, approximately 50 g subsamples of bedding material were collected from distinct areas in the barn: along water and feeder lines; between water and feed lines; near the wall corners; near the barn entrance and near the ventilation fans. From each of these areas, litter samples were collected from the surface to the cement floor with sterile gloves and Whirl-Pak sample bags (Whirl-pak, Madison, WI, USA). Subsamples were sealed and transported to the laboratory on ice. Immediately after arriving the laboratory, samples from the same barn were pooled (resulting in ~ 200 g of litter/barn) in a sterile sampling bag containing 250 ml of 0.1% buffered peptone water and homogenized by a Seward Stomacher 400 (Seward, Worthing, West Sussex, UK) for 1 min.

To collect feeding pan and drinker swabs, Whirl-Pak Speci-Sponge® Sampling Bag (Whirl-pak) were used. For feeding pan swabs, 10 ml of 0.1% buffered peptone water (Oxoid, Basingstoke, Hampshire, UK) was added to the sampling bag to re-hydrate the sampling sponge. About 50 cm^2 of the feeding pan surfaces were carefully swabbed using the re-hydrated sampling sponge. For drinker swabs, ~ 5 ml of the water coming from the drinking nipple was collected using the de-hydrated sponge followed by swabbing approximately 15 cm of the waterline on both sides of the drinking nipple. In each barn, 10 feeding pans and 10 drinkers in multiple locations

were randomly selected to subsample. Each sampling bag was used to swab 1 drinking nipple or 1 feeding pan. Sterile gloves were changed before each swab. Subsamples were sealed and transported to the laboratory on ice. Immediately after arriving at the laboratory, the 10 subsamples of drinker/feeding pan from the same barn were pooled together in a sterile stomacher bag aseptically. Buffered peptone water (0.1%) was added to a 1 in 10 dilution. Pooled samples were homogenized for 1 min at 260 using a Seward Stomacher 400 before processing for *Campylobacter* and *Salmonella* detection.

To collect shoe-cover samples, sterile DuPont Tyvek® shoe covers (Wilmington, DE, USA) were used. Briefly, three layers of sterile shoe-covers were put on at the entrance of the barns before environmental sampling. Shoe-covers were used to walk through the barn following the feeder and water lines. After walking through the whole barn, the outer layers of the shoe-covers were collected using a Whirl-Pak sample bag (Whirl-pak). Sample bags were sealed and put on ice before arriving at the laboratory. In the laboratory, the shoe-cover sample was transferred to a sterile stomacher bag with 100 ml of 0.1% buffered peptone water, followed by homogenization for 1 min using a Seward Stomacher 400 before processing for *Campylobacter* and *Salmonella* detection.

At broiler placement day and day 30, five broilers per flock were euthanized using cervical dislocation for sampling. To ensure representative sampling, the five broilers were each randomly selected from different areas within each barn.

Broiler cecal content collections were conducted aseptically. Briefly, the sampling table was cleaned with 70% ethanol before and between every broiler dissection. Tools and collection tubes were autoclaved and tubes were sealed until samples were added. Approximately 100 mg (at

placement) and 300 mg (day 30) of cecal contents were collected and placed in sterile 2 ml Eppendorf tubes containing 1 ml of 0.1% buffered peptone water (Oxoid) for detection of cecal *Campylobacter* and *Salmonella* by enrichment. In addition, approximately 500 mg of cecal contents were frozen on dry ice immediately until transported to the lab, and stored at -80 ℃ for subsequent DNA extraction.

2.2.3 *Campylobacter* and *Salmonella* enrichment

For genus *Campylobacter* enrichment and detection, ~50 mg of cecal contents were homogenized with 950 µl sterile peptone water. Homogenized cecal contents, litter samples, feeding pan and drinker swabs, as well as shoe-cover samples were incubated at 37°C overnight followed by inoculation in Bolton *Campylobacter* selective broth in 1:10 ratio (Oxoid) at 42°C for 24 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Aliquots (100 µl) were serially diluted and spread onto Preston *Campylobacter* selective agar (Oxoid,). For *Salmonella* enrichment, universal pre-enrichment broth (Sigma-Aldrich, Oakville, ON, Canada) was used to homogenized cecal contents and the environmental samples in 1:10 ratio. All homogenized samples were incubated aerobically at 35°C for 24 h. The cultured broth was transferred to 10 ml of tetrathionate broth (Difco, Becton, Dickinson and Company Sparks, MD, USA) and to 10 ml of selenite cystine broth (Difco). The tetrathionate broth and selenite cystine broth were incubated for 24 h at 42°C and 35°C, respectively. After incubation, tetrathionate and selenite cystine broths were streaked onto xylose-lysine-tergitol 4 agar (**XLT-4**; Difco) and brilliant green sulfa agar (**BGS**; Difco) plates, respectively. Plates of XLT-4 and BGS agar were incubated at 35°C for 24 h.

Occurrence score for detected pathogens was calculated to evaluate the effect of cleaning method on pathogen occurrence in the chicken ceca. Occurrence score was defined as the number of positive broilers divided by the total number of broilers sampled from the same flock within each barn.

2.2.4 DNA extraction and microbiome analyses

Total DNA was extracted from homogenized litter samples and cecal contents using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) with an additional bead-beating step with ~200 mg of garnet beads at 6.0 m/s for 60 s (FastPrep-24 5G instrument; MP Biomedicals Inc., Santa Ana, CA, USA). Amplicon libraries were constructed according to the manufacture protocol from Illumina (16S Metagenomic Sequencing Library Preparation) targeting V3-V4 region of the 16S rRNA gene (primers: Forward: 5′-TCGTCGG CAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; Reverse: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACT ACHVGGGTATCTAATCC-3′). An Illumina MiSeq Platform (2×300 cycles; Illumina Inc., San Diego, CA, USA) was used for a paired-end sequencing run. All sequences were submitted to NCBI Sequence Read Archive under BioProject ID: PRJNA767330. The quality of sequencing reads was assessed using FastQC. Sequenced reads were processed using Quantitative Insight into Microbial Ecology2 (**QIIME2**) - 2020.6 (26). Divisive Amplicon Denoising Algorithm 2 was used to denoise and generate paired-end representative read with truncation lengths of 280 bp forward and 260 bp reverse reads (27). Amplicon sequence variant (**ASV**) feature table was created based on the denoised results. Qiime2's q2-feature-classifier was used to assign taxonomy (28) with a pretrained classifier "Greengenes 13_8" (99% identity) (29). Analyses of diversity were done by the 'diversity core-

metrics-phylogenetic' command normalizing to a sampling depth set by the sample with the lowest number of reads (17,309). Chao1 and Shannon diversity indices were calculated with 'diversity alpha-phylogenetic'. Significance of alpha diversity was determined by 'diversity alpha-group-significance'. Beta diversity was determined in QIIME2 using the unweighted- and weighted-Unifrac distance metric and a principal coordinate analysis (**PcoA**) was plotted using phyloseq package in R (version 3.6.1). Permutational Multivariate Analysis of Variance (**PERMANOVA**) based on the unweighted- and weighted-UniFrac distance matric was used to determine whether there were significant differences in community structures between treatments (adonis function). Differentiate taxa relative abundance between treatments was determined by the analysis of composition of microbiome (**ANCOM**) in QIIME2 (26).

2.2.5 Quantitative PCR (qPCR)

A qPCR assay was used to quantify *C. jejuni*, *C. perfringens* as well as total cecal bacteria using hippurate hydrolase (***HipO***), necrotic enteritis B-like toxin (***NetB***) and the targeted 16s rRNA gene, respectively (Table 2.2). Total genomic DNA was extracted from cecal contents as described above. The concentration of DNA was determined by a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). PerfeCTa SYBR Green Supermix (Quantabio, Beverly, MA, USA) was used for qPCR assays which were conducted on an ABI StepOne real-time System (Applied Biosystems, Foster City, CA, USA) following the setup of 95°C for 3 min and 40 cycles of 95°C for 10 s, 60°C for 30 s. To generate targeted gene standards, the 16s rRNA gene, *HipO* and *NetB* were amplified by PCR with primers as listed. Concentrations of the amplified gene fragments were determined by a Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Waltham, MA, USA) and used for standard curve. Gene copy numbers were

determined using the Relative Standard Curve Method, and normalized to the weight of cecal content used for bacterial DNA extraction.

2.2.6 Short chain fatty acid (SCFA) analysis

Approximately 30 mg per sample of snap frozen cecal content was weighed, followed by homogenization with 25% phosphoric acid. Samples were centrifuged at 21,130 x g for 10 min and the supernatant was collected and filtered using a 0.45 μm filter. Isocaproic acid (23 μmol/ml) was added at a 1:4 ratio to samples as an internal standard. Samples were analyzed on a Bruker Scion 456-Gas Chromatography instrument (Bruker, Billerica, MA, USA).

2.2.7 Statistical analyses

Unless otherwise stated, statistical analyses were conducted using GraphPad Prism 8 (Graphpad Software, San Diego, CA, USA). Because no location effects were observed for any measurements (i.e. chicken performance and gut microbial structure), all data were analyzed based on treatment across location. Statistically significant differences were determined ($p < 0.05$) by an unpaired student's t-test for parametric data (i.e., analyses of performance, qPCR and SCFAs). The Kruskal–Wallis test was used to determine the significance of non-parametric data (i.e., microbiome alpha diversity indices). The Spearman correlation was used to correlate SCFA concentration and bacterial relative abundance. Correlation significance was determined by psych package and visualized using corrplot package in R (version 3.6.1).

2.3. Results

2.3.1 Chicken 32-day body weight and mortality were not affected by the cleaning methods

The 32-day average body weight in the WW group was comparable to the FD group (Figure 2.1a, $p = 0.22$). In addition, no difference in 30-day mortality was observed between the two barn cleaning treatments (Figure 2.1b, $p = 0.91$), suggesting that the cleaning method had a minimal impact on the flock performance.

2.3.2 Chicken barn FD resulted in increased *Campylobacter* occurrence in the 30-day-old chicken ceca

Salmonella was not detected through enrichment in any of the samples collected through the study, therefore, the impact of treatment on *Salmonella* shedding could not be assessed. To evaluate the effect of cleaning method on the pathogen occurrence, an occurrence scoring method was used. In the *Campylobacter* enrichment assay, no *Campylobacter* were detected from the litter samples, feeding pan and drinker swabs, or the shoe-cover samples before broiler placement. At the broiler placement day, the *Campylobacter* occurrence scores were not different between the two treatments (Figure S1). However, at day 30, the WW group exhibited a significantly lower *Campylobacter* occurrence score compared to the FD group ($p < 0.05$) (Figure 2.2). *Campylobacter* was detected in at least one sample from each FD flock. Therefore, the WW rearing environment reduced the occurrence of *Campylobacter* colonization in the 30-day-old broiler ceca.

2.3.3 Chicken barn FD increased *C. jejuni* abundance

The quantification of cecal microbial load using qPCR showed that the WW group did not differ from the FD group in cecal *C. perfringens* load (5.81 and 6.06 $\log_{10}$ copies/g of *netB* for WW and FD group, respectively, $p = 0.20$) (Figure 2.3a). Consistent with the *Campylobacter* enrichment assay results, the WW group exhibited approximately 0.9-$\log_{10}$ lower *hipO* copy numbers compared to the FD group (Figure 2.3b, 8.13 and 9.03 $\log_{10}$ copies/g of *hipO* for the WW and FD group, respectively, $p < 0.05$), indicating that the decreased sanitation stringency reduced *C. jejuni* colonization in the mature chicken ceca. Furthermore, the barn cleaning method did not affect the total bacterial load in the chicken ceca (Figure 2.3c, $p = 0.15$).

2.3.4 Barn cleaning methods had subtle impacts on the 30-day-old chicken microbiome

On average, 42,943.3 ± 2,757.8 (mean ± SEM) reads per cecal sample were generated and processed by QIIME2 pipeline, resulting in a total of 3,845 ASVs. For litter samples, an average of 7,847.8 ± 1,209.2 (mean ± SEM) reads per sample were generated and processed, resulting in 1,780 ASVs. When focusing on the effect of different cleaning methods, the cecal microbiomes of broilers from the WW group and FD group had comparable richness and evenness indicated by alpha diversity indices (chao1, $p = 0.71$; and Shannon, $p = 0.25$) (Figure 2.4). Beta diversity analyses based on both weighted- and unweighted-Unifrac matrices suggested that cecal microbial communities in WW group differed from the FD group (adonis $p = 0.05$, $R^2 = 0.012$ and adonis $p < 0.01$, $R^2 = 0.013$ for weighted-Unifrac and unweighted-Unifrac matrix, respectively) (Figure 2.5). Differences in abundances of two bacterial taxa were also suggested by ANCOM (Figure 2.6). The genus *Helicobacter* was more predominant in the WW group (W = 85), whereas the family *Bacillaceae* was more predominant in the FD group (W = 66). These results suggested that

the barn cleaning treatments influenced the relative abundance of two bacterial taxa, and in turn led to a modest but significant impact on overall structure of the chicken gut microbiota. However, the cleaning method did not lead to changes in microbial structures of the litter samples (Figure S2, Figure S3).

2.3.5 Cecal SCFA profile differed by cleaning methods

Cecal contents were subjected to gas chromatography to measure cecal SCFA concentration. The WW group showed significantly greater total SCFAs than the FD group ($p < 0.01$) (Figure 2.7a). Specifically, acetate (Figure 2.7b), propionate (Figure 2.7c) and butyrate (Figure 2.7d) concentration in the WW group were higher than that in the FD group. A trend for higher valerate concentration (Figure 2.7e, $p=0.06$) was also observed in WW broilers. Spearman correlation between SCFA concentration and bacterial relative abundance suggested a series of microbes that are correlated to the altered SCFA profile between treatments (Figure 2.8). Total SCFAs and acetate concentration were negatively correlated with the genus *Campylobacter,* and members from orders RF32 and YS2 ($p < 0.05$). On the other hand, an unclassified genus belonging to the order of *Clostridiales* was positively associated to total SCFAs, acetate and butyrate concentrations ($p < 0.05$). Propionate concentration was negatively associated to the genus *Lachnospira* ($p < 0.01$) and an unclassified genus of the family *Enterobacteriaceae* ($p < 0.05$), whereas positively associated to the genus *Odoribacter* ($p < 0.05$), and an undetermined genus of the family *Clostridiaceae* ($p < 0.05$).

2.4 Discussion

This study was the first to characterize the impact of FD and WW on chicken gut microbiota in a commercial setting. Previously, de Castro Burbarelli et al. examined the effects of poultry barn cleaning using neutral detergent versus a protocol using acidic and alkaline detergent with chemical disinfectants (2). In accordance with their results, we found that compared to FD, the WW did not have compromised flock mean body weight or increased mortality rate at day 32 in production. Unfortunately, we were not able to collect feed consumption data and are therefore not able to comment on any potential impacts on feed conversion.

Although limited research has explored how barn cleaning practices affect the development of chicken intestinal microbial communities, especially in the context of commercial production, efforts have been made to study the effect of reused litter on chicken gut microbiota. Some laboratory-scale research suggested that reused litter mainly influences broiler gut microbiota at early ages. Cressman et al. reported that compared to the reused litter group, broilers provided fresh litter had greater bacterial alpha diversity in ceca at 7 days of age (16). However, no treatment effects on the gut microbiota were observed in the later timepoints. In addition, broilers reared on fresh litter were colonized by microbes identified in fresh litter including *Lactobacillus*, unclassified *Lachnospiraceae* and *Enterococcus*, whereas broilers reared on reused litter were colonized by typical poultry intestinal bacteria, such as members from the order *Clostridiales* (16). Similarly, Wang et al. reported that at both day 10 and day 35 of age, broiler gut microbiota was altered by the reused litter treatment with increased predominance of *Faecalibacterium prausnitzii* in ceca (17). In the current study, no treatment effect on alpha diversity of the 30-day-old broiler gut microbiota was observed, and only a modest effect was shown on the beta diversity between FD and WW treatments. This may be explained by the fact that fresh litter, which acts as a physical

barrier, was placed in both WW and FD barns. In the current study, we did not observe differences of the litter microbiota between treatments. In addition, reused litter may provide a more functional ecological niche compared clean litter. Generally, reused chicken litter is a mixture of bedding material and excreta, which offers more surfaces and available nutrients for microbes to attach and survive on. In addition, as chickens are coprophagic, the consumption of litter material likely increases the opportunity for successful microbial transmission from one flock to the next (30). In the current study, only 5 broilers per flock were selected for 16S rRNA gene amplicon sequencing and *Campylobacter* and *Salmonella* enrichment. While a larger sample size would strengthen the conclusions, previous microbiome studies report that individuals housed together, particularly coprophagic animals, show less variation in the intestinal microbiota with strong co-housing effects (31, 32). A clear flock effect was also observed in the current study (data not shown). Rather than sampling more broilers from each barn, we chose to sample more barns. In addition, the cross-over design of the animal trial can also help eliminate bias brought by management and/or housing facilities.

Helicobacter was found to be less abundant in the FD group at day 30 compared to the WW group. Interestingly, *Helicobacter* is a genus identified as a disappearing member of the human gut microbiome, and may also be associated with increased use of disinfectants (33). In avian species, members in genus *Helicobacter* have been detected in wild birds (34). Studies on the relationship between *Helicobacter* spp. and chicken host are highly variable. Some members of genus *Helicobacter* are considered opportunistic pathogens in chickens. For example, *Helicobacter pullorum* infection was found to cause mild lesion in the chicken ceca (35). However, the effects of *Helicobacter* on host health can vary between different bacterial strains within species (36). Yin et al. reported that *Helicobacter* abundance increased in response to α-amylase,

amylopectase and glucoamylase supplementation in a corn-based diet, and was associated with increased starch digestibility and higher mature bodyweight (37). In the current study, *Helicobacter* positively correlated to branched-chain fatty acids (**BCFA**s) isobutyrate and isovalerate (Figure 2.7). BCFAs are often used as indicators of protein catabolism (38). Currently, the direct relationship between BCFAs and their impact on host health is still unclear (39). It is reported that BCFAs modulate adipocyte lipid and glucose metabolism, and contribute to increased insulin sensitivity (40). With 16S rRNA gene amplicon sequencing, it is difficult to discriminate bacteria to the species or strain level. Therefore, our identification of *Helicobacter* as the genus of increased predominance in the WW group needs to be further studied. In addition, information on metabolic functionality is also warranted to understand the role of *Helicobacter* in the chicken gut.

Interestingly the FD broilers showed increases in the relative abundance of the family *Bacillaceae*. Members of the *Bacillaceae* family, such as *Bacillus* are Gram-positive, rod-shaped bacteria that can form endospores to survive in harsh physical and chemical environments (41). It is possible that the chemical conditions given by FD treatment provided a selective pressure that led to the increased level of *Bacillaceae*. Some members in this order have the ability to produce antimicrobial peptides, and are recognized as potential beneficial bacteria (42). Some *Bacillus* species, such as *Bacillus licheniformis* and *Bacillus subtilis*, have been commercially added into poultry feed as probiotics (43). However, not all *Bacillus* species are beneficial. *Bacillus cereus* is a food-borne pathogen that causes diarrhea (44). Recently, the prevalence of the *B. cereus* group made up 50% of *Bacillus* spp. isolates from retail chicken products (45). In addition, some non-*B. cereus* species were found to carry virulence genes and exhibited the same phenotypic virulence characteristics as *B. cereus* (46). Furthermore, tests of antimicrobial resistance have identified multi-drug resistant isolates regardless of virulence factors, indicating that further evaluation of

the impact of *Bacillus* on food safety and public health is needed (45). In this sense, the effect of barn disinfection on increasing *Bacillaceae* may need to be carefully assessed.

The reduced *Campylobacter* load in the current study with WW treatment is consistent with previous studies showing that reused litter has the potential to reduce gut pathogen abundance in broiler chickens. It has been reported that reused litter reduced the colonization of *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis in infected chickens (13, 47). In the current study, although *Salmonella* was not detected in any of samples by the enrichment assay, our results supported that the WW treatment did not increase *Salmonella* occurrence in the chicken intestine. Changing from FD to WW for within-year washing would be associated with lower labor and material costs for cleaning. To date, limited data is available regarding the effects of cleaning treatments and disinfectants on *Campylobacter* occurrence and abundance in the chicken intestine. De Castro Burbarelli et al. reported a trend that *Campylobacter* was more frequently detected in the intestine of the stronger disinfection group (2), although the study was conducted in a controlled research setting. Furthermore, it has been shown that colonization of young chicks with bacterial cocktails of mature chicken commensal isolates reduced colonization by *C. jejuni* (48). Therefore, the FD treatment in the current study may have eliminated some microbes from the previous flock which can potentially compete with *Campylobacter*.

While the effects of cleaning method on the gut microbial composition were relatively modest, changes in concentrations of microbial metabolites, SCFAs, were observed. SCFAs enhance intestinal integrity as direct energy sources to enterocytes (49). Moreover, complex interactions between SCFAs, gut microbes, and the host immune system have been well documented (50). Briefly, SCFAs from intestinal microbe fermentation are imported into

enterocytes and tissues via transporters and paracellular transport. SCFA receptors expressed on enterocytes and immune cells in the lamina propria and mucosal lymphoid tissue can activate signaling pathways to regulate host immune response according to the SCFA concentration to maintain intestinal homeostasis (50). Butyrate signaling through G-protein coupled receptors can confer anti-inflammatory properties in colonic dendritic cells by down-regulating the expression of cytokines and chemokines (19). In the present study, there were negative correlations between the relative abundance of *Campylobacter* and total SCFAs, acetate, and butyrate concentrations in the ceca. It has been suggested that SCFAs, especially butyrate, had shown bactericidal effect on *Campylobacter in vitro* (51). More recently, Awad et al. reported that *C. jejuni* infection led to reduced acetate and butyrate concentration in the chicken ceca (52). Adding microencapsulated butyrate to feed was also found to reduce *Campylobacter* colonization in the chicken intestine (53). Together with our results, it is reasonable to suggest that the FD treatment discriminated against beneficial commensals in the gut environment, which could compete with or inhibit *Campylobacter* by producing SCFAs.

2.5 Conclusion

Compared to FD, we found that WW can be beneficial to broiler chicken production by inhibiting *Campylobacter jejuni* colonization in the chicken gut with reduced cleaning costs. Further studies examining other barn disinfection practices and testing for other pathogens are warranted to identify the best practices to minimize pathogen load and maintain animal performance.

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Table 0.1 Production cycles, barns and cleaning treatment schedule.

	Barn A	Barn B	Barn C	Barn D	Barn E	Barn F	Barn G
Cycle 1	FD	FD	FD	FD	FD	WW	WW
Cycle 2	FD	FD	WW	WW	FD	WW	WW
Cycle 3	WW	WW	WW	WW	WW	FD	FD
Cycle 4	WW	WW	FD	FD	WW	FD	FD

WW, water-wash; FD, full disinfection

Table 0.2 Primers used for qPCR assay of broiler chicken cecal samples collected at 30 days of age.

Target gene	Primer orientation	Primer Sequence	Reference
Total bacteria 16S rRNA gene	Forward	5′-CGGYCCAGACTCCTACGGG-3′	(54)
	Reverse	5′-TTACCGCGGCTGCTGGCAC-3′	
HipO (124bp)	Forward	5′-TCCAAAATCCTCACTTGCCATT-3′	(55)
	Reverse	5′-TGCACCAGTGACTATGAATAACGA -3′	
NetB (196bp)	Forward	5′-TGATACCGCTTCACATAAAGGTTGG-3′	(56)
	Reverse	5′-ATAAGTTTCAGGCCATTTCATTTTTCCG-3′	

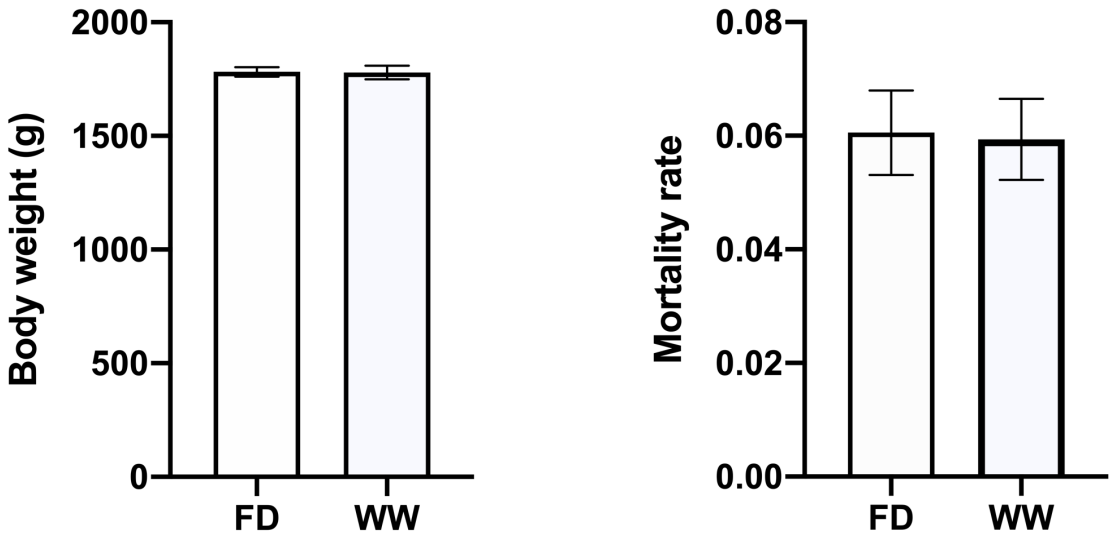

Figure 0.1 Broiler chicken flock performance at 32 d of age.

(a) Flock mean body weight at day 30, 1782 ± 30.09 and 1780 ± 20.59 g for FD and WW, respectively (n=12 flocks/treatment, mean ± SEM; FD, full disinfection; WW, water-wash); (b) Flock mean mortality rate at day 30, 0.061 ± 0.0074 and 0.059 ± 0.0071 for FD and WW, respectively (n=14 flocks/treatment, mean ± SEM; FD, full disinfection; WW, water-wash).

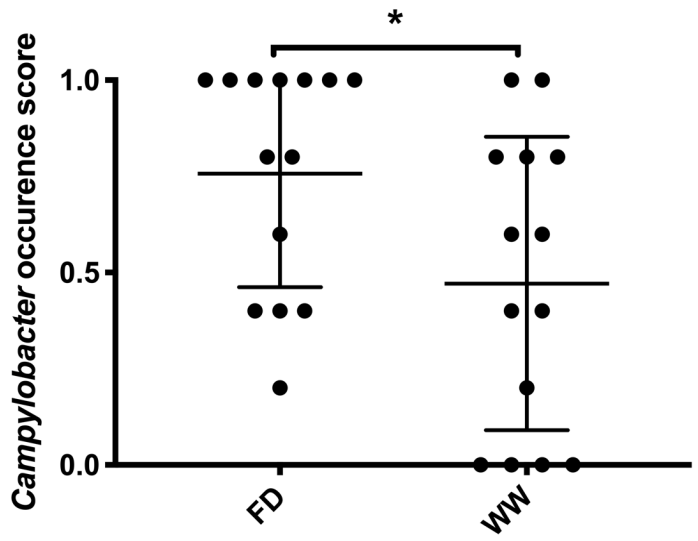

Figure 0.2 Broiler chicken cecal Campylobacter occurrence score at 30 d of age.

Results showed the mean flock score ± SEM (n = 14/treatment, *, $p < 0.05$). Campylobacter occurrence score = number of pathogen positive broilers/total number of broilers sampled per barn. WW, water-wash; FD, full disinfection.

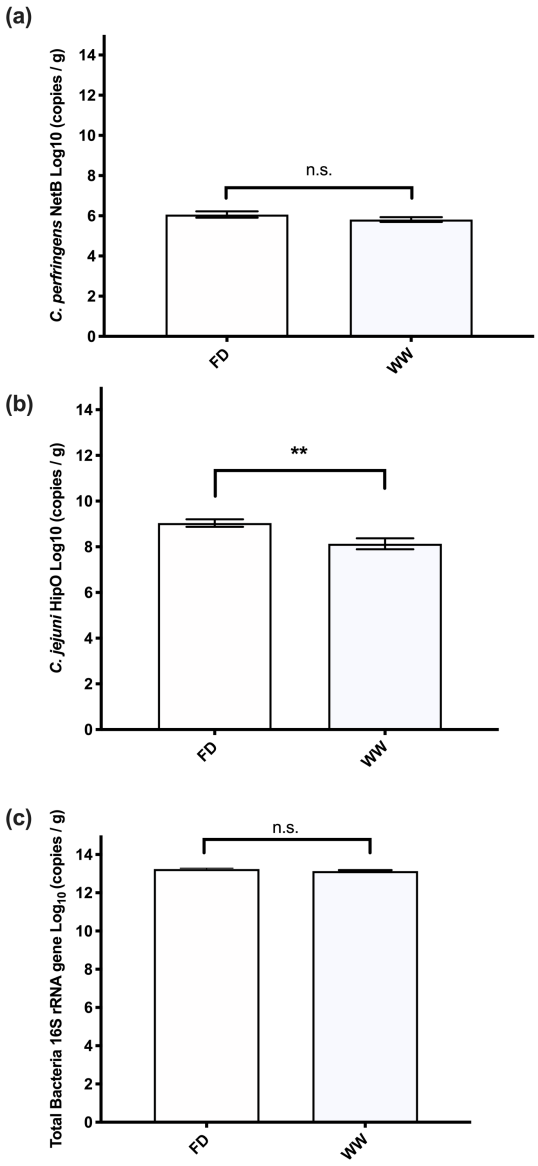

Figure 0.3 Cecal bacteria qPCR quantification.

Broiler chicken (30 d of age) cecal qPCR targeting C. perfringens netB gene (a), C. jejuni hipO gene (b), and bacterial 16S rRNA gene (c). Results are the average copy number of each target gene (mean ± SEM, n=70/treatment, **, $p < 0.01$, n.s., $p > 0.05$). WW, water-wash; FD, full disinfection.

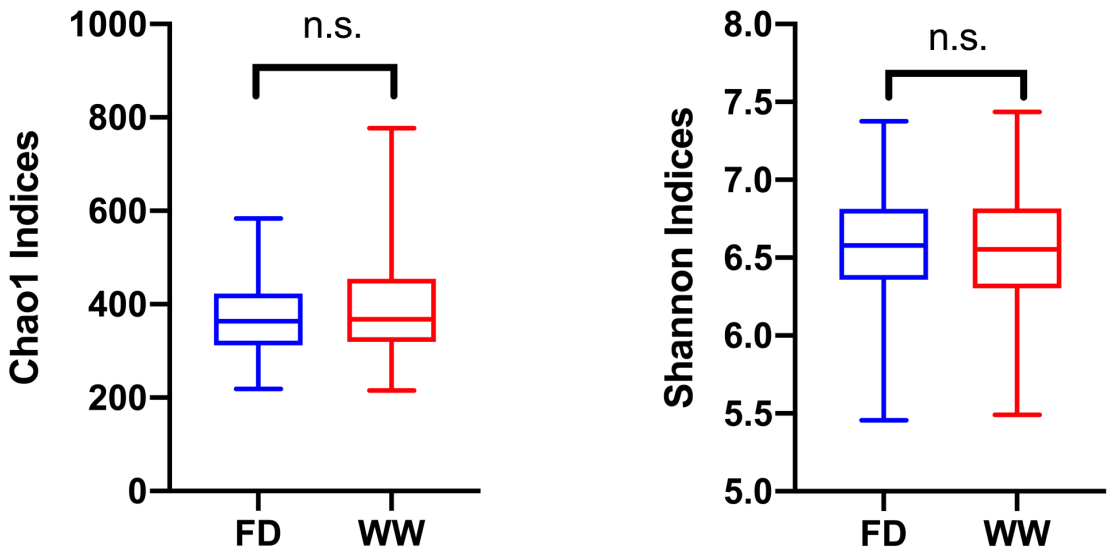

Figure 0.4 Alpha diversity of broiler chicken cecal microbiome at 30 d of age.

Box-plots showing alpha diversity in samples using Chao1 index and Shannon index (n=70/treatment, n.s., $p > 0.05$). WW, water-wash; FD, full disinfection.

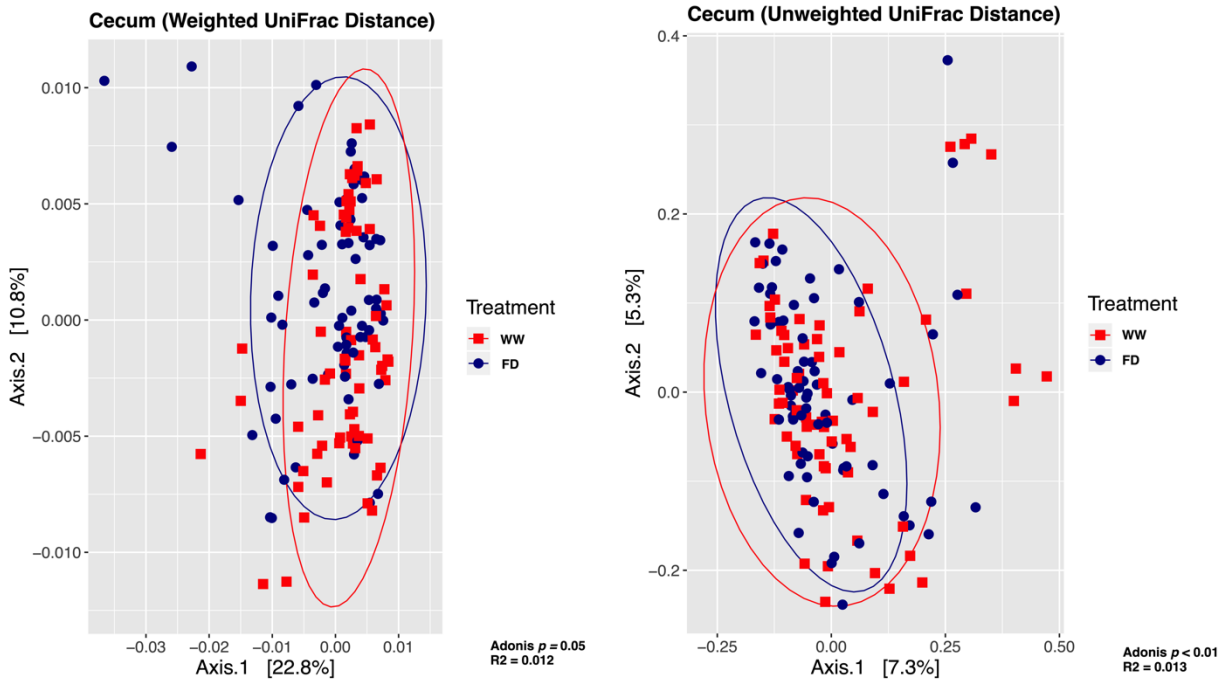

Figure 0.5 Principal coordinate analysis plots based on weighted- and unweighted- Unifrac distance matrices.

Barn cleaning treatments had modest but significant effects on microbial community structure in the chicken ceca at 30 d of age (n=70/treatment). WW, water-wash; FD, full disinfection.

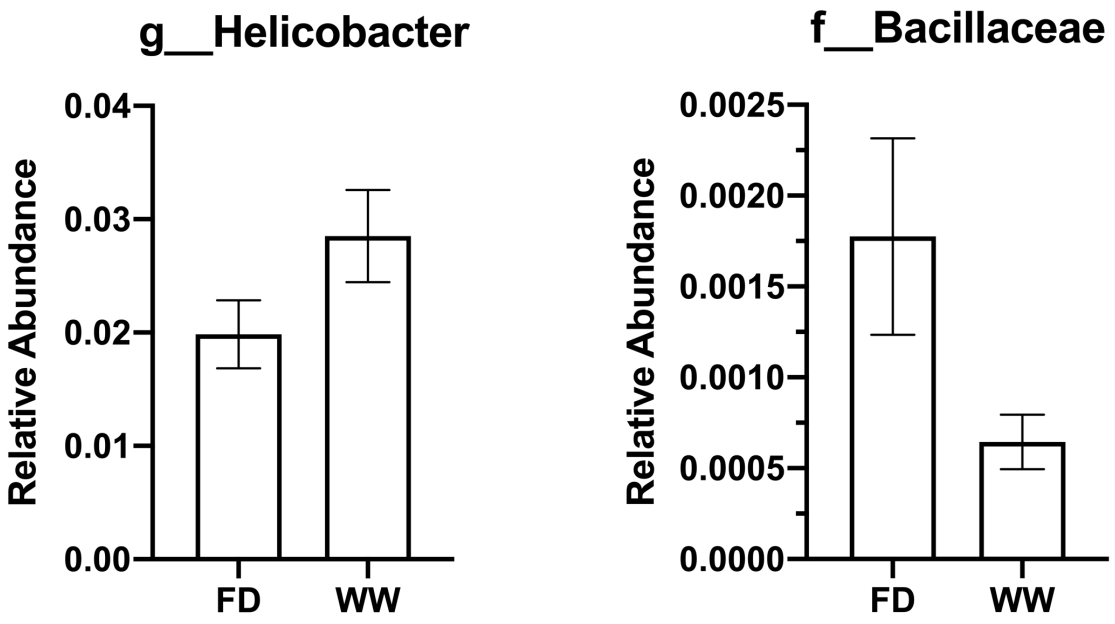

Figure 0.6 Relative abundance of genus Helicobacter and family Bacillaceae.

The bar plot shows the relative abundance of taxa of interests of each treatment with individual values (n=70/treatment, mean ± SEM). G__, genus; f__, family

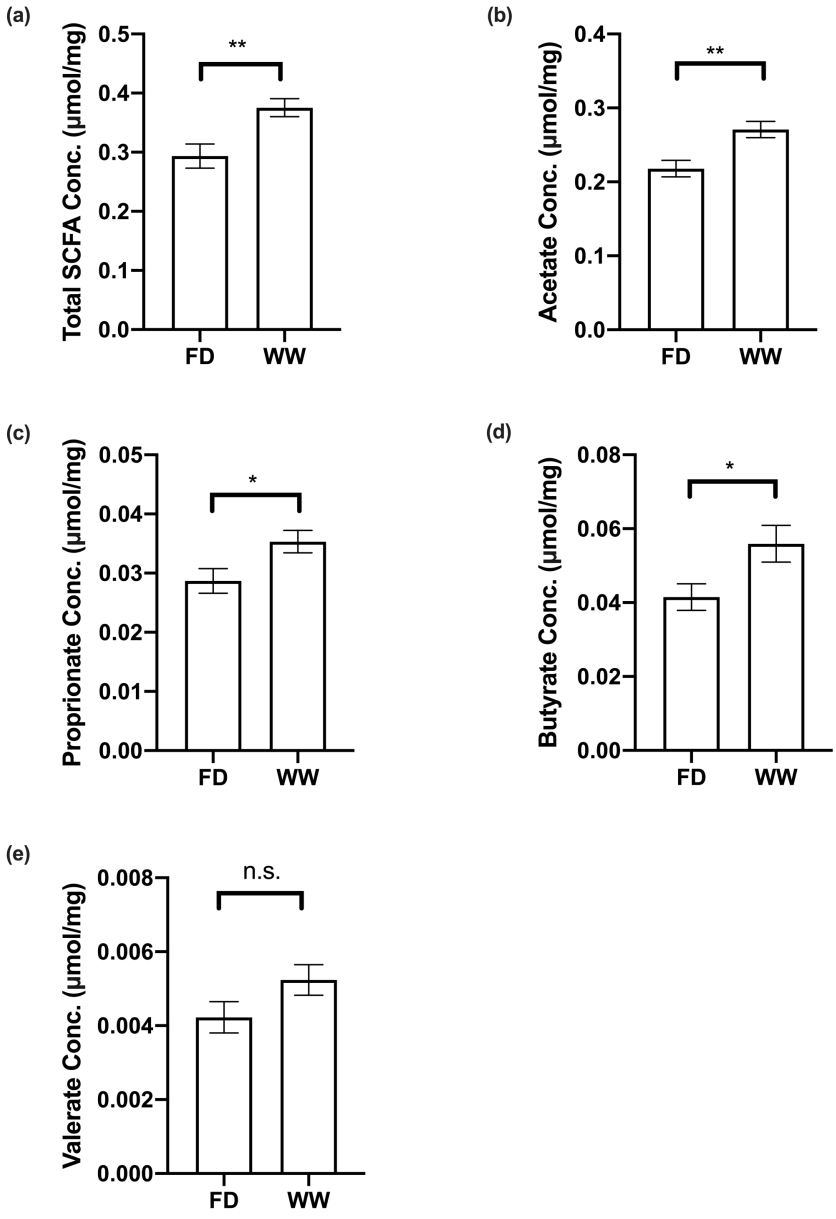

Figure 0.7 Cecal short chain fatty acid production in broiler chickens at 30 d of age.

Results were shown as the average of (a) total SCFA concentration, (b) acetate, (c) propionate, (d) butyrate, and (e) valerate (mean ± SEM, n = 20/treatment, *, p <0.05, **, p < 0.01, n.s., p > 0.05). FD, full disinfection; WW, water-wash; Conc., Concentration.

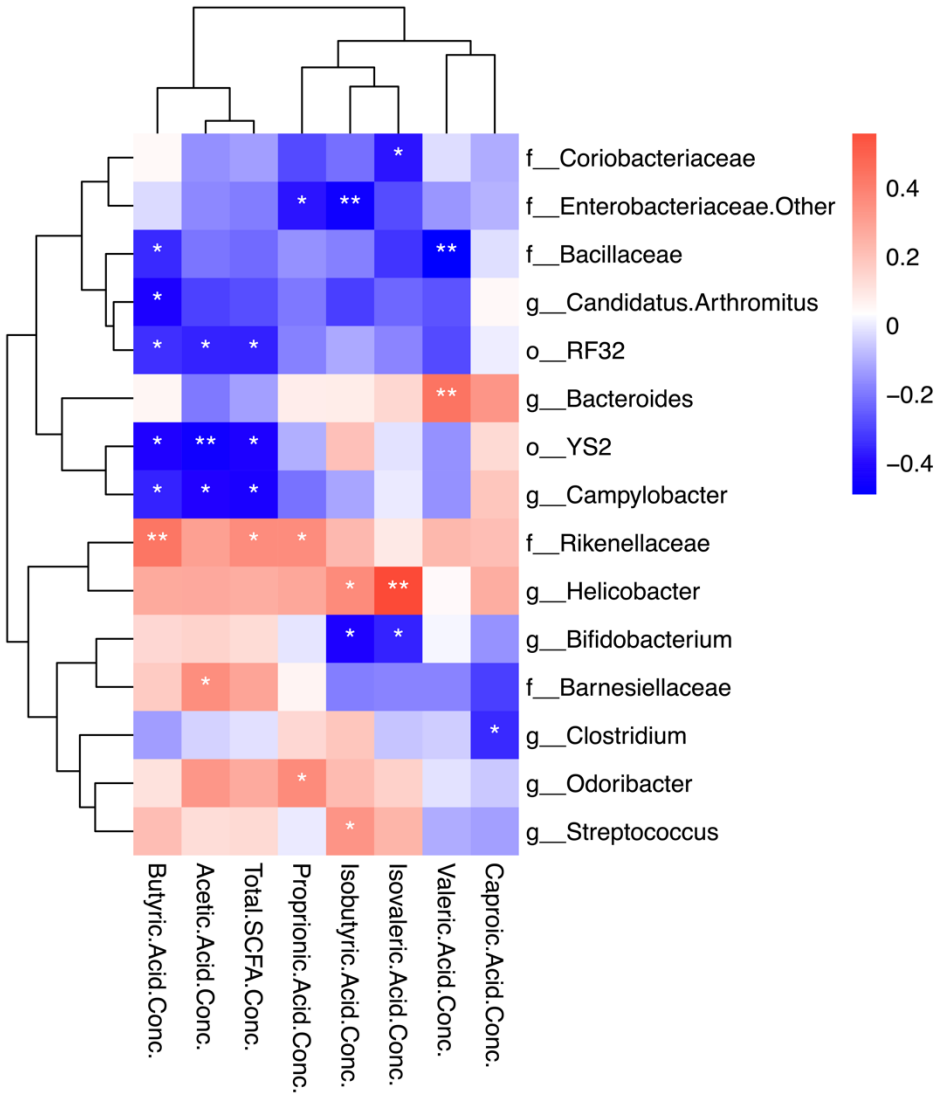

Figure 0.8 Spearman correlation heatmap

Heatmap showing Spearman correlations between cecal bacterial abundance and short chain fatty acid concentrations in broiler chickens at 30 d of age. *, $p < 0.05$; **, $p < 0.01$; Conc., Concentration.

CHAPTER 3. THE IMPACT OF BARN DISINFECTION AND AGE ON THE CECAL MICROBIAL FUNCTIONAL CAPACITY AND RESISTOME OF BROILER CHICKENS

3.1 Introduction

Barn disinfection between flocks has been used as a strategy to prevent the transmission of disease from one flock to the next in broiler chicken production. In practice, both barn full sanitation with chemical disinfectants (FD) and water-wash (WW) are widely employed in poultry production in many parts of the world. We have recently shown that the use of disinfectants in cleaning barns between broiler chicken flocks can result in increased carriage of *Campylobacter jejunii,* impacts the composition of the cecal microbiota, and results in reduced cecal SCFA levels (1). Changes in community composition were determined by 16S rRNA gene amplicon sequencing, therefore, provided limited ability to understand functional changes the microbiome induced by barn disinfection. It has been reported that chemical disinfectants can trigger the proliferation of antibiotic resistant genes (ARGs) (2), which may pose a threat to public health. Therefore, examining the effects of barn cleaning practices on the chicken gut microbial metabolic functionality and the ARG profile (also termed as resistome) can offer insightful information to both the poultry industry and the general public.

Livestock farming accounts for over 50% of all antibiotic usage globally (3). Due to the high stocking density during production as well as the short production cycle, chickens were reported to have the highest densities of ARGs compared to other livestock species (4). Presently, there is no data available evaluating how barn cleaning practices affect the intestinal ARG profiles of the broiler chickens. As well, no consensus had been reached regarding the

effect of chemical disinfectants on ARG abundance. Previous studies looked at the chemical disinfectant-treated facility/device surfaces, soil, or livestock manure, and reported very different results. In some studies, chemical disinfectants were reported to stimulate microbial ARG proliferation in the environment, because some disinfectants share similar modes of actions with antibiotics (5-7). For example, benzalkonium chloride (BAC), a widely used quaternary ammonium compound, was shown to select bacterial taxa in the waste-water bioreactors that were multi-drug resistant by stimulating the expression of ARGs encoding multi-drug efflux pumps (7). Zeng et al. studied agricultural soils amended with BAC and reported that exposure to BAC led to increased incidence of ARGs in the agricultural soil (8). However, after assessing the effect of a series of chemical disinfectants on inhibiting ARG abundances in swine manure storage, Hall et al. reported that disinfectants such as BAC and sodium hypochlorite significantly reduced the abundance of some ARGs (e.g. *ermB, ermC,* and *tetX*) in the swine manure (8). Consequently, from the perspective of food safety and environmental sustainability, it is important to explore the influence of chemical disinfectants usage in barn cleaning, particularly the gut microbial resistome of the broiler chickens raised in these barns.

This study was designed to explore the functional capacities of the chicken cecal microbiota derived from FD and WW. Shotgun metagenomic sequencing was used to characterize the response of the cecal microbiome, microbial metabolic functionalities and ARG profiles to different barn cleaning practices. We aimed to gain further insight into changes in microbial functionality that led to higher SCFA levels. Furthermore, we predicted that the use of chemical disinfectant in barn cleaning would increase ARG carriage in the cecal microbiome.

3.2 Materials and methods

3.2.1 Broiler production facilities and barn cleaning practices

As a follow-up study of the previous chapter, a subset of broiler chicken cecal samples collected previously were used. Broiler chicken management and barn cleaning practices were previously described (1). Briefly, broiler chicken samples were collected from a commercial broiler company in Alberta, Canada. To focus on the effects of cleaning methods without introducing other confounding factors and bias, production cycles 2 and 4 were chosen (Table. 2.1 from chapter 2), in which most barns had been through 2 consecutive repeated cleaning practices with both barn cleaning treatment applied on each barn. The cross-over design resulted in a total of 14 production flocks (sampled at day 7 and day 30), which 7 flocks were assigned to each treatment. In detail, the sample set of this study included a total of 140 chickens (70 chickens from each age), and 35 chickens were sampled from each treatment (FD or WW) at each age (day 7 or day 30).

For FD, manure and litter were completely removed from the barn after chickens were sent for slaughter. Subsequently, chemical disinfection were performed using foam containing 7% sodium hydroxide, 7% 2-(2-2-butoxyethoxy) ethanol, 6% sodium laureth sulfate, 5% sodium N-lauroyl sarcosinate, and 5% tetrasodium ethylenediaminetetraacetic acid (EDTA) on all surfaces of the facilities, followed by high-pressure and low-pressure water rinse with the water temperature set at 35℃; after the facilities were air-dried, foam containing 10% glutaraldehyde, 10% benzalkonium chloride (BAC), and 5% formic acid were applied to the surfaces of the facilities for 60 mins followed by high-pressure water rinse, overnight air-dry and fresh litter placement.

For WW, manure and used litter were removed, followed by low-pressure water rinse with the water temperature set at 35℃ for all facility surfaces, air-dry overnight, and fresh litter placement.

3.2.2 Chicken management and sample collection

The current study was performed according to the guidelines of the Canadian Council on Animal Care (9) with approval of the University of Alberta Animal Care and Use Committee (AUP00002377). In each production flock, Ross 308 broiler chicks were placed within 12 h post-hatch and confined to half of the house, then allowed access to the entire house starting at D7. Flock size was 14,000, with a final stocking density of 30 kg/m^2. All chickens were fed *ad libitum* without antibiotics and from placement through processing at about 32-35 days of age when the average target live weight of 1.8 kg was reached.

To collect cecal digesta, at D7 and D30, five broilers per flock were each randomly selected from different areas within each barn and euthanized using cervical dislocation. Tools and collection tubes were pre-autoclaved and kept sealed before sampling. To collect cecal contents aseptically, sampling table was cleaned with 70% ethanol before and between every broiler dissection. Approximately 300 mg of cecal contents were collected into sterile 2 ml Eppendorf tubes, and were frozen on dry ice immediately after collection until transported to the lab, and stored at -80 ℃ for subsequent DNA extraction.

3.2.3 Shotgun metagenomic sequencing and microbiome analyses

Total DNA extraction was as previously described (1). Library preparation and shotgun sequencing were performed at the Genome Quebec Innovation Centre (Montreal, Canada) using the NovaSeq 6000 S4 PE150 system (Illumina Inc., San Diego, CA, USA). FastP v0.23.2. was used for quality control. Low quality reads, sliding windows, adaptors, polyG and duplicated sequences were removed (10). Kneaddata v0.10.0 was used to remove host DNA contaminants (https://github.com/biobakery/kneaddata). Briefly, a chicken host reference database was built using bowtie2 v2.4.1 with genome *Gallus_gallus* 105 release from Ensembl (11), followed by subsequent removal of reads aligned to the host. Cecal microbiota taxonomic classification was performed profiled using kraken2 (v2.1.2) (12), followed by subsequent relative abundance estimation using Bracken2 (v2.6) (13) Bacterial taxa appeared less than 5% of the samples were filtered out for subsequent analyses. Assembly was performed via megahit (v1.2.9) with default parameters (14). The abundance of functional genes and enriched pathways were estimated using HuMAnN3 (v3.0.1) based on the UniProt 90 database followed by subsequent annotation using the Metacyc database (15, 16). The relative abundance of aligned genes and pathways were normalized to copy numbers per million reads using the HuMAnN3 utility scripts (16). Antibiotic resistance-encoding genes were annotated by the Comprehensive Antibiotic Resistance Database (CARD, version 3.1.4) via Resistance Gene Identifier (RGI, version 5.1.0) with cut-off set at 95% identity (17). To reveal the distributions of microbial taxa, functional genes, and ARGs across samples, principal coordinate analysis (PCoA) based on Bray-curtis distance metric was calculated via vegan package in R (version 3.6.1). To evaluate dispersion, the R package “betadisper” was used to calculate distance to centroid. Two-way ANOVA was used to assess total ARG reads between different barn sanitations practices and different

sampling timepoints. Because no differences were suggested between different barn sanitation practices or sampling timepoints (details shown in 3.3.2), to avoid bias introduced by variations of the relative abundance of bacterial taxa, variations of 16S rRNA copy numbers harbored by different bacterial species as well as fluctuation caused by ARGs located on mobile genetic elements, identified ARGs were normalized to reads per million total ARG reads for subsequent comparisons. Permutational Multivariate Analysis of Variance (PERMANOVA) tests were used to determine clustering significance using the adonis function in the vegan package in R (version 3.6.1). Significance of PERMANOVA and betadisperse were set to FDR $P < 0.05$. Differentially abundant microbial taxa, gene pathways and ARGs associating with treatments or sampling timepoint were identified using LDA effect size (LEfSe) implemented in the lefser R package (Bioconductor version 3.15) with significant cut off set at LDA score > 2 and FDR $P < 0.05$. Differentiate abundant analysis of profiled gene pathways and ARGs for pairwise comparisons was performed using R package DESeq2 (Bioconductor version 3.15) (18). Significance of differential abundance was determined with FDR $P < 0.05$ and log2 fold change > 1.

3.2.4 Statistical Analyses

Except for the statistical analyses mentioned above, GraphPad Prism 8 (Graphpad Software, San Diego, CA, USA) was used to conduct statistical analyses. To determine significance of the microbiome alpha diversity indices, the Kruskal–Wallis test was used with significance set at $P < 0.05$. The Spearman correlation was used to correlate ARG abundance and the abundance of bacterial species. Correlation significance was determined by the corr.test function (false-discover-rate adjusted $P > 0.05$), and a moderate association was determined by $|R| > 0.4$. Correlation was visualized using the corrplot package in R (version 3.6.1).

3.3 Results and discussion

3.3.1 The cecal microbial structures and functional capacities were impacted by barn cleaning methods and sampling timepoints

Shotgun metagenomic sequencing was used to provide a more comprehensive profile the cecal microbial structures to further assess the effects of barn cleaning methods on the cecal microbiota. Overall, an average of 52,133,725.06 ± 1,265,988.36 quality-controlled reads per sample (mean ± SEM) were processed by kraken2 for cecal microbial structure profiling, resulting in 34,252,401.09 ± 6,541,062.17 reads per sample aligned to the RefSeq bacteria database by Kraken2. The overall day 7 and 30 cecal microbiota in this study is consisted of 43.36% Firmicutes, 39.42% Bacteroidetes, 11.90% Proteobacteria, 3.34% Actinobacteria, and 1.98% of other phyla with relative abundance lower than 0.1% (D7: 62.14% Firmicutes, 25.57% Bacteroidetes, 8.21% Proteobacteria, 0.76% Actinobacteria, 0.48% Tenericutes, and 2.84% of other phyla; D30: 24.59% Firmicutes, 53.27% Bacteroidetes, 15.59% Proteobacteria, 5.92% Actinobacteria and 0.63% other phyla). Generally, Bray-Curtis distance metric revealed that sampling timepoints had a major impact on the beta diversity of the chicken cecal microbiota (Figure 3.1a, $R^2 = 0.15$, adonis $P < 0.001$); whereas the cleaning methods had limited impact ($R^2 = 0.02$, adonis $P = 0.23$) on the D7 microbiota and a modest impact ($R^2 = 0.03$, adonis $P = 0.005$) on D30 beta diversity (Figure 3.1b and 3.1c for D7 and D30, respectively). Betadisper analysis showed that the cecal microbiotas of D7 chickens had greater distance to centroid compared to D30 (distance to centroid = 0.56 and 0.48 for D7 and D30, respectively, FDR $P < 0.01$), indicating that the 30-day cecal microbiota had greater homogeneity. In addition, microbial alpha diversity was impacted by the sampling timepoints, but not cleaning methods. Species richness and evenness, indicated by Shannon diversity index, increased with age ($P < 0.05$) (Figure 3.1 d-

f). Shotgun metagenomics offered better resolution of changes at the species level than previously reported by 16S rRNA gene sequencing (1). At D7, LEfSe analysis did not indicate any taxa associating with treatment (FDR $P > 0.05$, Figure 3.2). However, at D30, the relative abundance of *Ruminococcus torques, Faecalibacterium prausnizii, Barnesiella viscericola,* and *Helicobacter pullorum* were enriched in the WW group, whereas the relative abundance of *Megamonas funiformis* was higher in the FD group (FDR $P < 0.05$, LDA >2, Figure 3.2).

To date, very limited information is available regarding how cleaning methods affected chicken cecal microbiota. Using 16S rRNA sequencing technique, we previously reported that at D30, genus *Helicobacter* was enriched in the WW group (1). Shotgun metagenomic sequencing was able to provide taxonomic information to the species level with confidence. In accordance with the last chapter, shotgun metagenomics results revealed that the cecal composition of *H. pullorum* was increased by the WW treatment at D30. Although some researchers suggested that *H. pullorum* may be an opportunistic pathogen (19), the role of *H. pullorum* in poultry and its pathogenicity to human and chickens remains unclear. In fact, to date, no concrete evidence has shown that it is related to either human or poultry diseases (20, 21). Previously, it was reported that the prevalence of *Faecalibacterium* increased in the chicken ceca of the recycled litter group compared to the fresh litter group, which was concluded as a beneficial effect using recycled litter (22). As a commensal member also found enriched in the conventionally grown chickens (23), *F. prausnizii* was previously identified as a promoter of epithelial health for its strong metabolite production, such as butyrate (24). Moreover, it was found to have anti-inflammatory activity in broilers (25). In line with previous study (22), we found that the predominance of *F. prausnizii* was found increased by WW, indicating that WW may confer potential beneficial effects to the chickens. *B. viscericola* has been characterized as an important representative

commensal of barn-raised chickens (26), that could be considered as a potential propiogenic probiotics (27-29). Whereas *Ruminococcus* are important butyrate producers in the gut that degrades mucins (24, 30). Mucolytic bacteria were widely found in the gut microbiota (31). Some studies had reported that mucolytic bacteria, such as *Clostridium perfringens* could be pathogenic as degradation of the mucous layer might be detrimental by impairing gut barrier function (32, 33). However, mucolytic bacteria (e.g. *Akkermansia* spp.) may provide beneficial metabolites (e.g. SCFAs) to stimulate goblet cells to secrete more mucus, and thus contribute to the development of a healthy mucus-associated microbiota (33, 34). In this case, the effect of increased *R. torques* in the chicken gut needs to be further studied.

Amongst the D30 FD chickens, *M. funiformis* was recognized as a strong biomarker of the cecal microbiota. Previously, genus *Megamonas* was identified as an important producer of acetate and propionate in avian species (35), and *M. funiformis* was characterized as one of the most efficient colonizers in early life of chickens (36). Clavijo et al. and Roth et al. assessed the chicken microbiota and reported that *Megamonas* accounted to the core microbiota (Clavijo et al. defined taxa presenting in more than 50% of samples and Roth et al. set the coverage to 97% of samples) (37, 38). Interestingly, Clavijo et al. found that *Megamonas* mainly appeared in the broiler ceca (37), whereas Roth et al. studied the microbiome of laying hens and reported that *Megamonas* colonizes the upper intestinal tract, but could not be detected in the ceca, where other SCFA producers normally inhabit (38). Although reasons behind the differences reported needs to be further explored (e.g. host determinant and diet compositions), it indicated that *Megamonas* can adapt to multiple sections of the chicken gut and may colonize niches with less competition.

To investigate the impact of barn sanitation practices on the cecal microbial functional consequences, gene families were profiled using HUMAnN3 and annotated according to the Enzyme Commission (EC) number annotations. An average of 64.63% ± 1.18% of the total reads per sample (mean ± SEM) were mapped to the UniProt 90 database by HUMAnN3, and were further annotated based on the MetaCyc database. The cecal microbial communities of broiler chickens harbored 449 metabolic pathways. Chicken age significantly impacted the cecal microbial functional capacities. DESeq2 revealed that the abundance of a total of 89 pathways that were different between D7 and D30 (Table S1, log2 fold-change > 1, FDR $P < 0.05$). However, surprisingly, LefSe did not identify associations between functional pathways and age (FDR $P > 0.05$ or LDA < 2).

The effects of the barn cleaning methods on the cecal microbial functional capacities were relatively modest compared to the age-effect. At D7, DESeq2 suggested that the abundance of 6 pathways were altered by the barn cleaning treatments (Figure 3.3a, log2 fold-change > 1, FDR $P < 0.05$), namely the sucrose degradation pathway IV (PWY-5384), the L-cysteine biosynthesis pathway VI (PWY-I9), the super-pathway of UDP-glucose-derived O-antigen building blocks biosynthesis (PWY-7328), the UDP-N-acetyl-D-glucosamine biosynthesis pathway (UDPNAGSYN-PWY), the phospholipase pathway (LIPASYN-PWY), and the stringent response guanosine 3'-diphosphate 5'-diphosphate metabolism pathway (PPGPPMET-PWY).

PWY-5384 is linked to polysaccharide utilization (sucrose degradation), and it was enriched in the D7 WW microbiome (FDR $P < 0.05$, log2 fold change >1). When assigned to the different bacteria, PWY-5384 was harbored by *Escherichia coli, Lactobacillus* spp., *Bifidobacterium* spp., and unclassified bacteria. In the cecal microbiome of the D7 FD group, it

was mainly harbored by *E. coli* and unclassified bacteria, whereas in the D7 WW group, it was contributed by *E. coli, Lactobacillus* spp., *Bifidobacterium pullorum* and the unclassified bacteria together (data not shown). This resulted in the overall increased abundance of PWY-5384 observed in the WW group. A similar effect was observed in the amino acid synthesis pathway (PWY-I9), which in the FD group microbiota was mainly harbored by *E. coli,* but possessed together by *E. coli, Bifidobacterium* spp., and *Lactobacillus* spp. in the WW counterparts.

The stringent response pathway was enriched in the cecal microbiome of the D7 FD group (FDR $P < 0.05$), and it was primarily harbored by *E. coli.* Interestingly, the stringent response regulates gene expression in response to nutrient starvation or environmental stresses (39), the enriched stringent response pathway in the FD group may be a sign of that the FD treatment had put stress to the barn environment and therefore impacted the assembly and development of the cecal microbiota of young chickens. Previously, it was reported that stringent response was important for bacterial virulence and persistence in the environment (e.g. resistance to antimicrobials) for a variety of taxa (39). Intestinal microbes such as *Bacteroides* shift from growth to stasis via the regulation of the stringent response (40). In addition, it was also reported that the stringent response can induce microbes to the viable but nonculturable state, which have strong tolerance to environmental stresses with minimum nutrient requirement (41). Thus, the enriched of the PPGPPMET-PWY may indicate that the chemical disinfection may select for tolerance to harsh environmental conditions.

At day 30, DESeq2 suggested the abundance of a series of pathways that were altered by cleaning method (Figure 3.3b). Specifically, the WW group had increased the relative abundance of 12 pathways, namely the pyruvate fermentation to acetate and lactate pathway II (PWY-

5100), the L-lysine biosynthesis pathway I (DAPLYSINESYN-PWY), the L-lysine biosynthesis pathway II (PWY-2941), the L-isoleucine biosynthesis pathway I (ILEUSYN-PWY), the L-methionine biosynthesis pathway III (HSERMETANA-PWY), the L-isoleucine biosynthesis pathway III (PWY-5103), the phosphatidylglycerol biosynthesis pathway I (PWY4FS-7), the phosphatidylglycerol biosynthesis pathway II (PWY4FS-8), the ADP-L-glycero- and β-D-manno-heptose biosynthesis pathway (PWY0-1241), the CMP-3-deoxy-D-manno-octulosonate biosynthesis pathway (PWY-1269), the super-pathway of phospholipid biosynthesis I (PHOSLIPSYN-PWY), and the super-pathway of branched chain amino acid biosynthesis (BRANCHED-CHAIN-AA-SYN-PWY). Whereas the FD group had enriched abundance of the hexitol fermentation to lactate, formate, ethanol and acetate pathway (P461-PWY).

At day 30, pathways linked to SCFA production (acetate and lactate) and amino acid biosynthesis (i.e. L-methionine, L-lysine, L-isoleucine, branched-chain amino acids) were enriched in the WW group indicating that the WW treatment had impacted the D30 microbiota that led to the increased nutrient utilization functionality of the gut microbial communities.

The pathway encoding production of acetate and lactate (PWY-5100) were increased by the WW treatments at D30. Previously, Gong et al. reported that chickens infected by *Clostridium perfringens* had significant drop of PWY-5100 in the cecal microbial functional capacities, and that was restored by the supplementation of probiotic *L. plantarum* (42). In the current study, PWY-5100 was mainly harbored by *H. pullorum* in both the FD and WW group, and to a lesser extent, *Lachnoclostridium* sp. *An76* and *Lactobacillus salivarius*. To further assess effect size of each microbial species and their contribution to the altered microbial functionality at D30, pathways were stratified to the strain level, and LEfSe was used to discover species contribution. As a result, the 13 altered pathways were attributed by 62 different bacteria

species. LEfSe results indicated that the enriched PWY-5100 in the D30 WW cecal microbial community could be considered as a direct consequence of the increased *H. pullorum* (Figure 3.4, FDR $P < 0.05$, LDA >2) by the WW treatment, whereas the contribution of *Lachnoclostridium* sp. *An76* was relatively smaller (FDR $P < 0.05$, LDA = 1.36). Similarly, the observed increased abundance of pathways linked to amino acid syntheses at the D30 WW group were all mainly attributed to *H. pullorum*. In addition, PWY-1269 encodes genes that produce acid sugar 3-deoxy-α-D-manno-2-octulosonate, which is a component of bacterial lipopolysaccharides (LPS). A wide variety of bacteria including *Campylobacter* and *Helicobacter* are able to synthesize 3-deoxy- D-manno-2-octulosonate and produce LPS as final products (43-45). At D30, PWY-1269 was shown to be mainly harbored by *H. pullorum* and *C. jejuni* in the chicken gut microbiome of the WW and FD, respectively (Figure 3.4). In the previous chapter, we reported that *Campylobacter jejuni* was decreased in the D30 WW group using qPCR techniques. In line with that, functional analyses showing that the WW-derived chicken cecal microbiome had decreased functionality of *C. jejuni*-derived LPS production.

Overall, functional genetics analyses suggested that the barn cleaning methods had altered the functional capacities of the chicken gut microbiota. Exposure to the FD barn at 1 day of life may impact the assembly of the early cecal microbiota of the chicks, and thereby led to altered microbial functionalities observed in later life, which possess decreased genetic potential for amino acid and SCFA synthesis. In addition, we reported that the relative abundance of *Helicobacter* was increased in the WW group at D30 compared to the D30 FD group, and it was positively correlated to cecal branched-chain fatty acid (BCFA) concentration (1). In accordance with that, in the current study, we confirmed that chicken cecal *H. pullorum* was significantly increased by the WW treatment. In particular, results suggested that the genetic potential to

synthesize amino acids were mainly harbored by *H. pullorum*, which may partially explained the increased the association of BCFA and the abundance of *Helicobacter* observed previously (1). In chapter 2, we also found that the cecal SCFA production was increased by WW, which may also be a consequent of the increased cecal *H. pullorum* in the D30 WW group.

3.3.2 The effect of barn cleaning practices and sampling timepoint on the chicken gut microbial resistome

An average of 0.133% ± 0.012% of the total reads per sample (mean ± SEM) were mapped to the CARD database. Overall, both the barn cleaning methods and the sampling timepoint had impacted the chicken cecal microbial resistome. Two-way ANOVA analyses revealed that, in relative to total reads, ARG read count percentage were higher at D7 compared to D30 (Figure 3.5a). The Bray-Curtis distance metric revealed that the cecal microbial resistome clustered significantly by treatment and age (Figure 3.5b, FDR P <0.05). Beta-dispersion analyses revealed that the cecal resistomes of the 7-day old broiler chickens had greater distance to centroid compared to the D30 broiler chickens (P < 0.01), indicating more diverse resistomes between individuals at D7 (Figure 3.5c). A total of 496 ARGs from 60 gene families and 386 ARGs from 52 gene families were identified from the D7 chickens and D30 chickens, respectively. A similar result was reported in chicken litter and cattle studies where fecal resistome diversity decreased with increasing age (46, 47).

At the single gene level, the tetracycline-resistant gene (*tet*) *tetW* was the most abundant ARG, followed by lincosamide nucleotidyltransferase (*lnu*) *lnuC*, *tet(44)*, *tet(W/N/W)*, *tetQ*, the erythromycin ribosomal methylation 23S ribosomal RNA methyltransferase (*erm*) *ermB*,

aminoglycoside resistance gene (*APH*) *APH(3)-IIIa*, *tetO*, and *tet32.* Gene families encoding tetracycline-resistant ribosomal protection proteins (RPPs) were the most abundant in the broiler chicken resistomes followed by the lincosamide nucleotidyltransferase (LNU, lincosamide-resistant) family, and the major facilitator superfamily (MFS) antibiotic efflux pump (multi-drug resistant) family. These ARG families collectively accounted over 90% of the broiler chicken cecal resistome (92.08% ± 11.03% mean ± SEM).

Previously, Munk et al. studied chicken fecal samples collected from multiple European countries and reported that the majority of ARGs were tetracycline-, aminoglycoside-, and macrolide-, lincosamide- and streptogramin- (MLS-) resistant genes (48). Similarly, chicken fecal samples collected from China were high in aminoglycoside, tetracycline, MLS, and β-lactam resistance (49). In the current study, the most abundant ARGs detected conferred resistance to tetracycline (*tetW, tet(44), tet(W/N/W), tetQ, tetO* and *tet(32)*), MLS (*lnuC, ermB*), and aminoglocoside (*APH(3)-IIIa*); whereas β-lactam resistance was found in relatively low prevalence in the chicken cecal resistome. The low abundance of β-lactam resistant genes observed in this study may link to the prohibition of prophylactic use of β-lactam in poultry farming since 2018 (50), whereas β-lactam was reported to be one of the most commonly used antibiotic in poultry production in China (51).

Many of the most abundant ARGs detected in the current study confer resistance to tetracycline through ribosomal protection proteins (i.e. *tetW, tet(44), tet(W/N/W), tetQ, tetO* and *tet(32)*). Tetracycline inhibits bacterial protein synthesis by binding to the 16S rRNA preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (52). Ribosomal protection mediated by RPPs is an ubiquitous mechanism conferring resistance against tetracycline in both Gram-positive and Gram-negative bacteria (53). Genes encoding tetracycline-resistant RPPs

were often found on transmissible elements (e.g. plasmids and conjugative transposons) (52). In addition, tetracycline-resistant genes, particularly *tetW,* were frequently detected in environments related to livestock farming (54-58) as well as bacteria isolated from the chicken gut (59) making it difficult to chase the sources of these genes. Thus, future studies are warranted to investigate the origins of the tetracycline-resistant RPP genes detected in the current study.

3.3.2.1 Barn cleaning methods had a modest impact on the chicken gut resistome

PERMANOVA analyses indicated modest differences of the microbial resistomes between treatments at the same-age group (D7 FD vs. D7 WW, D30 FD vs. D30WW, Figure 3.5b). However, LEfSe did not show significant association between AMR gene families and treatments between treatment groups at the same age (FDR $P > 0.05$ or LDA < 2).

To evaluate the barn cleaning treatment effects, DESeq2 was used to compare the abundance of AMR genes between FD and WW within timepoint. Interestingly, contrary to our hypothesis, WW increased the abundance of some ARGs in the chicken cecal resistomes. Specifically, five AMR gene families (i.e. the *erm* gene family, rifamycin resistant beta-subunit of RNA polymerase (*rpoB*), streptogramin vat acetyltransferase, ATP-binding cassette (ABC) - F subfamily RPPs (macrolide- and lincosamide- resistant), and *vanR* glycopeptide resistance gene cluster were found more abundant in the D7 WW treatment (Figure 3.6, FDR $P < 0.05$).

We expected that disinfection may increase selection of ARG, however, these data suggests that disinfection is associated with reduced ARG abundance, suggesting that it should be evaluated further for its ability to help control transmission of ARG between flocks. Previously, it was reported that poultry litter harbored high densities of AMR bacteria and antibiotic residuals (60-62). Although poultry litter was removed in the current study, without

chemical disinfectants, WW might preserve bacteria carrying ARGs in the barn. Therefore, it is reasonable to assume that compared to WW, the chemical disinfectants used in the current study, which destroys both bacteria cells and DNA structures, may be more effective in controlling ARGs like *erm* genes and the glycopeptide resistance gene clusters.

At the gene level, *cprR* (peptide antibiotic- resistant), *ermB* (MLS-resistant), *lnuA* and *lnuB* (lincosamide-resistant), *lsaE* (pleuromutilin-, streptogramin-, and lincosamide- resistant), *oleB* (macrolide-resistant), *tet(L)* and *tetM* (tetracycline-resistant), *vatE* (streptogramin-resistant), *Vibrio anguillarum* chloramphenicol acetyltransferase gene (phenicol-resistant), the *Bifidobacterium bifidum ileS* (mupirocin-resistant), the *Bifidobacterium adolescentis rpoB* (rifampicin-resistant), and *vanR* variants in the *vanA*, *vanG*, and *vanL* clusters (vancomycin-resistant) were more abundant in the WW group.

By D30, the impacts of cleaning methods on the chicken gut microbiome were subtle. No differentially abundant ARG gene families were identified by DESeq2. However, single genes including *ermG* (MLS-resistant) and *vanR* variant in *vanI* cluster (vancomycin-resistant) were enriched in the D30 WW group (Figure 3.7). Compared to the ARGs affected by the barn cleaning practices at D7 (e.g. *ermB,* 82,227.45 ± 8,067.27 reads per million total ARG reads, mean ± SEM), the ARGs affected by the treatments at D30 were the ones with low abundance indicating that the barn cleaning practices had greater impacts in younger chickens.

Notably, in both D7 and D30, ARGs from the *erm* gene family with high relative abundance (over 100,000 reads/million total ARG reads) and the glycopeptide resistant gene cluster (*vanR*) with lower relative abundance (less than 5,000 reads/million total ARG reads) were depleted with disinfection. More than 30 *erm* genes have been characterized, and a number

of them (e.g. *ermB, ermC, ermG, ermF, ermX*) frequently detected in livestock farming related environments (63-65). Frequent horizontal transfer of *erm* genes through mobile genetic elements has been reported within the gut microbiota (66, 67) and between intestinal bacteria and environmental bacteria (68, 69), accounting for their distribution among diverse taxa. Additionally, *erm* genes were found to persist stably both in the gut and in the environment 2-3 years after the removal of antibiotic selection pressure (65, 70). Furthermore, *erm* genes were also known to spread through poultry dust (58, 71). Transmission of persistent *erm* residues from the previous production cycles is therefore likely.

Vancomycin and other glycopeptide antibiotics inhibit bacterial cell wall biosynthesis by interfering with the formation of peptidoglycan (72). The *vanS/vanR* - two-component regulatory system is important in activating and regulating transcription of the glycopeptide gene cluster. As part of the two-component system, v*anR* can be activated by *vanS* and subsequently promote the cotranscription of other genes in the glycopeptide gene cluster (e.g. *vanI*) (73). Previously, vancomycin resistant genes were detected in poultry farms (74) and products (75). Similar to *erm* genes, a recent study revealed a glycopeptide resistant gene cluster persisting in the environment for 20 years (76). In addition, glycopeptide resistance gene clusters are also highly transferable via plasmids (75, 77) making it difficult to identify the main carriers.

3.3.2.2 The cecal microbial composition and resistome of broiler chickens were significantly affected by the sampling timepoint

At D7, the most abundant ARG families in the cecal resistomes were the tetracycline-resistant RPP family, the MFS antibiotic efflux pump family, the resistance-nodulation-cell

division (RND) antibiotic efflux pump (multi-drug resistant), the *erm* gene family and the LNU family. Whereas at D30, the 5 most abundant gene families were the tetracycline-resistant RPP followed by the LNU family, the MFS antibiotic efflux pump family, the aminoglycoside nucleotidyltransferase (*ant*) family *ant(6)* (aminoglycoside-resistant), and the *erm* gene family. When characterizing the resistant mechanism and distribution of the ARGs, we discovered that antibiotic target protection, which mainly consisted of tetracycline resistance via tetracycline-resistant RPPs, was the most common mechanism of antibiotic resistance. In addition, antibiotic efflux and antibiotic inactivation were the second most common resistance mechanism found at D7 and D30, respectively (Figure 3.8). LEfSe results supported the successional change of the chicken cecal resistome (Figure 3.9, FDR $P < 0.05$, LDA > 2). In accordance with the different antibiotic mechanisms between D7 and D30, LefSe analysis identified that 11 ARG gene families were associated with the age of the broiler chickens (Figure 3.7). Particularly, the MFS antibiotic efflux pumps and the RND antibiotic efflux pump showed highest LDA score at D7, indicating that antibiotic efflux plays a key role in the early life cecal resistomes. Whereas on D30, AMR gene families conferring antibiotic inactivation, such as the LNU family and the tetracycline inactivation enzyme, had increased predominance.

In the current study, the D7 and D30 cecal microbial compositions differed significantly as indicated by Bray-Curtis distance metric (adonis $P < 0.001$). Previously, successional changes of the chicken gut microbiota were recognized (78-80). Although it was speculated that the change of microbial composition would lead to alteration of the ARG profile (59), limited information was available on how the chicken gut resistome change as chickens grow. The relative abundance of total ARGs was higher in the ceca of D7 chickens compared to D30. Previously, Lebeaux et al reported similar results in human infants showing that the overall

relative abundance of ARGs was significantly higher at 6 weeks than 1 year (81). In addition, the change of antibiotic resistant mechanism associated with age was surprisingly similar to the current study. Antibiotic efflux pump (particularly RND antibiotic efflux pump) and tetracycline-resistant RPP, were reported in 6-week and 1-year infant fecal samples respectively (81). Antibiotic efflux pumps were also a strong biomarker of the D7 chicken cecal resistome (Figure 3.7).

Efflux pumps are ubiquitous transmembrane proteins conferring antibiotic resistance by transporting a wide spectrum of antibiotics (82). At D7, the MFS antibiotic efflux pump family were mainly consisted with tetracycline-resistant gene *tet(40).* Microbial community composition may have contributed to the composition of cecal ARGs. Particularly, LefSe analysis suggested the family *Lachnospiraceae* and *Enterobacteriaceae* were strong biomarkers of the chicken cecal microbiota at D7 (Figure 3.10, FDR $P < 0.05$, LDA >3). In addition, spearman correlation (Figure 3.11, FDR $P < 0.01$) revealed that the relative abundance of the MFS antibiotic efflux pump family was positively correlated to *Lachnospiraceae* (*Lachnoclostridium* sp *An76*), which further supported that members in *Lachnospiraceae* play important role in the gut microbial resistome. Previously, using bacteria isolates from chickens, Juricova et al. compared ARG sequences and bacteria genomes and reported that the family *Lachnospiraceae* was an important reservoirs for *tet(40)* (59). Thus, the predominance of the MFS antibiotic efflux pumps in the D7 cecal resistome may be partially explained by the predominace of *Lachnocpiraceae* in the early life chicken microbiota.

RND antibiotic efflux pumps are mainly encoded by gram-negative bacteria (83), and *Enterobacteriaceae* were well known to harbor the RND antibiotic efflux pumps (84, 85). In the current study, *Enterobacteriaceae* (*E. coli* and *E. albertii*) was positively correlated with the

abundance of the RND antibiotic efflux pumps (Figure 3.11). Recently, Gupta et al. reported genes encoding RND antibiotic efflux pumps (e.g. *mdtF, mdtC, mdtB, mdtE, mdtA,* and *sdiA*) were enriched with *Enterobacteriaceae* in chicken litter and cloacal samples (47). Thus, the enriched RND antibiotic efflux pump family at D7 may be a consequence of the early colonization of *Enterobacteriaceae* in the chicken ceca. In addition, among all taxa in the chicken ceca, *E. coli* was significantly associated with the highest number of ARG families (positively correlated to 20 different gene families, Figure 3.11, FDR $P < 0.05$) indicating great impact on the cecal resistome. Similarly, in the human infant resistome study, Lebeaux et al. concluded that the human early-life resistome composition was primarily driven by *E. coli* (81) indicating that *Enterobacteriaceae,* particularly genus *Escherichia,* is of great importance in shaping the early life resistome of the host.

Antibiotic inactivation genes, particularly genes encoding LNUs (mainly *lnuC* gene) and tetracycline inactivation enzymes (mainly *tetX* gene and its variants), were strongly associated with the D30 resistome (Figure 3.9). Previously, Noyes et al. reported that LNUs were more predominant in adult cattle fecal resistome compared to calves (46), however reasons behind still remained unclear. Lincosamide resistant gene *lnuC*, which is a transposon-mediated, was first found in genus *Streptococcus* (86), and was shared extensively between different phyla (87). Recently, emerging evidence showing that *C. jejuni and C. coli* also harbor *lnuC* (88-91). Particularly, examining *Campylobacter* isolates in broiler farms and slaughter plants, Tang et al. reported that *lunC* gene was detected in 19/20 sequenced *C. jejuni* isolates (91). In the current study, LEfSe results revealed that *Streptococcaceae* and *Campylobacteraceae* were representative of the D30 microbiome (Figure 3.10). In this case, the increased composition of *Streptococcaceae* and *Campylopbacteraceae* at D30 may partially explain the enriched LNU family observed in the

older chickens. Interestingly, *Bacillus subtilis* was positively correlated to the LNU gene family (Figure 3.11). Previously, *B. subtilis* exhibited ability to naturally activate the competence state and uptake foreign DNA (92), and this may make it to become an ideal host of LNUs. The degree to which microbial compositions affect ARG composition is still unclear (93), especially in the case of highly mobile ARGs like *lnuC*. Therefore, further research is highly warranted to reveal the reason behind the increment of LNUs in later life.

Tetracycline resistance conferred by inactivation enzymes were executed through the enzymatic modification or destruction of the tetracycline molecule (94, 95). In the current study, the detected genes encoding tetracycline inactivation enzymes were *tetX* and its variants (i.e. *tet(X1), tet(X3), tet(X4), tet(X5), tet(X6)*), with *tetX* being dominant. Spearman correlation showed a wide range of bacteria species that were positively correlated to the tetracycline inactivation enzyme genes (Figure 3.11). Coincides with that, many of these microbes were also associated with the D30 cecal microbiome (i.e. *Rikenellaceae Barnesiellaceae, Odoribacteraceae, Campylobacteraceae, Tannerellaceae, Helicobacteraceae,* and *Streptococcaceae*). It supports the fact that chicken cecal microbiota successional change leads to alteration of the microbial resistome.

3.4 Conclusion:

This is the first study to report that the impact of disinfectants in broiler production on microbial functional capacities and the resistome. We showed that the barn chemical disinfection may alter the composition of the chicken gut microbiota and thereby lead to decreased microbial functional capacity on nutrient production. In addition, compared to WW, FD was associated

with lower abundance and diversity of ARGs between flocks, potentially by destroying both bacteria carrying ARGs.

3.5 References

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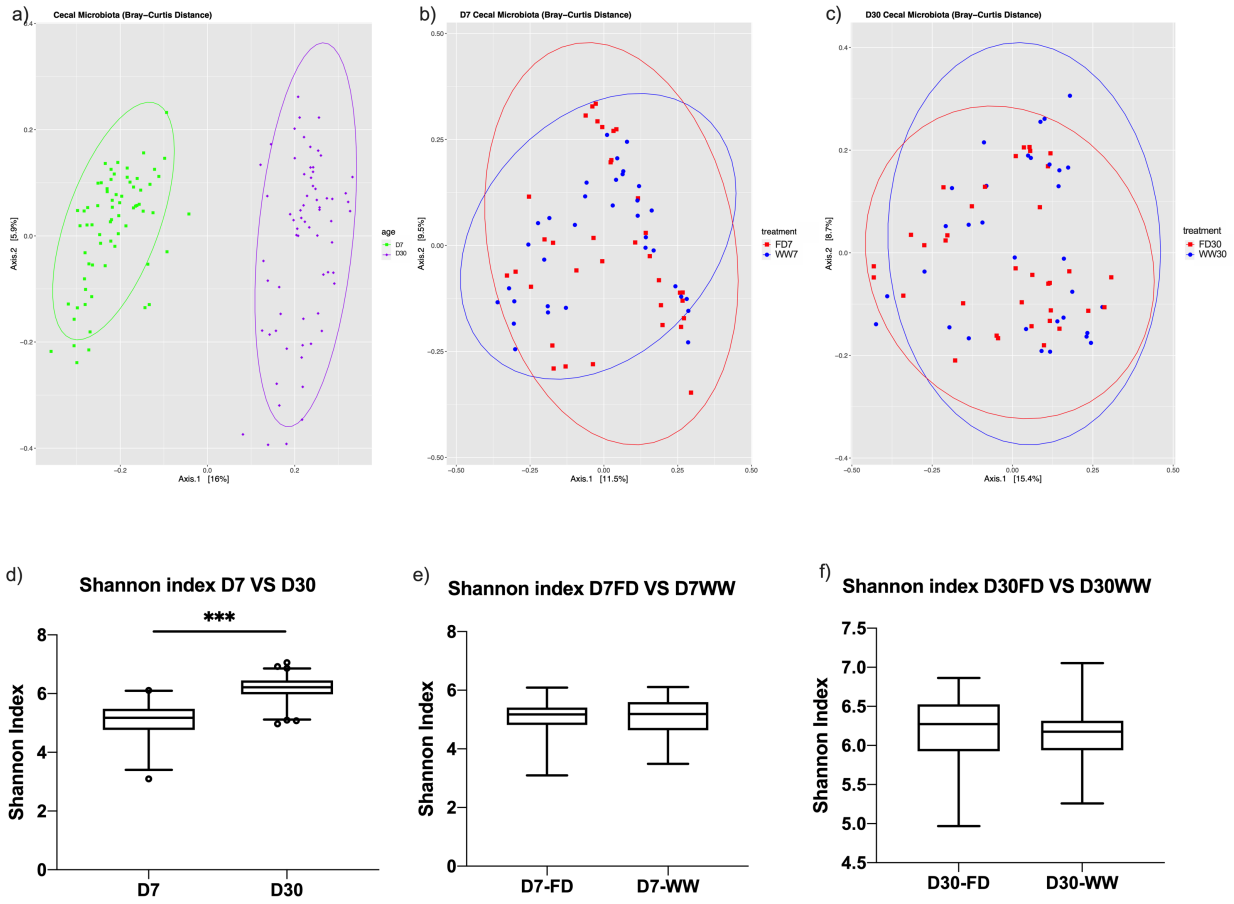

Figure 0.1 Broiler chicken cecal microbial structure affected by the barn cleaning practices and sampling timepoints.

Figure a-c show factors impacting the cecal microbial beta-diversity. Sampling timepoint had a major impact on the microbial compositions, where the cleaning methods had a modest effect on the D30 microbiota. Figure d-f show factors affecting the cecal microbial alpha-diversity (Shannon index). At D30, the richness and evenness of the cecal microbial spices significantly increased compared to D7. FD, full disinfection; WW, water-wash; ***, $P < 0.001$.

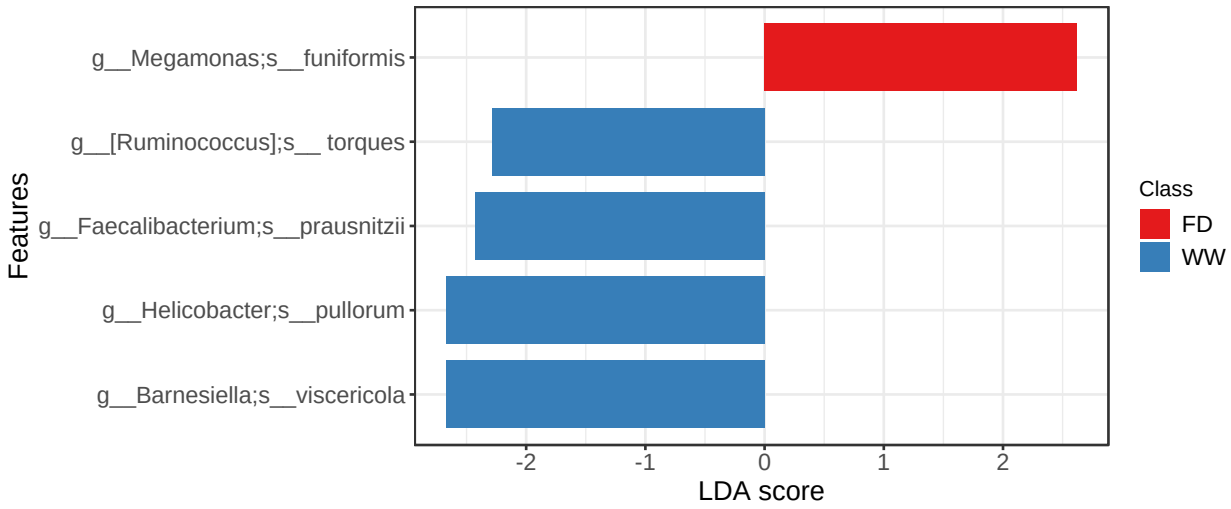

Figure 0.2 Differential abundant bacterial species between barn cleaning practices at day 30 suggested by LEfSe analysis.

At day 30, *Ruminococcus torques, Barnesiella viscericola, Helicobacter pullorum*, *Faecalibacterium prausnitzii* were more abundant in the ceca of the chickens from the water-washed treatment group, whereas *Megamonas funiformis* was more abundant in the chicken cecal microbiota of full disinfection group.

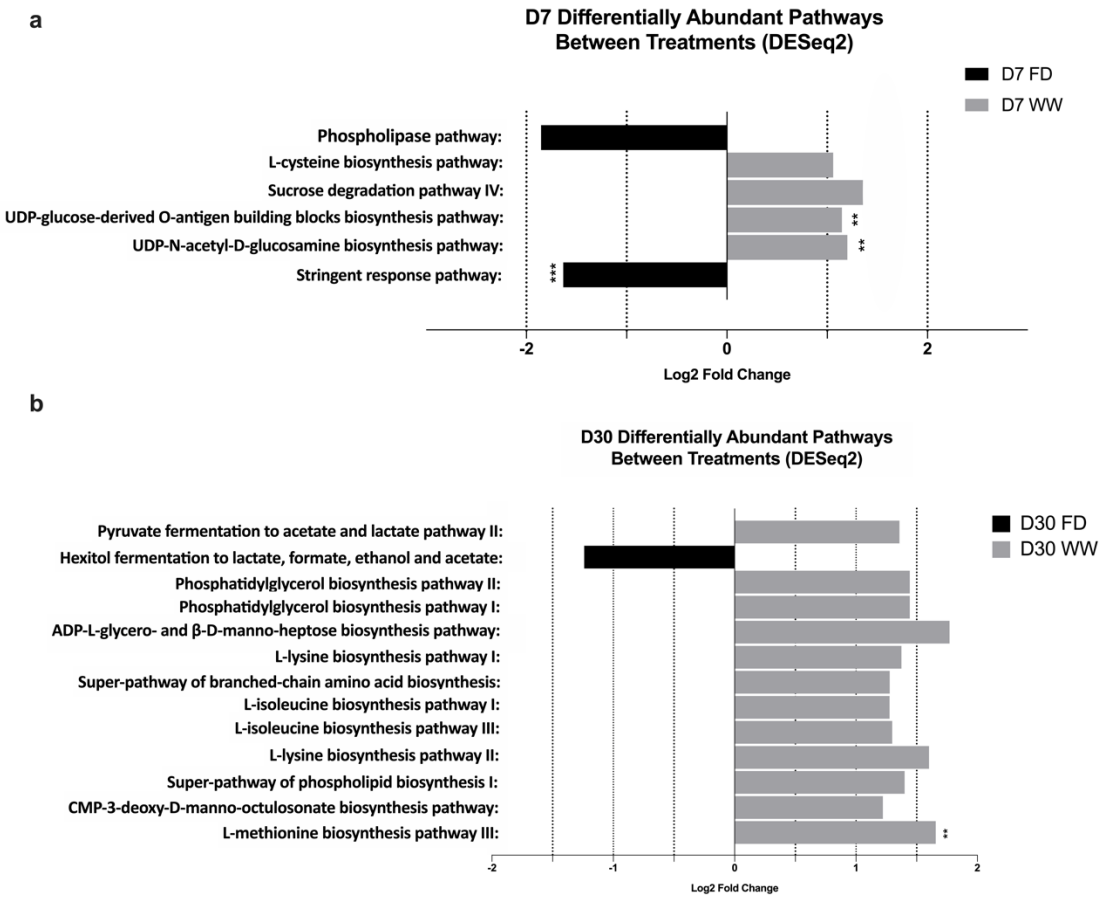

Figure 0.3 The microbial functional pathways that were significantly impacted by the barn cleaning treatments at day 7 (a) and day 30 (b) revealed by DESeq2.

The graph shows differentially abundant genetic pathways at harbored by the chicken cecal microbial communities at day 7 and day 30 suggested by DESeq2 (FDR $P < 0.05$, log2 fold-change >1). a) At day 7, the FD-derived chicken gut microbiome had enriched stringent response pathway coupled with decreased abundance of pathways linked to amino acid synthesis, saccharide degradation, and bacterial cell wall synthesis. b) At day 30, the FD-derived chicken gut microbial functional capacity had decreased abundance of genetic pathways linked to multiple amino acid syntheses. D7, day7; D30, day 30; FD, full disinfection; WW, water-wash **, FDR $P < 0.01$; ***, FDR $P < 0.001$.

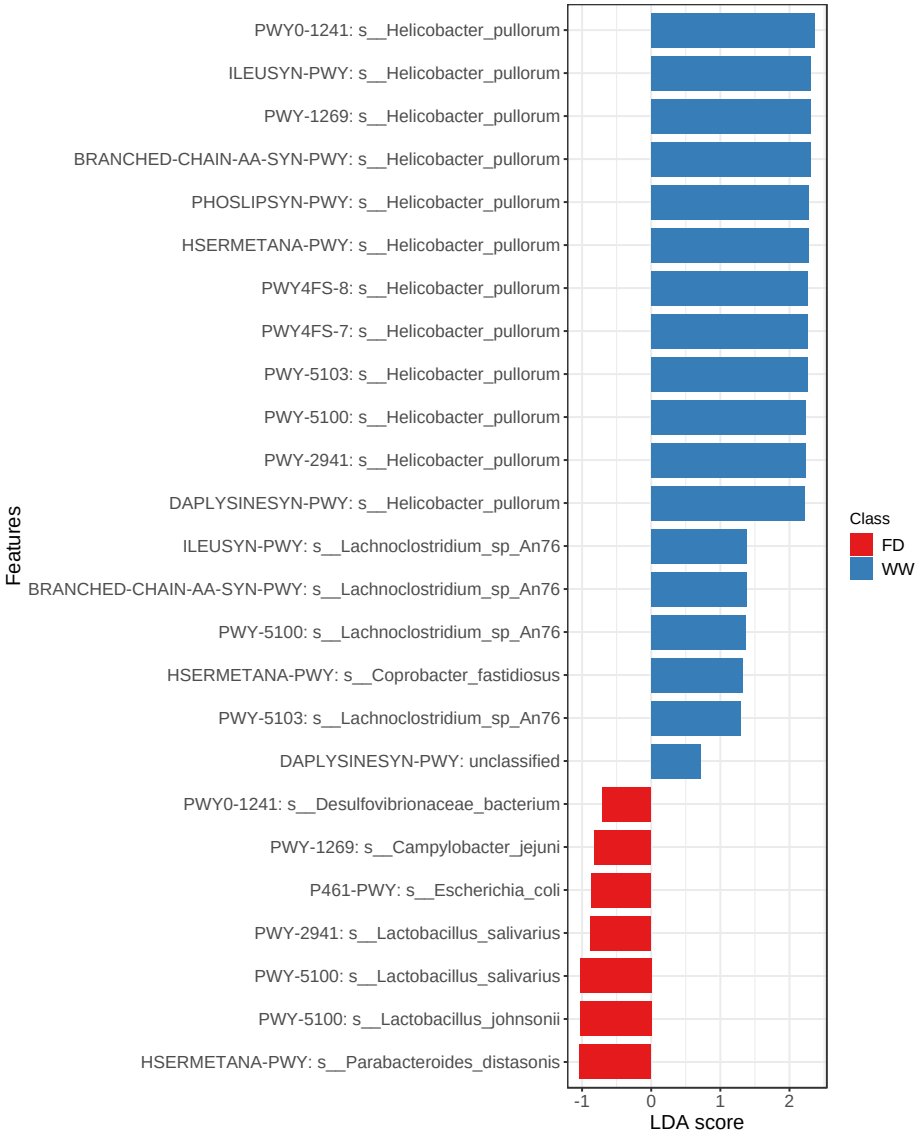

Figure 0.4 LEfSe results of metabolic pathways harbored by specific bacteria species and the association with treatments at day 30.

LEfSe result suggested that the increased abundant pathways in the WW group were mainly contributed by *Helicobacter pullorum.* FD, full disinfection; WW, water-wash.

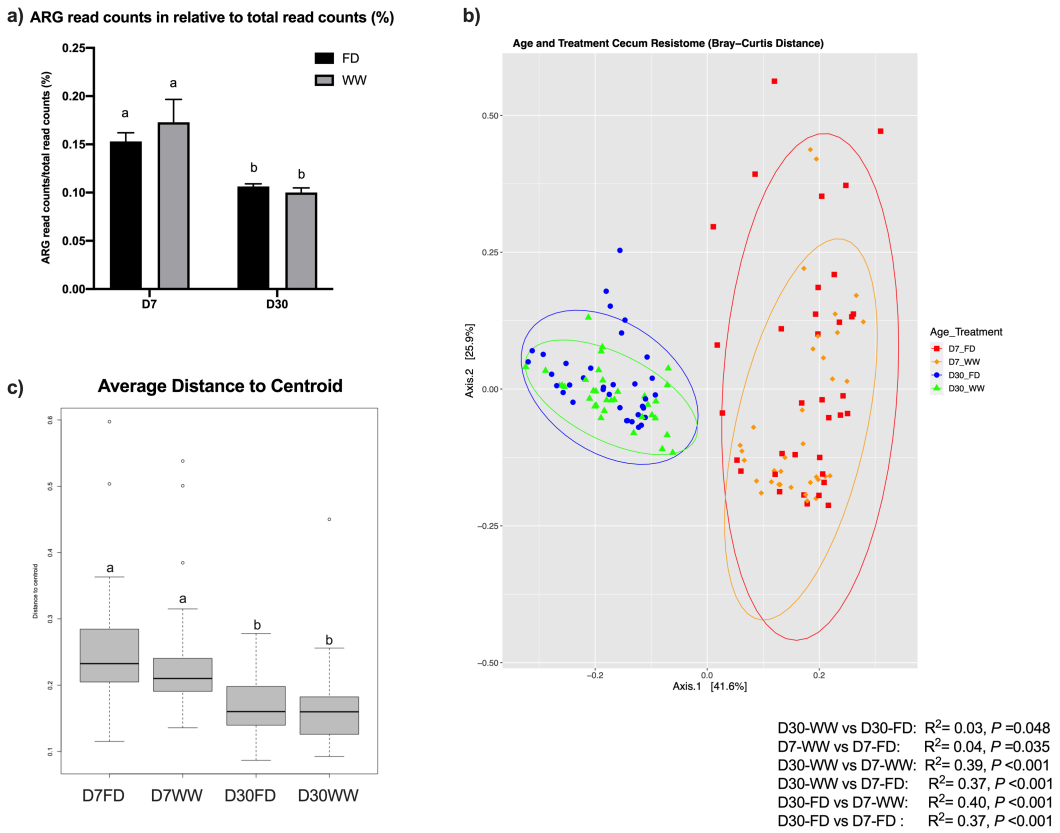

Figure 0.5 The chicken cecal resistome was affected by the barn sanitation practices and sampling timepoints.

a) ARG read counts in relative to total read counts after quality control. Generally, at D7, higher percentage of ARG read counts were detected in the chicken cecal microbiota compared to D30. b) Bray-Curtis distance matric showing resistome clusters of treatments and age. Bray-Curtis distance matric revealed that cleaning methods had modest effect on the microbial resistome. Sampling timepoint was the main factor impacting the resistome. c) The average distance to centroid based on beta-disperse showed that the variation between resistomes was greater among the D7 chickens in comparison to the D30 ones. ARG, andibiotic resistant genes; D7, day7; D30, day 30; FD, full disinfection; WW, water-wash.

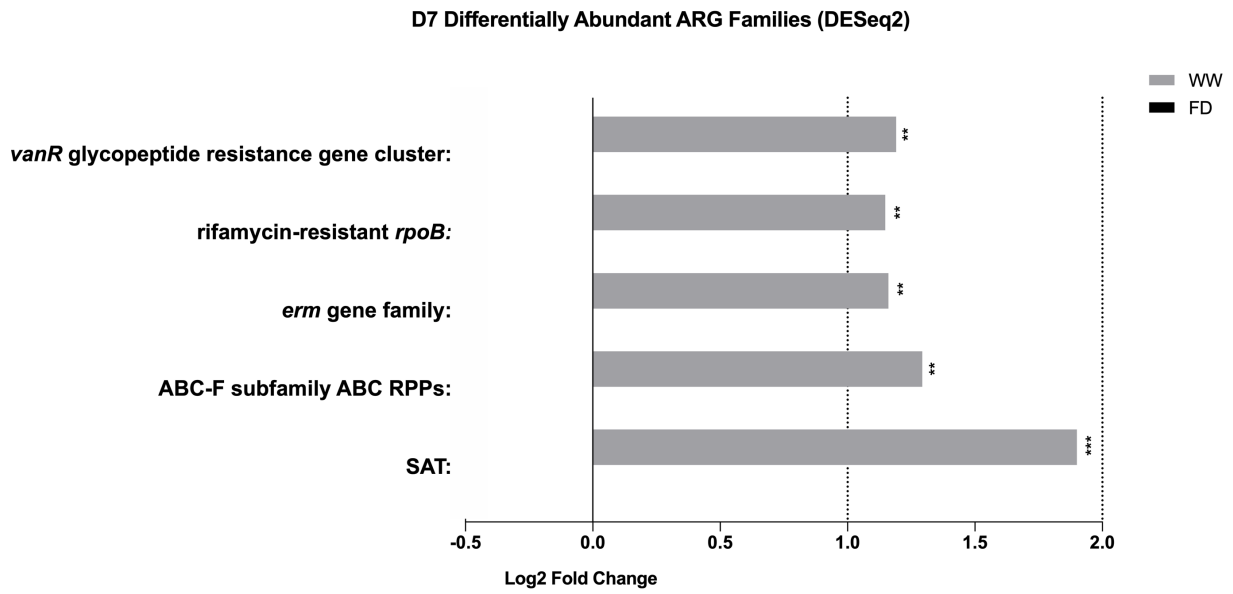

Figure 0.6 Differentially abundant antibiotic resistant gene families between FD and WW at D7 suggested by DESeq2.

Graph shows differentially abundant antibiotic resistant gene families between barn sanitation practices at day 7 suggested by DESeq2 (FDR $P < 0.05$, Log2 fold change > 1). Some persistent ARG gene families (e.g. *erm* gene family) were enriched in the WW-derived chicken cecal microbiome at day 7. ARG, antibiotic resistant gene; *vanR*, vancomycin resistant gene R component; *rpoB,* gene encoding β-subunit of bacterial RNA polymerase; *erm,* 23S ribosomal RNA methyltransferase; ABC-F subfamily ABC RPPs, ABC-F ATP-binding cassette ribosomal protection protein genes; SAT, Streptogramin A acetyltransferase genes, FD, Full disinfection; WW, Water-wash, ***, $P < 0.001$; **, $P < 0.01$.

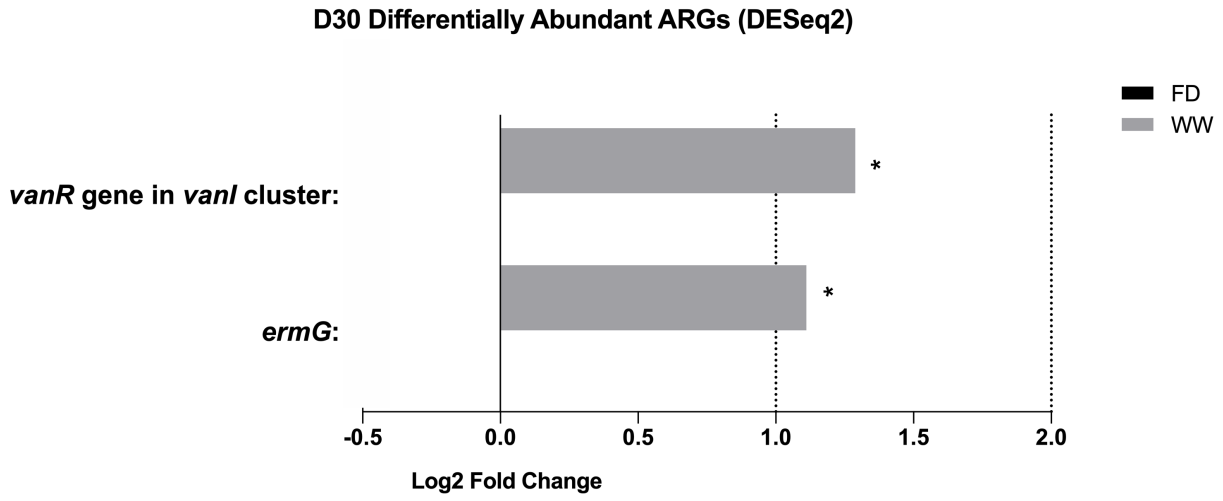

Figure 0.7 Differentially abundant antibiotic resistant gene between FD and WW at D30 suggested by DESeq2.

Graph shows differentially abundant antibiotic resistant genes between barn sanitation practices at day 30 suggested by DESeq2 (FDR $P < 0.05$, Log2 fold change > 1). Compared to the barn cleaning pracitces effects at day 7, the effects of the barn cleaning practices on the 30-day chicken gut microbial resistome was relatively smaller. Little treatment effect was observed on the gene family level, whereas on the gene level, *ermG* and *vanR* gene in the *vanI* cluster were enriched by the WW treatment. ARG, antibiotic resistant gene; *vanR*, vancomycin resistant gene; *erm,* 23S ribosomal RNA methyltransferase genes; FD, Full disinfection; WW, Water-wash, *, $P < 0.05$.

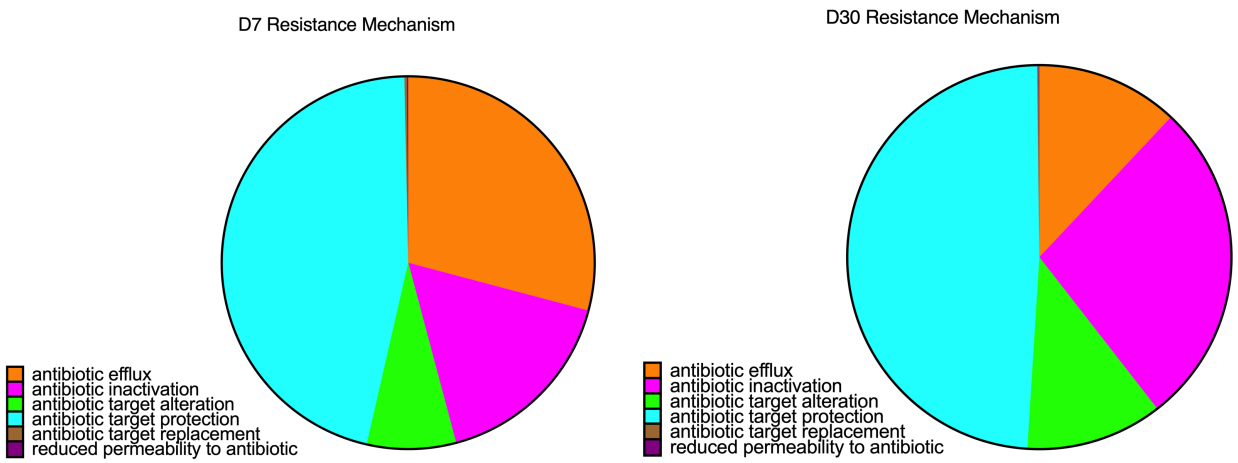

Figure 0.8 Antibiotic resistance mechanism conferred by detected ARGs in the cecal microbial resistomes of D7 and D30 chickens.

Differences on the major antibiotic resistant mechanisms harbored by the 7-day and 30-day chicken cecal microbial resistomes were observed. With the mechanism of antibiotic target alteration being dominant on both ages, genes conferring antibiotic efflux and antibiotic inactivation were representative of the day 7 and day 30 chicken cecal resistome, respectively.

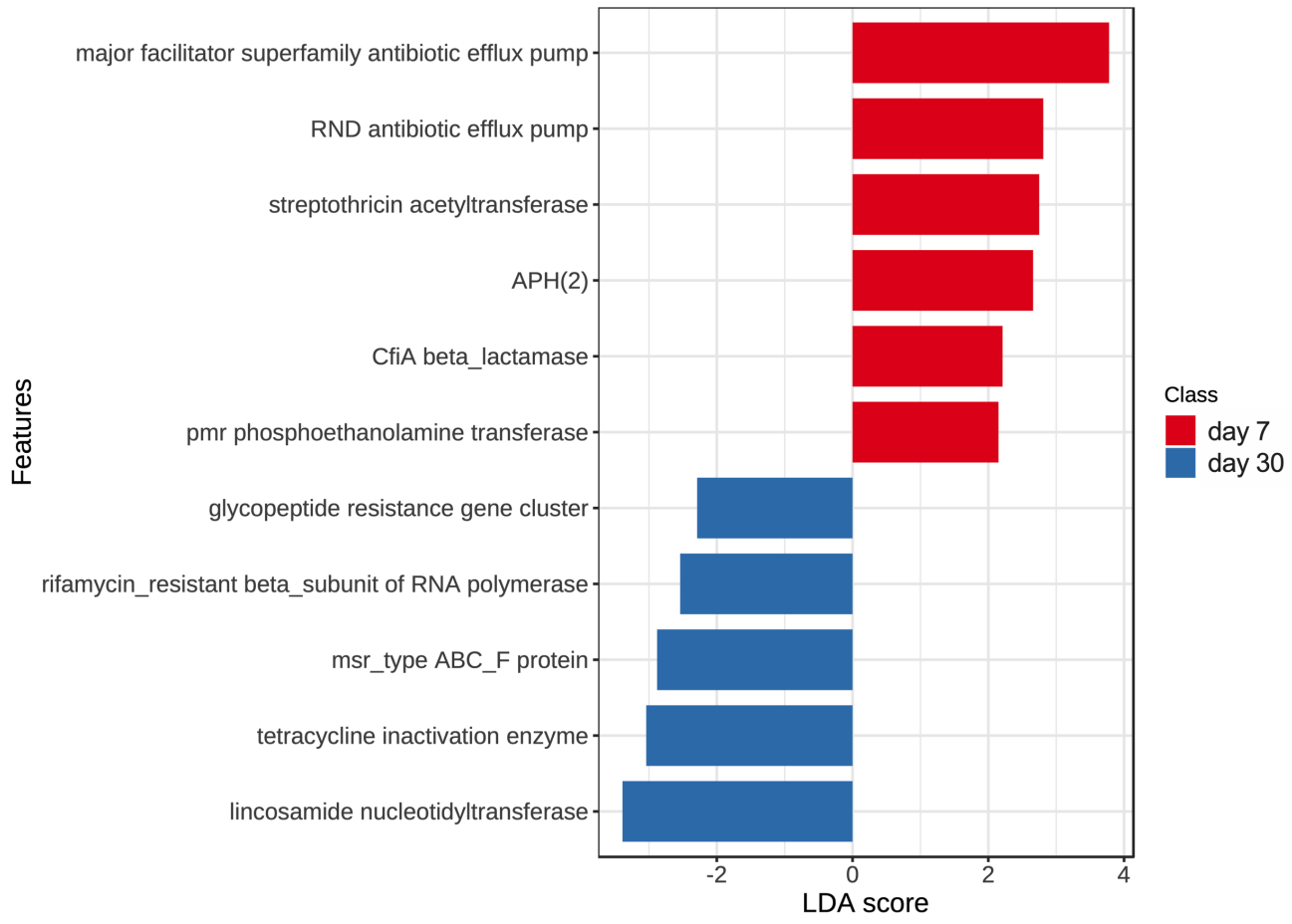

Figure 0.9 Differential abundant cecal microbial antibiotic resistant gene families between day 7 and day 30 suggested by LEfSe.

Graph showing antibiotic gene families associated with different chicken sampling timepoints (FDR $P < 0.05$, LDA > 2). At day 7, genes encoding antibiotic efflux pumps were representative to the chicken cecal microbial resistome. At day 30, antibiotic resistant genes conferring antibiotic inactivation (e.g. the lincosamide nucleotidyltransferases and tetracycline inactivation enzymes) were more predominant. RND, resistance-nodulation-cell division; APH, aminoglycoside resistance gene; pmr, polymyxin resistance; msr, macrolide resistance; ABC, ATP binding cassette.

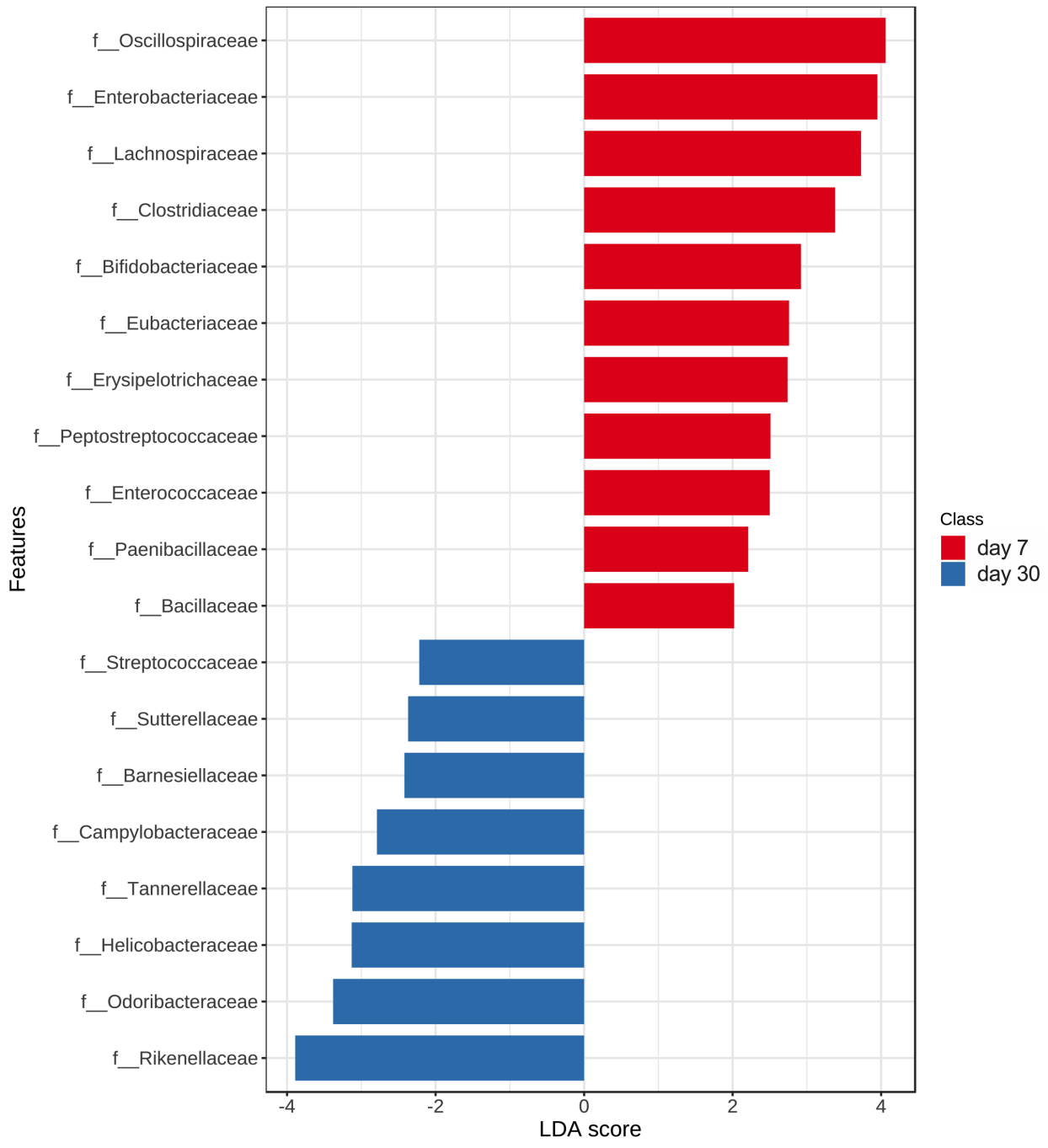

Figure 0.10 LEfSe results of differentially abundant bacterial taxa (on the family level) between the cecal microbiota of day 7 and day 30 chickens.

Result suggested 11 and 8 bacteria families associated with D7 and D30 chicken cecal microbiota, respectively. f__, family.

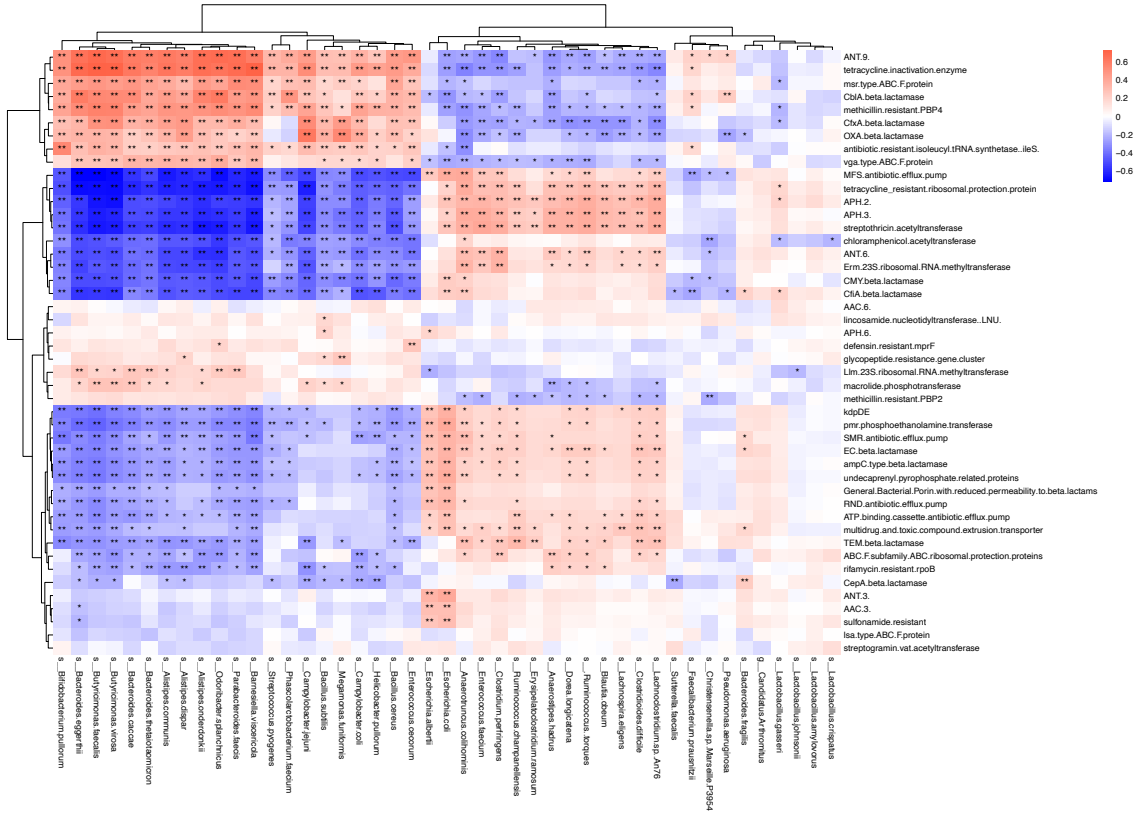

Figure 0.11 Spearman correlation between the relative abundance of bacterial taxa (species level) and ARG families.

Graph shows bacteria species that were correlated to antibiotic resistant gene families. Notably, among all bacteria species in the chicken gut microbiota, *Escherichia coli* was significantly positively correlated to the most families of antibiotic resistant genes. It indicates that *Escherichia coli* plays an important role in antibiotic gene proliferation and transmission. s__, species; *, FDR $P < 0.05$; **, FDR $P < 0.01$.

Chapter 4: WEEK-OLD CHICKS WITH HIGH *BACTEROIDES* ABUNDANCE HAVE INCREASED SHORT-CHAIN FATTY ACIDS AND REDUCED GUT INFLAMMATION[†]

4.1 Introduction

In the ceca of matured chickens, *Firmicutes* and *Bacteroidetes* are reported to be the most dominant phyla, where together these two phyla represent more than 90% of total cecal microbiota (1-3). It has been shown that *Bacteroides* have relatively low abundance in the ceca of newly hatched chicks (4), and become the predominant taxa at day 7 reaching the peak (40 - 45%) at 3 weeks of age (5). Great variation of *Bacteroides* abundance was reported in the ceca of young chickens ranging from 2 to 40% (6, 7). Members belonging to the genus *Bacteroides* are Gram-negative, rod-shape bacteria, which are highly adapted to the gut environment, especially the lower gastrointestinal tract. Encoding a high number of genes for polysaccharide and monosaccharide metabolism, *Bacteroides* are important complex carbohydrates degraders in the host gut (8). However, limited information is available regarding how differential abundance of this taxa affects gut immune state or functional capacity of the gut microbiota in broiler chickens.

In microbiome research, studying variations in microbial structure and composition can offer insight into complex host-microbe-metabolite interactions. Arumugam et al. (2011) first described 3 robust clusters in the human gut microbiota, indicating the importance of the population-level analysis of the gut microbiome variation (9). In chicken research, studies have also suggested the existence of distinct gut microbiomes among individuals (10, 11). Kaakoush

[†] This chapter has been accepted by Microbiology Spectrum as Yi Fan, Tingting Ju, Tulika Bhardwaj, Douglas R. Korver, Benjamin P. Willing. Microbiology Spectrum. 2022. Week-old chicks with high *Bacteroides* abundance have increased short-chain fatty acids and reduced markers of gut inflammation.

et al. (2014) reported that chicken fecal microbiomes could be seperated into four enterotypes, including elevated *Bacteroides,* and that microbial composition could be associated with pathogen carriage, however, the authors did not explore changes in metabolite profile or host responses (10). A more recent study identified high *Bacteroides* in the duodenum of mature chickens with less fat deposition and lower serum triglyceride levels (11).

The aim of the study was to understand how high and low *Bacteroides* abundance are associated with early life chicken gut microbial functional capacity and immune response. This was achieved by sampling and characterizing week-old broiler chickens from commercial production flocks with distinct cecal *Bacteroides* abundance.

4.2 Materials and methods

4.2.1 Chicken management and sample collection

Following the Canadian Council on Animal Care guidelines (12), the animal usage of this experiment was approved by the Animal Care and Use Committee administered by the University of Alberta (AUP00002377). A commercial broiler farm in Alberta, Canada provided facilities and all the chickens for this study. A total of 14 broiler flocks reared under the same feed and water, light exposure, and immunization condition in similarly engineered broiler production houses were sampled. Animal management and sample collection procedure was performed as descried previously (13). Briefly, for each flock, 14,000 Ross 308 broiler chicks were placed at 1 day of age and were fed *ad libitum* until the end of the production cycle. At day 7, five broiler chickens from each flock randomly selected from different areas in the barn were

euthanized by cervical dislocation for sampling. Approximately 300 mg of cecal contents and cecal tonsil tissue were collected, snap frozen, and stored at -80 ℃ for further analyses.

4.2.2 *Bacteroides* over-/under- representing sample identification

Total DNA was extracted from cecal contents using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) with an additional bead-beading step with ~200 mg of garnet rock at 6.0 m/s for 60 s (FastPrep-24 5G instrument, MP Biomedicals). Amplicon libraries were constructed according to the manufacturing protocol from Illumina (16S Metagenomic Sequencing Library Preparation) targeting V3-V4 region of the 16S rRNA gene (primers: Forward: 5′-TCGTCGG CAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; Reverse: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). An Illumina MiSeq Platform (2 × 300 cycles; Illumina Inc. San Diego, CA) was used for a paired-end sequencing run. The 16S rRNA sequences in the current study were submitted to NCBI Sequence Read Archive under BioProject PRJNA876288.

The quality of the reads was assessed using FastQC. Quantitative Insight into Microbial Ecology (QIIME2)-2020.6 was used to process the sequenced reads (14). DADA2 was used to denoise and generate paired-end representative reads (15), and samples with reads less than 14,000 reads were removed. An amplicon sequence variant (ASV) feature table was subsequently created. To assign taxonomy, the q2-feature-classifier in QIIME2 was used with a pretrained classifier "SILVA 132 99%" (16). The command “qiime feature-table relative-frequency” on taxa collapse level 6 was used to calculate genus relative abundance in QIIME2. The mean value and standard deviation of *Bacteroides* relative abundance was calculated. Based on the data distribution, to

screen for distinct cecal *Bacteroides* samples, cecal content with *Bacteroides* relative abundance falling inside one standard deviation were considered as non-assigned (n/a) samples, whereas the ones falling outside of a standard deviation was considered as LB or HB samples.

The 'diversity core-metrics-phylogenetic' command was used for diversity analyses on the screened samples. The Chao1 and Shannon diversity indices were computed using “diversity alpha-phylogenetic” and the significance was determined using “diversity alpha-group-significance”. The beta diversity was analyzed in QIIME2 using the Bray-Curtis distance metric, and a principal coordinate analysis (PCoA) was plotted in R utilizing phyloseq package. Pairwise Permutational Multivariate Analysis of Variance Using Distance Matrices (pairwise Adonis) based on the Bray-Curtis distance matrices was used to identify significant differences in community structures between treatments.

Microbial co-occurrence network was calculated using the NetCoMi package (version 1.0.2) in R with the Sparse Correlations for Compositional data (SparCC) as the sparsification method. The algorithm estimated pairwise association after 20 iterations, assuming an absence of a large number of co-occurring taxa with strong correlations. The taxa count data was resampled 100 times before being used to generate randomized correlation tables. For each pairwise correlation, the randomized correlation matrix was used to calculate bootstrapped *P* values. The resulting correlation matrix was utilized in network models to define links between taxa. If the absolute pairwise correlation between two taxa was greater than 0.25 and there was strong evidence for the association ($P < 0.001$), correlations between the two taxa were considered during network construction. Network features including degree, betweenness, closeness centrality and modularity computation enable identification of hubs (quantile set at 0.9). The community structure was constructed based on the fast greedy algorithm (17).

The extracted genomic DNA was also used to measure the abundance of *Bacteroides-Prevotella* group in the cecal content using quantitative PCR (qPCR) targeting the 16s rRNA gene (Table 1). PerfeCTa SYBR Green Supermix (Quantabio, Beverly, MA, USA) was used for qPCR assays which were conducted on an ABI StepOne real-time system (Applied Biosystems, Foster City, CA, USA) following the setup of 95 °C for 3 min and 40 cycles of 95 °C for 10 s, 68 °C for 30 s. A 10-log-fold standard curve for quantification of the target gene was created using PCR amplicon which concentration was determined by a Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Waltham, MA, USA). *Bacteroides-Prevotella* 16S rRNA gene copy numbers were determined using the relative standard curve method and normalized to the weight of cecal content used for DNA extraction.

4.2.3 Shotgun metagenomic sequencing and functional genomics analyses

Total genomic DNA extracted from the cecal contents as described above were used for Shotgun metagenomics sequencing. Library preparation and shotgun sequencing were performed at the Genome Quebec Innovation Centre (Montreal, Canada). Libraries were generated using NEBNext Ultra II DNA Library Prep Kit (New England Biolabs). Shotgun metagenomic sequencing was performed using the NovaSeq 6000 S4 PE150 system (Illumina Inc., San Diego, CA, USA). The shotgun metagenomic sequences in the current study were submitted to NCBI Sequence Read Archive under BioProject PRJNA902117.

FastP v0.23.2. was used for quality control. Low quality reads, adaptors, polyG and duplicated sequences were removed (18). To remove host DNA contamination, a chicken host reference database was built using bowtie2 v2.4.1 with genome *Gallus_gallus* 105 release from Ensembl (19). Kneaddata v0.10.0 were used to remove host contaminants with the built reference database (https://github.com/biobakery/kneaddata). Gene abundance and pathway analyses were

conducted using HuMAnN3 with default settings (20). Gene and pathway abundance were annotated by the Metacyc database and normalized to copy numbers per million reads using the HuMAnN3 utility scripts. Differentiate gene and pathway abundance were identified using LDA effect size (LEfSe) implemented in the Galaxy online tool (LDA score > 2). Differentiate gene network was constructed using the NetCoMi v1.0.2 package in R (17). Briefly, filter parameters were set to the 50 most frequent genes. Gene network was clustered based on the fast greedy algorithm. Gene association was determined by the SPRING method with correlation coefficient set at 0.3 (21). The corresponding similarities were used as edge weights. Eigenvector centrality was used to define hubs and scale node sizes. Properties of the constructed network were calculated based on the highest degree, betweenness and closeness centrality at the same time with hub quantile set at 0.9. The Jaccard index was used to assess the differences of the most central nodes between groups. Similarity between networks were assessed based on the adjusted Rand index.

4.2.4 Reverse-transcription (RT) - qPCR assay

To examine host response to different *Bacteroides* relative abundance, cecal tonsils were subjected to RNA extraction followed by cDNA synthesis and qPCR assay. Primers targeting selected genes were used to assess host responses between chickens with high and low cecal *Bacteroides*. Specifically, the expression of the pro-inflammatory cytokine genes interleukin (*IL*)-*1β, IL-6,* and the anti-inflammatory cytokine gene *IL-10* in the cecal tonsil was measured to evaluate the immune status of the young chickens. To assess gut integrity, the expression of the tight junction protein genes claudin-1 (*CLDN1*) and zonula occludens 1 (*ZO1*) was measured. In addition, to investigate how the chicken ceca respond to the SCFA production of the high and low *Bacteroides* groups, the expression of the sodium coupled monocarboxylate transporter gene (*SMCT*), which facilitates transcellular transfer of fatty acids from the lumen into lamina propria,

was measured. Primers targeting the selected genes are as listed in Table 4.1. To extract RNA, approximately 30-50 mg of snap-frozen cecal tonsil tissue was cut and weighted. Tissue was ground by pre-chilled RNAse-free mortar and pestle in liquid nitrogen. RNA was extracted using the GeneJET RNA Purification Kit (Thermo Scientific) with modifications. Specifically, ground tissues were homogenized in 600 µl of lysis buffer followed by bead beating in nuclease-free tubes with three metal beads at 4 m/s for 20 s (MP Biomedicals, Solon, OH, USA). Prior to elution, DNase I (Qiagen) was used to treat samples for 15 min to remove DNA. RNA concentration was determined by a NanoDrop 2000c spectrophotometer (Thermo Scientific) and were normalized to 1 µg of RNA for reverse transcription. QScript Flex cDNA Synthesis Kit (Quanta Biosciences) was used for RNA reverse-transcription following the random primer and oligo (dt) protocol. qPCR was performed using PercfeCTa SYBR Green Supermix (Quantabio) and conducted on an ABI StepOne real-time system following the cycles: 95 °C for 3 min and 40 cycles of 95 °C for 10 s, 60 °C for 30 s. Glyceraldhyde-3-phosphate dehydrogenase (*GAPDH*) was used as the housekeeping gene for calculating the fold change of gene expression relative to LB birds using the $2^{-\Delta\Delta Ct}$ method.

4.2.5 Short-chain fatty acids (SCFAs) analysis

Cecal contents used for 16S rRNA gene amplicon sequencing and shotgun metagenomic sequencing were also used for SCFA analysis. Approximately 30 mg per sample of snap-frozen cecal content was weighed, followed by homogenization with 25% phosphoric acid. Samples were centrifuged at 21,130 x g for 10 min and supernatant was collected and filtered using 0.45 µm filter. Isocaproic acid (23 µmol/ml) was added at a 1:4 ratio to samples as an internal standard. To measure SCFA concentrations in samples, a standard solution containing acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and caproic acid mixed with 25% phosphoric acid was

prepared. Samples were analyzed on a Scion 456-GC instrument. Final concentrations of SCFAs were normalized to sample weights.

4.2.6 Statistical analyses

If not otherwise stated, statistical analyses were conducted using GraphPad Prism 8 (Graphpad Software, CA, USA), and mean values were presented as mean ± the standard deviation. Statistically significant differences were determined ($P < 0.05$) by an unpaired student's t-test for parametric data (i.e., gene expression and SCFA concentrations). The Kruskal–Wallis test was used to determine the significance of non-parametric data (i.e., microbiome alpha-diversity indices). The Spearman's correlation was used to correlate SCFA concentration and bacterial relative abundance as well as to determine correlations between cecal microbial taxa. Correlation significance was determined by psych package and visualized using corrplot package in R (version 3.6.1).

4.3 Results

4.3.1 Bacterial composition of early life chicken cecal microbiome

On average, 24,647.56 ± 7632.78 reads per sample were generated and processed by the QIIME2 pipeline, resulting in 1,798 ASVs. Filtered reads were taxonomically classified to represent 4 major phyla (Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria) and 106 genera. The 3 most abundant phyla made up over 98% of the population and included Firmicutes (76.16 ± 15.72 %), Bacteroidetes (17.54 ± 16.59 %), and Proteobacteria (5.07 ± 6.83 %)).

4.3.2 *Bacteroides* over-/under- representing sample identification

On the genus level, various levels of *Bacteroides* relative abundance were observed (16.3 ± 15.6%; range, 0 - 52.2%). Low *Bacteroides* samples were defined as samples with *Bacteroides* relative abundance lower than 0.7% (mean - SD); whereas high *Bacteroides* samples were defined as samples with *Bacteroides* relative abundance higher than 31.9% (mean + SD). As a result, chickens from 11 different flocks were assigned to either the LB or HB group. Specifically, 18 birds with low *Bacteroides* levels from 6 flocks and 15 birds with high *Bacteroides* from 6 flocks were identified. Chickens that were not assigned to either group were marked as not assigned (n/a) (Table 4.2). Bodyweights were not collected at day 7 terminations; however, the average 32-day flock bodyweight ($P = 0.91$) and mortality rate ($P = 0.93$) were similar between flocks that had the majority of birds identified as LB, HB, or n/a (Table 4.2).

Beta diversity analyses revealed that LB, HB, and n/a groups were significantly separated based on Bray-Curtis distance metric ($P < 0.05$ for HB vs. LB, HB vs. n/a, and LB vs. n/a, as shown in Figure 1). Observed ASV index revealed that the richness of observed taxa between the HB, LB and n/a group was comparable. However, the Shannon index showed that the HB had decreased evenness due to the high relative abundance of *Bacteroides* in the cecal microbiota. qPCR assay using primers targeting the *Bacteroides-Prevotella* group showed that the LB group had a lower absolute abundance of *Bacteroides-Prevotella* group (5.96 and 9.04 $\log_{10}$ copies/g cecal contents of *Bacteroides-Prevotella* 16S rRNA gene for LB and HB group, respectively, $P < 0.01$).

The generated cecal microbiome network (Figure 2) included taxa that were involved either in coabundance (positive associations represented by green line) or coexclusion (negative associations represented by pink line) based on a threshold of $P < 0.05$ and an absolute pairwise

correlation of > 0.30. Nodes were categorized as hubs or non-hubs based on the within-module degree and eigenvector centrality. Eigenvector considers both the importance of the node and the degree of connectivity of its neighbors. The hub nodes were further classified as network hubs (modularity > 0.63) and module hubs (modularity between 0.60 and 0.63) based on the degree of connectivity. Based on both eigenvector centrality and modularity, 5 centered genera (*Lactobacillus, [Clostridium]_methylpentosum_group, Acinetobacter, Phascolarctobacterium* and an uncultured member from the family *Erysipelatoclostridiaceae)* were identified as hubs. This rendered the potential of identified hubs in diverse species interactions. Possible competition interactions were identified in Figure 2, where *Bacteroides* relative abundance was negatively correlated with *Lactobacillus*, the *[Clostridium] methylpentosum* group, and an uncultured member of the *Erysipelatoclostridiaceae* family. In addition, genera *Alistipes* showed a positive correlation to *Bacteroides* indicating mutualism between these genera.

Spearman correlation between cecal microbial taxa also revealed positive association between *Bacteroides* and the genera *Faecalibacterium, Anaerofilum, Anaeroplasma, Alistipes,* and an undetermined genus from the order *Oscillospirales*; whereas negative correlations between *Bacteroides* and the genera *Lactobacillus, Escherichia-Shigella, Blautia, Subdoligranulum, Anaerostipes, Negativibacillus,* the [*Ruminococcus*]-torques-group, and an uncultured genus belonging to the family *Ruminococcaceaea* were also suggested (Figure S4).

4.3.3 HB individuals have higher SCFA concentrations in cecal contents

Gas chromatography was used to measure SCFA concentrations in broiler cecal contents. The HB group had increased concentrations of total SCFAs ($P < 0.01$), acetate ($P < 0.01$), propionate ($P < 0.05$), butyrate ($P < 0.05$), and valerate ($P < 0.05$) compared with the LB group

(Figure 4.3). Spearman correlation between SCFA concentrations and bacterial relative abundance suggested a series of microbes that were correlated with the altered SCFA profile between HB and LB (Figure 4.4). Notably, an uncultured member belonging to the family *Lachnospiraceae* and *Faecalibacterium* were found positively associated with most of the detected SCFAs. In addition, acetate concentration was positively correlated with *Clostridia* vadinBB60 group and negatively correlated with *Tyzzerella.* Butyrate was positively correlated with *Bacteroides* and negatively correlated with *Blautia*. *Anaeroplasma,* a member from the order Oscillospirales, and an undetermined genus from the family *Ruminococcaceae* were associated with propionate concentration, whereas [*Ruminococcus*] torques group showed a negatively correlation with propionate levels. Branched-chain fatty acid, isobutyrate and isovalerate, were associated with *Merdibacter* and an undetermined member of *Ruminococcaeae.*

4.3.4 Shotgun metagenomics sequencing suggested differentiated functional capacities between the HB and LB group

To investigate functional capacities of the HB and LB gut microbiome, we performed shotgun metagenomics sequencing. Briefly, a total of 1,860 genes were annotated based on the Metacyc database (22). Gene networks were constructed based on the annotated genes from shotgun metagenomics sequencing. Genes that represented characteristics of the HB group and the LB group were predicted based on eigenvector centrality (Figure 4.5). The properties of the networks can be found in Table 4.3 (a-c) and Table S2. The Jaccard index was significantly close to 0 for betweenness centrality, closeness centrality, and eigenvector centrality (Table 3c), suggesting that the sets of most central were considerably different between the HB and LB group (Jaccard index ranging from 0-1, where 0 being two completely different sets and 1 being exactly equal sets). Different hub nodes were identified between HB and LB group based on

eigenvector centrality (Figure 4.5) that indicated nodes not only important by itself but also sharing high connectivity with important neighbors. Specifically, in the HB network the acetylxylan esterase (EC3.1.1.72), the type I arylsulfatase (EC3.1.6.1), the non-reducing end beta-L-arabinofuranosidase (EC3.2.1.185), and the licheninase (EC3.2.1.73) were identified as hubs, whereas only the histidine kinase (EC2.7.13.3) was identified as a hub in the LB group.

4.3.5 Shotgun metagenomic sequencing suggested enriched pathways related to complex carbohydrate degradation and SCFA production in the HB group

Shotgun metagenomics sequencing and functional genomics analyses identified 12 pathways that were different between the HB group and the LB group (LefSe LDA score > 2, Figure 4.6). The gut microbiota of the HB group harbored more abundant pathways including the Stickland reaction pathways (PWY-8190), the superpathway of UDP-N-acetylglucosamine-derived O-antigen building blocks biosynthesis (PWY-7332), the dTDP-β-L-rhamnose biosynthesis (DTDPRHAMSYN-PWY), the 1,5-anhydrofructose degradation pathway (PWY-6992), the β-(1,4)-mannan degradation pathway (PWY-7456), and the γ-aminobutyric acid degradation pathway (PWY-5022). The LB microbiota were more abundant in the L-carnitine respiration pathway (CARNMET-PWY), the superpathway of glycerol degradation to 1,3-propanediol (GOLPDLCAT-PWY), the heterolactic fermentation pathway (P122-PWY), the oleate β-oxidation pathway (PWY0-1337), the D-erythronate degradation II pathway (PWY-7873), and the superpathway of pyrimidine ribonucleosides degradation pathway (PWY-7209), which exerts reductive pyrimidine degradation in bacteria.

4.3.6 HB and LB cecal tonsil exhibited different expression levels of genes involved in immune tolerance and gut integrity

To investigate how different levels of *Bacteroides* population affected host responses, cecal tonsil RNA was extracted from the HB and LB group and subjected to RT-qPCR assay to examine immune-related genes including *IL-1β, IL-6* and *IL-10*, as well as tight junction protein gene *CLDN1* and *ZO1,* and the fatty acid transporter *SMCT*. The cecal tonsils of the HB group showed a decreased *IL-1β* with an increased *IL-10* compared with the LB group (Figure 4.7). In addition, *CLDN1,* which has previously been associated with improved barrier function (23), was also higher in the cecal tonsil tissues of the HB group ($P < 0.05$; Figure 4.7). *SMCT* expression was not different between groups ($P = 0.103$; Figure 4.7).

4.4 Discussion

While several studies have shown significant variations in *Bacteroides* populations in the chicken gut microbiome in early life (6, 7), this is the first study to investigate how distinct *Bacteroides* compositions associate with cecal SCFA profiles, host responses, as well as microbial functional capacity. Bodyweight of day-7 chickens were not collected, limiting a connection to growth performance, however, 32-day bodyweight and flock mortality rate were similar between flocks where the majority of birds were identified as LB, n/a, or HB. With regards to the cecal microbial composition of chickens close to the end of production, the variability of *Bacteroides* was much less pronounced (data not shown), therefore the LB/HB phenotype was only explored in day-7 chickens. Moreover, in the current study, only 5 young broilers per flock were sampled. Previously, microbiome studies showed that individuals housed

together, particularly coprophagic animals like mice and chicken, exhibited high similarity in the intestinal microbiota (24, 25). Therefore, to better explore the variability of the cecal microbiome among commercial broiler chickens, instead of sampling more broilers from each flock, we chose to increase the number of flocks sampled.

What caused the distinct *Bacteroides* levels was not investigated in the current study. Previous studies showed that cecal *Bacteroides* abundance in young broiler chickens could be increased by exposure to cecal contents from 40-week-old healthy chickens via oral gavage (26), the use of recycled litter (27) or hen contact at hatch (4). The absence of contact with the parent flock in modern broiler production likely limits the transmission of mature chicken-derived commensals (2, 28). In this sense, the alternate initial exposures (e.g. parent flock, hatching environments, or handling crew) may play an important role in shaping the early-life broiler microbiota. It has been reported that the relative abundance of *Bacteroides* was positively correlated with chicken cecal SCFA profiles (29). In accordance with previous findings, our results showed that the over-representation of *Bacteroides* in ceca was associated with increased concentrations of SCFAs, particularly acetate, propionate, butyrate, and valerate. In the chicken intestine, SCFAs are products of the gut microbiota fermentation from partially- or non-digestible polysaccharides mainly derived from plant biomass. Functional gene network analyses showed that the microbial functional capacity of the HB group was centered by a series of complex carbohydrate degradation enzymes. Specifically, acetylxylan esterase (EC3.1.1.72) contributes to xylan utilization (30) and β-L-arabinofuranosidase (EC3.2.1.185) helps digest glycoproteins that are widely found in plant cell wall fractions (31). The licheninase (EC3.2.1.73) can degrade β-glucans which have been used as chicken feed additives (32), and were found to modulate the host gut microbiota and thus decrease intestinal inflammation (33,

34). In the current study, Spearman correlation analyses showed that the abundance of the microbe-encoded acetylxylan esterase, β-L-arabinofuranosidase and licheninase were significantly associated with cecal total SCFA, acetate, propionate, butyrate, and valerate concentrations (Figure S5). Therefore, harboring microbial functional capacity centred by these key enzymes, the microbiota of the HB group showed potential for increased utilization of plant-derived biomass to promote SCFA production and thereby improve gut health.

Van der Hee and Wells (2021) recently reviewed the complex interactions between SCFAs, gut microbes, and the host immune system (35). Briefly, enterocytes can absorb SCFAs via passive diffusion or protein-mediated transport, and elevated levels of lumen SCFAs enhance associated transporter and receptor expression (35). Nastasi et al. (2015) reported that butyrate can confer anti-inflammatory properties in colonic dendritic cells via G-protein coupled receptors pathway, which inhibits the expression of cytokine and chemokine genes (36). In the current study, the elevated butyrate in HB birds coincided with lower *IL-1β* and higher *IL-10* expression in the cecal tonsil. In addition, tight junction protein levels are important indicators of gut integrity as they contribute to epithelial cell adhesions and act as a barrier. Generally, decreased expression of tight junction proteins may lead to diffusion of antigens or bacterial macromolecular (e.g. endotoxin) from the intestinal lumen into circulation (37). Decreased level of tight junction protein claudin 1 was reported in chronically stressed and repeatedly corticosterone-injected rats (38). In addition, gut inflammation caused by *Salmonella enterica* serovar Typhimurium challenge was also found to decrease the expression of chicken intestinal claudin 1 (23). Therefore, in the current study, the decreased expression of *CLDN1* mRNA level found in the LB group may indicate decreased gut integrity and may have contributed to the increased expression of *IL-1β*.

Microbial co-occurrence networks provided an opportunity to explore the impact of elevated *Bacteroides* on cecal microbial communities and types of interactions with other connected microorganisms. The analysis included both positive and negative links, considering the possibility that both types of associations could influence network stability. To circumvent the limitations of sparsity and high dimensionality of microbial data, the correlation principle was utilized to understand the pairwise associations among microbes and interactions. Further, network features were computed to identify biologically significant patterns and community keystone taxa. In the current study, the SparCC correlation method evaluated the variance of the log-ratio for modified data to infer pairwise relations. *Lactobacillus* was negatively associated with *Bacteroides* in the cecal microbial community. Similarly, previous human studies have demonstrated that *Lactobacillus* can competitively exclude commensals including *Bacteroides* (39). Competition is often observed between taxa sharing similar nutrient sources (e.g. nitrogen and carbon source). It might partially explain the negative correlations between *Bacteroides* and *Lactobacillus* in the current study since members from these two genera are efficient and important complex carbohydrate degraders. Particularly, our results of functional genetic analyses indicated that the 1,5-anhydrofructose degradation pathway (PWY-6992) and the β-(1,4)-mannan degradation pathway (PWY-7456) were more abundant in the HB cecal microbiota. The 1,5-anhydrofructose degradation pathway catalyzes the degradation of glycogen (40), whereas the β-(1,4)-mannan degradation pathway is involved in the hydrolysis of mannans, a major group of hemicellulose (41). The enriched pathway PWY-7456 in the HB cecal microbiota indicated that the microbial members harbored greater genetic potential in utilizing complex carbohydrates derived from plant cell wall which were contained in chicken feed. In the LB group, the heterolactic fermentation pathway (P122-PWY) was more abundant in the gut

microbiome. Possessed mainly by lactic acid bacteria, the heterolactic fermentation pathway ferments starch to lactates. The difference in the predominant carbohydrate utilization pathways between LB and HB groups further identified nutrient competition between *Bacteroides* and *Lactobacillus*, particularly regarding complex carbohydrate fermentation. Currently, a good number of studies have considered *Lactobacillus* as probiotics in poultry and reported potential beneficial effects. However, many of these studies also found that the abundance of *Lactobacillus* in the chicken ceca was not affected by *Lactobacillus* supplementation suggesting that the potential beneficial effects conferred by *Lactobacillus* may not be a consequence of cecal colonization (42, 43). In fact, Chen et al. (2016) studied the effect of the supplementation of *Lactobacillus* spp. and/or yeast with bacteriocin on broiler performance and reported that supplementation with *Lactobacillus* culture alone (without bacteriocin) was the only treatment group that increased cecal *Lactobacillus* colonization (44). Consistent with our study, the increase in *Lactobacillus* coincided with decreased SCFA production with no improvement on performance compared to the control (44). Thus, although supplementing *Lactobacillus* had been shown to exert beneficial effects on poultry, the effects of *Lactobacillus* colonization in the chicken ceca needs to be carefully assessed in the future.

In addition, results from the current study indicated that *Alistipes* may share a mutualistic relationship with *Bacteroides.* To date, little is known about the genus *Alistipes,* which is a sub-branch genus of the phylum Bacteroidetes (45). *Alistipes* are bile-resistant microorganisms with an ability to produce acetic acid by digesting gelatin and fermenting carbohydrates (46). Correlations between health outcomes and *Alistipes* indicated that *Alistipes* may exert protective effects against diseases such as liver fibrosis and colitis (47, 48). However, the causal effect of the microbial taxa in diseases and its prevalence remains unclear. Studies have shown that

Alistipes are more abundant in host gut with an anti-inflammatory background (49, 50). In the current study, the lower *IL-1β* expression in the ceca of HB broilers may have favored *Alistipes* growth.

The higher *Bacteroides* abundance in ceca may reflect a further progression of microbial succession, with the transition from facultative anaerobes, such as *Lactobacilli*, to strict anaerobes, such as *Bacteroides, Ruminococcaceae* and *Lachnospiraceae*. An anaerobic gut environment and undigested carbohydrates entering ceca are essential for the production of SCFAs (51), in turn, increased SCFAs help make the gut more anaerobic (52). In congruence, our results showed that obligate anaerobes from the families *Ruminococcaceae* and *Lachnospiraceae* were positively correlated with SCFA production. On the other hand, more inflammation could provide reactive oxygen species that could delay colonization of strict anaerobes (53). Although previously both *in vitro* (54) and *in vivo* (55) studies have shown the immunomodulatory effects of *Bacteroides* strains, the direction of causality between high *Bacteroides*/SCFA levels and inflammation has yet to be determined. Therefore, future studies assessing how the host intestine respond to increased SCFA (e.g. via histone deacetylation) is warranted. Further, while this study points to a beneficial impact of high *Bacteroides* colonization, future research with the introduction of *Bacteroides* strains to chickens in microbially controlled conditions will be needed to demonstrate causal contributions of *Bacteroides* in improving health outcomes and to support their development as potential probiotics.

To conclude, the current study identified distinct *Bacteroides* populations in the ceca of commercial broiler chickens in early life. Our results revealed that elevated level of cecal

Bacteroides in young chickens had led to altered microbial functional capacity of the gut microbiome, which promoted the production of SCFA. Coincided with that, compared to the LB group, chickens from the HB group had lower expression of pro-inflammatory cytokines coupled with and higher expression anti-inflammatory cytokine and tight-junction protein gene. Consequently, it indicated that elevated cecal *Bacteroides* may be beneficial to commercial broiler chickens in suppressing gut inflammation through the increment of short-chain fatty acid production.

4.5 Conclusion

The current study for the first time illustrated the effects of *Bacteroides* under-/over-represented gut microbiota to the broiler chickens in early life, particularly in the aspects of microbial compositions, the microbial functionality, and host immune responses. Our results indicated that the genus *Bacteroides* are important in maintaining chicken gut homeostasis in early life by promoting SCFA production. While this study points to a beneficial impact of high *Bacteroides* colonization, future research with the introduction of *Bacteroides* strains to chickens in microbially controlled conditions will be needed to demonstrate causal contributions of *Bacteroides* in improving health outcomes and to support their development as potential probiotics.

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Table 0.1 Primers used to quantify cecal *Bacteroides* and the expression of selected gene markers.

Primer†	Sequence	Product size (bp)	Reference
Bacteroides-Prevotella Forward	GGTGTCGGCTTAAGTGCCAT	140	(56)
Bacteroides-Prevotella Reverse	CGGACGTAAGGGCCGTGC		
IL-1b Forward	GGGCATCAAGGGCTACAA	88	(57)
IL-1b Reverse	CTGTCCAGGCGGTAGAAGAT		
IL-6 Forward	GAGGGCCGTTCGCTATTTG	67	(58)
IL-6 Reverse	ATTGTGCCCGAACTAAAACATTC		
IL-10 Forward	GCTGAGGGTGAAGTTTGAGG	121	(59)
IL-10 Reverse	AGACTGGCAGCCAAAGGTC		
SMCT Forward	GGCTTCAGCGTTTGGGACTA	235	(60)
SMCT Reverse	TGCAGAAGATGGCACCGTAG		
CLDN1 Forward	CCAGGTGAAGAAGATGCGGA	129	
CLDN1 Reverse	GGTGTGAAAGGGTCATAGAAGGC		
ZO1 Forward	CAACTGGTGTGGGTTTCTGAA	101	
ZO1 Reverse	TCACTACCAGGAGCTGAGAGGTA A		
GAPDH Forward	CTACACACGGACACTTCAAG	244	(61)
GAPDH Reverse	ACAAACATGGGGGCATCAG		

†: *IL*, interleukin; *SMCT*, sodium coupled monocarboxylate transporter; *CLDN*, claudin; *ZO,* zonula occludens; *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase.

Table 0.2 Distribution of chickens assigned to high Bacteroides (HB) group, low Bacteroides (LB) group, or unassigned (n/a) †

Chicken distribution	Flock mean bodyweight (gram)	Flock mean mortality rate (%)
HB (1) + n/a (4)	1,793	4.8
LB (5)	1,746	4.7
LB (4) + n/a (1)	1,810	3.3
LB (4) + HB (1)	1,836	5.7
LB (2) + n/a (3)	1,790	6.5
LB (1) + n/a (4)	1,850	3.1
HB (1) + n/a (4)	1,793	4.8
n/a (5)	1,744	5.2
HB (1) + n/a (4)	1,750	5.1
HB (3) + n/a (2)	1,762	7.0
n/a (5)	1,850	6.1
HB (3) + n/a (2)	1,886	3.7
HB (3) + n/a (2)	1,790	3.5
n/a (5)	1,744	5.2

†: Each row represents a single production flock. In each production flock, 5 young broiler chickens were randomly sampled. Numbers in the brackets refers to the number of broiler chickens assigned to each specific group.

Table 0.3 Properties of networks constructed for the HB group and LB group.

a) Jaccard index†

	Jacc	*P* (≤ Jacc)	*P* (≥ Jacc)
degree	0.048	0.002 **	1.000
betweenness centrality	0.000	< 0.001 ***	1.000
closeness centrality	0.091	0.009 **	0.998
eigenvector centrality	0.050	0.003 **	1.000
hub taxa	0.000	0.296	1.000
Adjusted rand index = 0.109 (ARI, measuring similarity between clusterings ranging from 0 - 1. ARI =1, perfect agreement between clusterings; ARI=0, two random clusterings; *P* <0.001)			

b) Hub nodes

HB	LB
3.1.1.72: Acetylxylan esterase	2.7.13.3: Histidine kinase
3.1.6.1: Arylsulfatase (type I)	
3.2.1.185: Non-reducing end beta-L-arabinofuranosidase	
3.2.1.73: Licheninase	

c) Eigenvector centrality

Gene	HB	LB
Highest values in the HB group		
3.2.1.185: Non-reducing end beta-L-arabinofuranosidase	1.000	0.267
3.2.1.73: Licheninase	0.949	0
3.1.1.72: Acetylxylan esterase	0.866	0.558
3.1.6.1: Arylsulfatase (type I)	0.839	0
3.2.1.52: Beta-N-acetylhexosaminidase	0.665	0.132
Highest values in the LB group		
2.7.13.3 histidine kinase	0	1.000
2.7.7.7: DNA-directed DNA polymerase	0	0.912
6.3.5.5: Carbamoyl-phosphate synthase (glutamine-hydrolyzing)	0	0.752
2.7.7.6: DNA-directed RNA polymerase	0	0.737

†, Jaccard index measured the similarity between sets of most central nodes. Jaccard index ranged from 0 (completely different) to 1 (sets equal), **, P <0.01; ***, P <0.001.

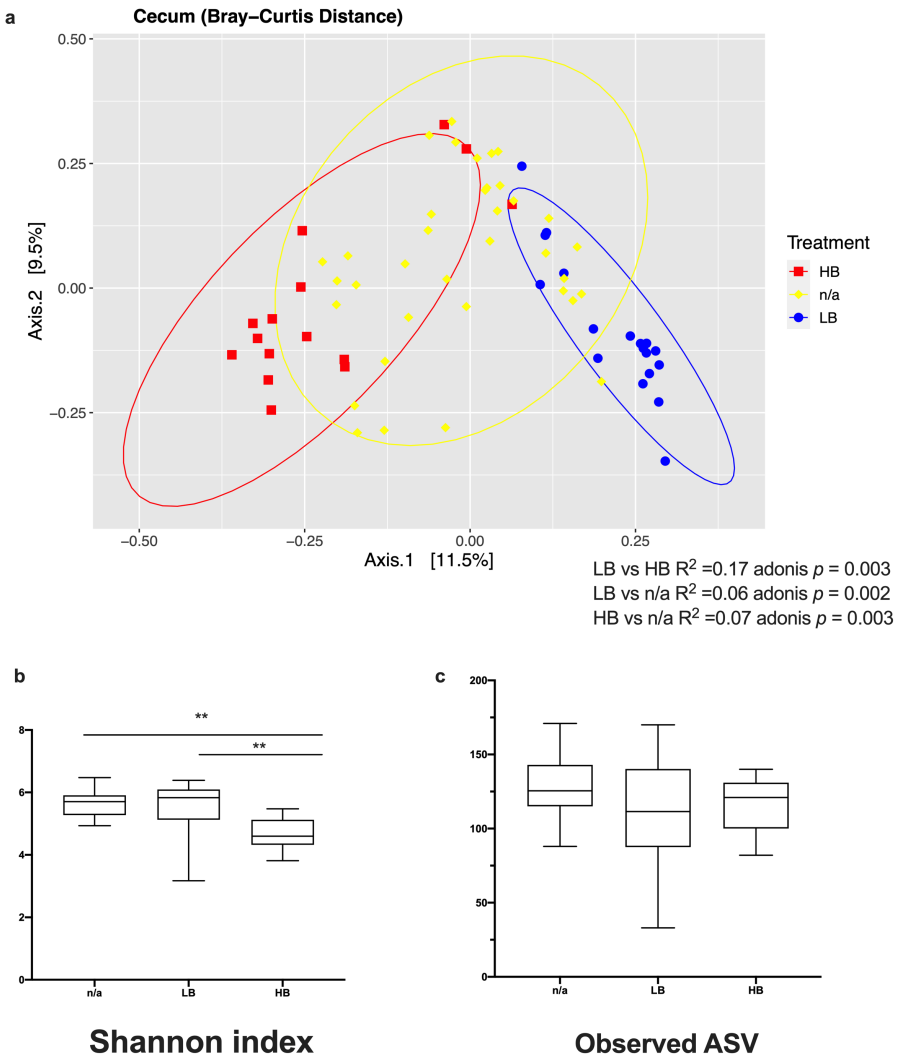

Figure 0.1 Diversity of the gut microbiota of the 7-day-old commercial broiler chickens

(a) Principal coordinate analysis plot based on Bray-Curtis distance metric. Distinct pattern of *Bacteroides* populations resulted in significantly different clusters between LB , HB and chickens not assigned to either HB or LB groups (n/a) (adonis $P < 0.05$). (b) The HB group had decreased bacterial species evenness compared with the LB group and the n/a group (Shannon index, $P < 0.01$). (c) Comparable bacterial species richness was observed between groups (observed ASV, $P > 0.05$). LB: low *Bacteroides,* n = 18; HB: high *Bacteroides,* n = 15; n/a: not assigned, n = 37. *, $P < 0.05$; **, $P < 0.01$.

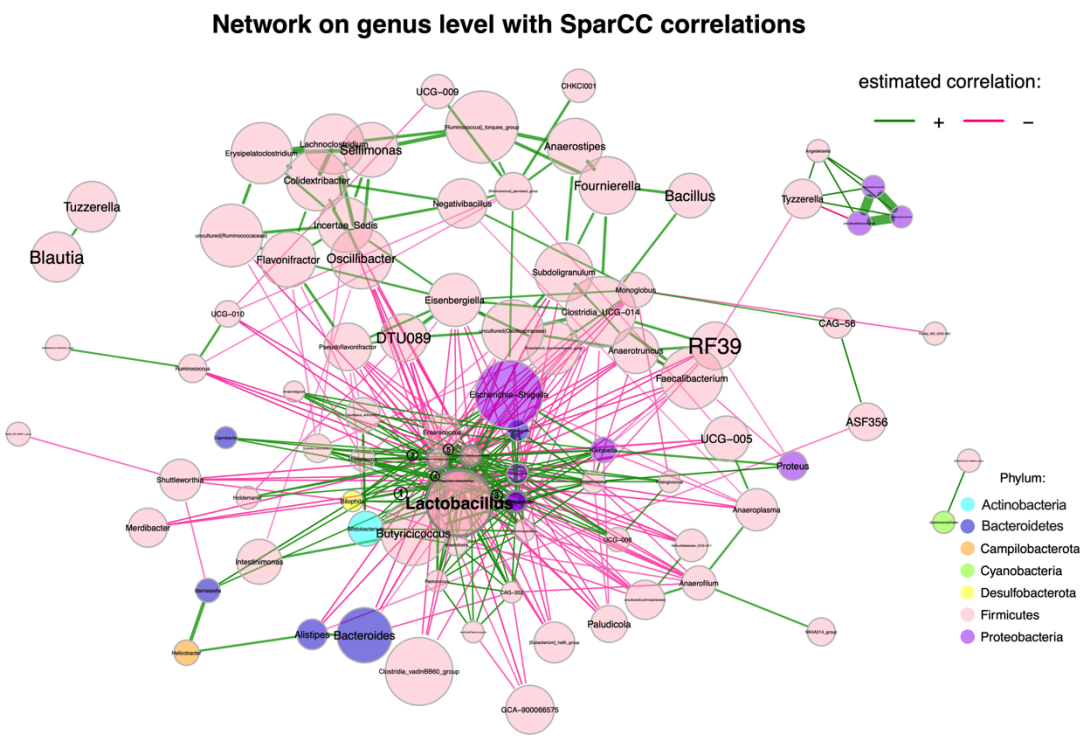

Figure 0.2 Cecal microbiome co-occurrence network based on SparCC correlation on the genus level.

Only significantly correlated ($P < 0.05$) taxa in the cecal microbiome with coefficient greater than 0.3 are shown. Estimated correlations were transformed to dissimilarities via the "signed" distance metric and corresponding similarities were used as edge weights. Node sizes were scaled based on eigenvector centrality. Hubs were defined using eigenvector centrality with a centrality value above the empirical 90% quantile. To increase visibility, hubs were highlighted by bold text and borders and marked as ①,②, ③,④and ⑤. Node colors represented phyla. ①, *Lactobacillus*; ②, *[Clostridium]_methylpentosum_group;* ③, *Acinetobacter;* ④, *Phascolarctobacterium*; ⑤, an uncultured member from the family *Erysipelatoclostridiacea.*

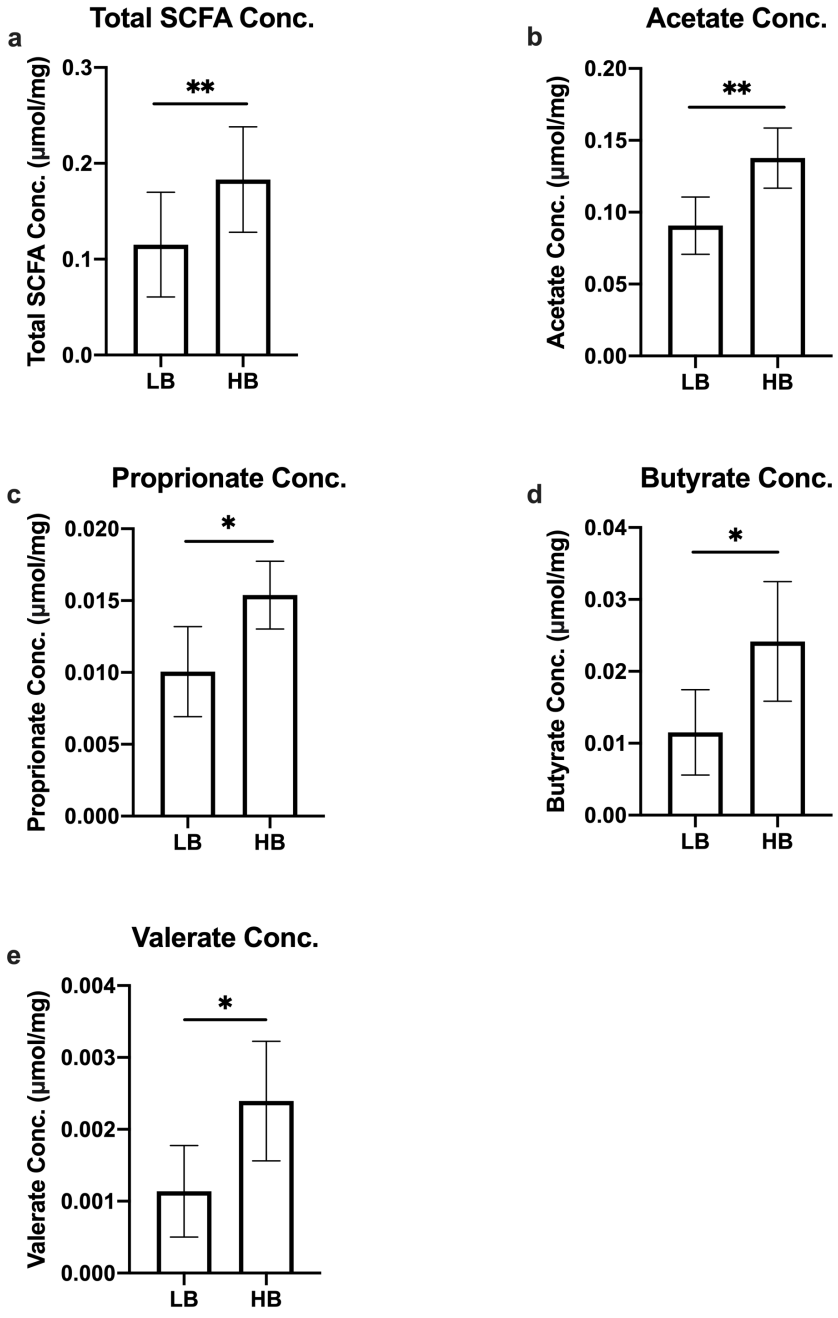

Figure 0.3 Cecal short-chain fatty acid (SCFA) concentrations in LB and HB groups.

Results were shown as (a) total SCFA concentrations, (b) acetate, (c) propionate, (d) butyrate, and (e) valerate (mean ± standard error of the mean, LB: n = 18, HB: n =15, *, P <0.05, **, P < 0.01). LB: low *Bacteroides*; HB: high *Bacteroides*; Conc., Concentration.

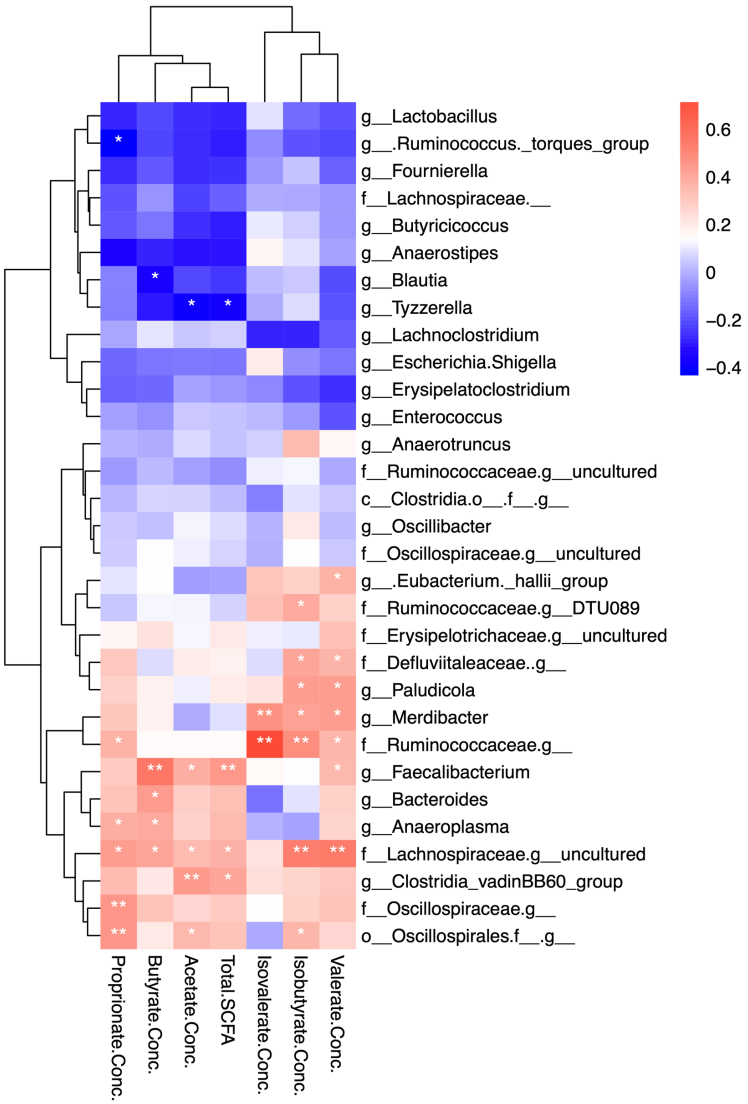

Figure 0.4 Spearman correlation heatmap

Heatmap showing Spearman correlations between cecal bacterial abundance and short-chain fatty acid (SCFA) concentrations in broiler chickens from low- and high-*Bacteroides* groups. *, $P < 0.05$; **, $P < 0.01$; Conc., Concentration.

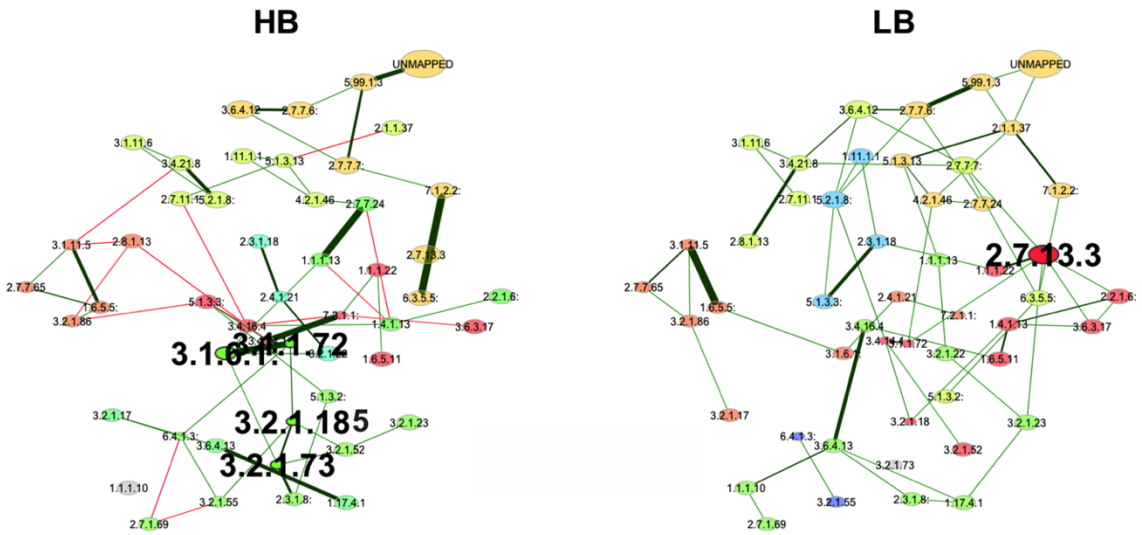

Figure 0.5 The comparison of the functional network harbored by cecal microbial communities.

Green edges corresponded to positive associations and red edges to negative associations. Colors of nodes represented clusters determined by the fast greedy modularity optimization. Node sizes were scaled according to eigenvector centrality. Nodes with bold text were identified hubs in the networks. Hubs were nodes with eigenvector centrality greater than 90% quantile of the empirical centrality distribution (LB: low *Bacteroides*; HB: high *Bacteroides,* LB: n = 18; HB: n = 15).

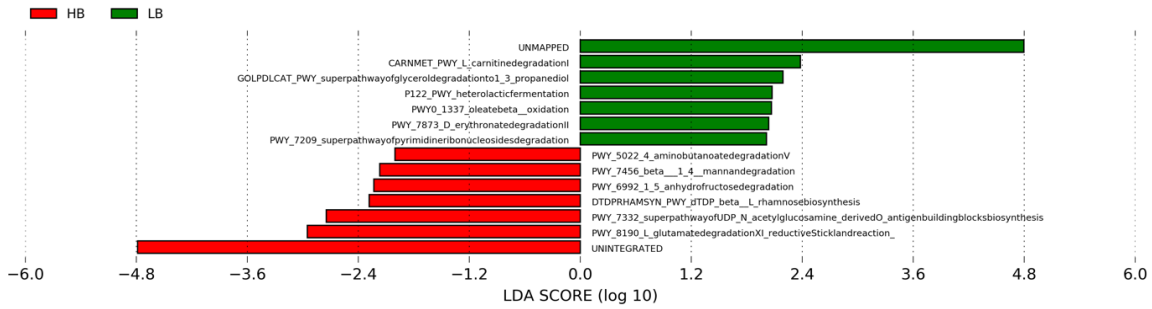

Figure 0.6 Differentially abundant pathways of the cecal microbiota

Linear discriminant analysis (LDA) effect size (LEfSe) showing differentially abundant pathways of the cecal microbiota (LDA score ≥ 2.0; $P < 0.05$; LB: low *Bacteroides*; HB: high *Bacteroides,* LB: n = 18; HB: n = 15).

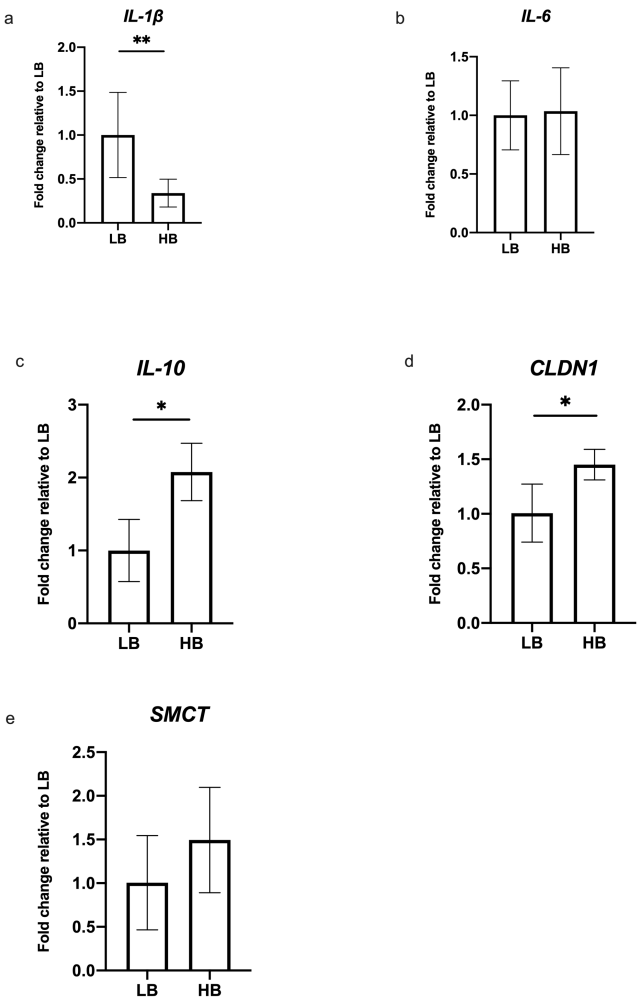

Figure 0.7 Gene expression in chicken cecal tonsil.

(a) Pro-inflammatory cytokine *IL-1β* showed lower expression levels in the HB group compared to LB broiler chickens. (b) *IL-6* expression was not differed between groups. (c) Increased anti-inflammatory cytokine *IL-10* was observed in the HB group. (d) The expression of the tight-junction protein *CLDN1* was slightly increased in the HB group. (e) Compared to the LB group, a trend of increased expression of short-chain fatty acid transporter *SMCT* was seen in the ceca of HB chickens (LB: n = 18; HB: n = 15, mean ± standard deviation, *, $P < 0.05$; **, $P < 0.01$, n.s., not significant; LB: low Bacteroides; HB: high Bacteroides).

Chapter 5: GENERAL DISCUSSION AND FUTURE WORK

5.1 Conclusions and General Discussions

This thesis had shed light on how barn cleaning practices in poultry production affecting the chicken gut microbiome and pathogen abundance. Our study also provided new insights into understanding the effects of possessing a microbiome with elevated *Bacteroides* in early life on the chicken gut microbiology and intestinal immune status.

In the past few decades, the poultry industry has relied on antibiotics to keep the prevalence of pathogens low during production. With the global goal of eliminating growth promotion using medically important antimicrobials and reducing prophylactic antimicrobial use, it is foreseeable that in the future, biosecurity measures such as barn chemical disinfection will be continuously applied frequently in production. As discussed, in the setting of the modern broiler production, the barn is one of the most important microbial sources for chickens that can shape the development of the gut microbiome. With the intent to control pathogen transmission, barn chemical disinfection also removes commensals from the previous flocks, leading to drastically reduced diversity and density of microbes for the chicks to be exposed to. Without competitive exclusion offered by a diverse gut microbiota, one possible consequence of this is that the commercial chickens are sensitive to pathogens (1). In this sense, using chemical disinfectants in barn sanitation may run contrary to its intent. Currently, the effects of the chemically disinfected barns on the chicken gut microbiome (e.g. the microbial composition, the microbial functionality, the resistome) as well as the related host responses were poorly understood. In addition, distinct early-life microbial compositions in the chicken gut were previously reported (2, 3). Perhaps it may also be a consequence of the lack of exposure to a stable, parental-flock-derived microbial community caused by the production mode, because

chickens could develop mature microbiome in less than a week if provided adult-chicken-derived microbes (4). *Bacteroides* is an important chicken commensal to have distinct colonization speed in young chicks with/without contact with adult hens (5). Therefore, understanding the effects of chicken early-life distinct *Bacteroides* compositions and the functional consequences can offer important scientific insights to the poultry industry, particularly with respect to establishing intestinal homeostasis in commercial broiler chickens early in their lives.

In chapter 2, we conducted studies to exam the effects of using chemical disinfectants in barn cleaning on the chicken gut microbiome and the host responses in production. Our results suggested that the barn cleaning methods did not affect chicken bodyweight or flock mortality rate. Using 16S rRNA amplicon sequencing technique and qPCR assays, for the first time, we reported that the chemically disinfected barns had modest but significant effects on the chicken cecal microbial communities. Decreased *Helicobacter,* which was further identified as *Helicobacter pullorum* in chapter 3*,* was observed in the chicken ceca at day 30 in response to the chemically disinfection barn treatment (FD).

In addition, compared to water-washed barn treatment (WW), the chickens from the FD group had decreased cecal short-chain fatty acid (SCFA) concentration associating with almost 1 log increased *Campylobacter jejuni hipO* copy numbers at the end of the production cycle. Previously, de Castro Burbarelli et al. conducted a study in research facility to examine the effects of poultry pen cleaning using neutral detergent versus acidic and alkaline detergent with chemical disinfectants (6). They assessed the effects the cleaning practices on chicken performance as well as the abundance of selected pathogens in the chicken ceca. Their results indicated no treatment effects on the *Campylobacter* occurrence frequency in the chicken gut at the end of the production cycle. However, they observed a trend ($P = 0.076$) that *Campylobacter*

were more frequently detected in the intestine of the stronger disinfection group at slaughter (6). In this thesis, the animal trial was performed in commercial broiler production facilities, with 28 flocks from 7 barns, whereas the previous study by de Castro Burbarelli et al. focused on a laboratory-scale setting and drew conclusions examining only 2 flocks. The increased power in the current study likely decreased the probability a false negative. In addition, instead of using an enrichment assay, the study by de Castro Burbarelli et al. used PCR techniques to test the presence and absence the selected *Campylobacter,* regardless of whether bacteria were dead or alive. Moreover, without assessing the composition of other bacteria and/or nutrients such as SCFAs, it is difficult to further address the reasons led to the observed trend of the different *Campylobacter* detection frequencies (6). Over the years, numerous efforts had been made by the poultry industry to control *C. jejuni*. One promising strategy reducing *Campylobacter* in the chicken gut could be altering the chicken cecal microbiome toward a composition that has increased microbial competition. Our study showed that after a previously healthy flock, WW helped chickens to develop cecal microbiota that did not favor *C. jejuni* growth, which could be made immediate practical by the industry during production. In addition to *C. jejuni,* this thesis also assessed the effects of barn sanitation practices on other pathogens in the chicken gut, such as *Salmonella* and *Clostridium perfringens,* and showed no differences on detection frequency or abundance between treatments. In fact, during the study period for almost 1 year, *Salmonella* was not detectable in both the barns and the chicken gut while cecal *C. perfringens* was at a very low level. These results can provide confidence to the industry that using WW can assure food safety while maintaining productivity.

Further in Chapter 3, with the help of shotgun metagenomic sequencing techniques, we were able to gain bacterial taxonomic information to the species level and assess the cecal

microbial functional consequences affected by the barn cleaning treatments. We found that both the cecal microbial composition and functionality were significantly altered by the barn cleaning methods. At day 7, the cecal microbial functionality of the FD chickens had enriched stringent response pathway with decreased amino acid synthesis capacities. At day 30, WW had increased the relative abundance of *H. pullorum, Barnesiella viscericola, Faecalibacterium prausnitzii* and *Ruminococcus torques* in the cecal microbiota at D30 compared to FD. The increased *H. pullorum* composition directly resulted in the increased functional compacity of SCFA and amino acid synthesis in the cecal microbiome of the D30 WW chickens. In line with that, it has been shown that *H. pullorum* was associated with increased starch digestibility and mature bodyweight in chicken (7). It supported that WW could benefit broiler production by increasing some important commensals in the chicken gut and thereby improve the microbial functional capacity of nutrient utilization and decrease pathogen colonization. *H. pullorum* has been frequently detected in poultry world-wide (8). Although being frequently detected in poultry worldwide and considered as opportunistic pathogens by some researchers, very few disease cases were reported directly linked to *H. pullorum* infection (8, 9). Therefore, the role of *H. pullorum* in poultry and human health needs to be further assessed. Regarding the chicken gut microbial functional capacity, to date, little is known about how it was affected by chemical disinfectants in barn cleaning. Previous studies on chemical exposure reported that disinfectants modulate environmental microbes by affecting their functions through directly inducing metabolic change (such as activation of the stringent response) (10), or by triggering bacteria to enter competent state to acquire foreign DNA to overcome the harsh environment (11). Therefore, to animals exposed to these environments, their gut microbial functionality will also be altered in response to the change of the environmental microbial functionality (10, 12).

Surprisingly, data on the cecal microbial resistome did not support our original hypothesis; instead of increasing antibiotics resistant gene (ARG) incidence in the chicken gut. FD decreased the abundance of some persistent ARGs in the chicken ceca, such as the *erm* gene family and glycopeptide resistance gene clusters. Although some chemical disinfectants exhibited selection stress to stimulate ARG proliferation and/or transmission (13-17), these studies assessed the effects of single chemical disinfecting agent. In this thesis, the chemical disinfectants included sodium hydroxide, 2-(2-2-butoxyethoxy) ethanol, sodium laureth sulfate, sodium N-lauroyl sarcosinate, tetrasodium EDTA, glutaraldehyde, benzalkonium chloride (BAC), and formic acid. The combination of these chemicals may have worked together as a broader spectrum antimicrobial agent, which was more effective in removing bacterial genetic elements carrying ARGs.

Chapter 2 and 3 represent the first studies in Canada, and amongst a few globally, directly comparing WW and FD and evaluating their effects on the chicken gut microbiome. These studies provide important information to the poultry industry. In the context of helping chickens to develop a gut microbiome with improved pathogen defense and microbial nutrient utilization, in barns without a previous diseases outbreak, WW should be considered with priority in production. In the context of controlling antibiotic resistance, especially the persistent ARGs, FD were more effective.

Interestingly, we observed drastic differences between the cecal microbial resistomes of the day 7 and day 30 chickens. Comparing the effect size of the barn cleaning practices on the chicken resistome, we found that the treatments had greater impact at day 7 than day 30. Further focusing just on the age differences, the antibiotic efflux pumps were identified as strong biomarkers of the 7-day chicken resistome, whereas ARGs with higher specificity on targets (e.g.

tetracycline inactive enzymes) became more predominant at day 30. In addition, the day 7 chicken resistome showed higher abundance and diversity in comparison to day 30. Previously, similar age-dependent pattern was reported in a human study (18). In poultry, far less is known about the effect of age on the gut microbial resistome. Thus, our results form a basis for future studies investigating chicken gut microbial resistome that chicken age should be taken into consideration when assessing treatment effects.

In chapter 4, we identified cecal microbiota with distinct *Bacteroides* compositions among the 7-day-old commercial chickens, namely the *Bacteroides*-under-represented (LB) and *Bacteroides*-over-represented (HB). Interestingly, we found that the cecal abundance of *Lactobacillus* was negatively correlated to *Bacteroides,* whereas an obligate anaerobe *Alistipes* was associated with *Bacteroides*. While numerous studies have considered *Lactobacillus* as probiotics, many of these studies also reported that the cecal *Lactobacillus* abundance was not affected by their treatment (19, 20) indicating that the beneficial effects by *Lactobacillus* may not be a consequence of cecal colonization. Chen et al. studied the effect of the supplementation of *Lactobacillus* spp. and/or yeast with bacteriocin on broiler performance and looked at the cecal microbiota (21). They reported that supplemented *Lactobacillus* culture alone (without bacteriocin) was the only treatment group that increased the level of cecal *Lactobacillus,* but it also led to decreased SCFA production with no improvement on performance. Thus, although supplementing *Lactobacillus* had been shown to exert beneficial effects on poultry, the effects of cecal *Lactobacillus* colonization needs to be carefully assessed in the future.

In addition, chicken cecal tonsil gene expression revealed that the HB group had altered cecal tonsil immune profile, which showed decreased of pro-inflammatory cytokine expression and increased anti-inflammatory expression. Perhaps due to the fact that using 16S rRNA

amplicon sequencing was sufficient to comprehensively study microbial functionality, although great variation of the cecal *Bacteroides* composition was observed previously, very little information is available about how distinct *Bacteroides* population influence the gut microbial functional capacities. In our study, we found that the functionality of the HB cecal microbiota, which was centered by a series of enzymes linked to poly-saccharide utilization, were significantly different from the LB counterparts that had a histidine kinase centre. In addition, microbiota of the HB chickens exhibited a pronounced increase in capacity for mono- and poly-saccharide utilization, which was further reflected by the increased cecal SCFAs. By far, high variations of *Bacteroides* had been reported from some studies without being further assessed (22, 23). Without investigation on the microbial functional consequences and host responses, it was difficult to have a comprehensive understanding of what this variation in the gut microbiota means to the commercial broiler chickens. Our results compared and characterized HB- and LB-microbiotas that exist in early life of the commercial broiler chickens. Given all the positive outcome exhibited by the HB-microbiota, it suggested that early colonization of *Bacteroides* in the chicken gut may be a sign of the advanced development of a more mature chicken microbiota, which favors other obligate anaerobic co-colonizers, such as *Alistipes*.

Modern broiler chickens are believed to be domesticated from the red jungle fowls thousands of years ago (24). Over 70 years of commercial practices in poultry breeding, humans have greatly changed and improved their genetic potential for meat production (25). The modern broiler industry also changed the ways that chickens have been living for millions of years by hatching eggs separately without hens in clean hatcheries and housing chickens in barns with limited contact to the wild. The change of both host genetics and living environments may have resulted in interruptions of the chicken gut microbiota, which have co-evolved with chicken for

millennia. In fact, the lack of contact with adult chickens raised concern over narrowing the microbial exposure for commercial broiler chickens to assemble and develop a balanced microbiota (5, 26).

5.2 Limitations and Future Work

Studies included in the thesis are based on commercial broiler chickens obtained from a mass-production system. Although most findings supported the hypothesis that the barn cleaning practices drives changes in the chicken gut microbiome and subsequent host physiology, there are still some limitations that should be acknowledged.

In these studies, individual chicken performance data, such as individual body weight, feed conversion ratio were not measured. Particularly in Chapter 4 studying early-life microbial structure and related host responses, the lack of individual performance limited our ability to interpret the positive outcomes brought by the elevated cecal *Bacteroides* population. In production, chicken producers focus mainly on chicken performance at the flock level. When assessing the effects of barn cleaning methods, barns were considered as experimental units, therefore chicken performance data in these studies were presented as the average body weight and mortality of the production flock. Although other individual host-related data, such as individual cecal SCFA concentration and cecal tonsil gene expression were measured, individual performance data are still of great importance, especially with respect to understand the host-related consequences in response of altered microbial community and functionality and nutrient metabolism.

Although the chickens sampled in these studies were treated with the same diet management, detailed diet information could not be provided by the chicken producers. It is noteworthy to mention diets play an influential role in shaping the chicken gut microbiota and is an important piece of information in gut microbiome studies (27-30). To improve our ability to interpret the observed effects of the barn cleaning practices on the chicken gut microbiome, future studies examining the effects barn cleaning practices in combination with different diet compositions are highly expected. Similarly, although these studies were performed within the same production system as a crossover design trying to eliminate bias, it was still difficult to strictly control animal management between flocks (e.g. handling crew, flock density, barn temperature, and feed/water quality) during production. Therefore, it was hard to comment on the effects of these confounding factors on the chicken gut microbiome. For example, we found that the cecal resistome of chickens sampled from the two timepoints (day 7 and day 30) had significant differences. However, we were not able to make conclusion that what is the main driver of the observed differences. Factors such as the chicken diets, the physiological changes of the chicken gut, the stoking densities, the feed/water quality differences, and even the temperature of the chicken barn might each/collectively contribute to the alteration of the gut resistome between the two sampling timepoints. Therefore, future studies in controlled environments and control diets to assess how these factors affect the chicken gut resistome should be conducted.

Limited by logistics, in these studies, only 5 chickens were sampled in each flock. As discussed in chapter 2 that to avoid "co-housing effect", we decided to increase replicates of the treatment barns in our studies instead of sampling more chickens from the same barn. In addition, in the studies of the barn cleaning practices, the animal trial was designed as a cross-over

experiment to help further eliminate bias brought by the housing facilities. However, since increased sample size can increase statistical power (31), future studies with more samples are warranted.

In chapter 4, selected host immune-related genes were measured. Although the altered expression of a couple of immune-related genes can be indicative of the host immune status, to more comprehensively understand the host-immune response, transcriptomic analyses using RNA-seq on the chicken cecal tonsils may be necessary in the future. In addition, what this immune status truly means in the context of pathogen defense was not studied. Therefore, future studies using a pathogen to challenge young chickens with distinct cecal *Bacteroides* will be of great value.

In these studies, microbial functional characteristics of the transcriptomic and proteomic levels remain to be investigated. High throughput metagenomic sequencing used in these studies offered valuable information about the microbial taxonomic compositions and their genetic potentials. However, it is not possible to identify the expression of these genes. Since not all encoded genes lead to the translation as functional proteins, our results were not able to identify if the enriched functional pathways were actively being expressed. Therefore, in the future, microbial transcriptomic and proteomic analyses should be performed.

In chapter 3, we have used the Comprehensive Antibiotic Resistance Database (CARD) to annotate ARG genes. However, other databases such as the Antibiotic Resistance Genes Database (32), the Antibiotic Resistance Gene-ANNOTation (33), the MEGARes database (34), National Database of Antibiotic Resistant Organisms (NDARO) (35) and ResFinder (36) are also available for ARG annotation. Very recently, Papp and Solymosi comprehensively reviewed and

compared multiple databases and concluded that both NDARO and CARD are prominent among other databases tested (37). With comparable numbers of ARGs, NDARO focuses more on acquired resistance genes whereas CARD focuses more on mutation conferring resistnace (37). Although in general, Papp and Solymosi suggested that CARD should be the first choice for ARG studies, they also suggested that differences could be observed in ARG nomenclature due to the differences of the databases and thereby using only one database may introduce bias. Therefore, to comprehensively understand the chicken gut resistomes in our studies, data analyses using other ARG databases (e.g. NDARO) is necessary in the future.

In addition, although the concentrations of selected cecal SCFA were measured, other metabolites such as cecal amino acids and saccharide concentrations remains unknown. While increased genetic potential of amino acids and saccharide utilizations were shown accompanying changes of cecal SCFAs, it would be more insightful if the concentrations of the related saccharides were unveiled. In future studies these could be measured by metabolomic methods such as high-performance liquid chromatography or hydrophilic interaction liquid chromatography coupled tandem mass spectrometry (HILIC-tandem MS) (38). Thus, future metabolic analyses on the cecal contents are warranted.

In chapter 4, we identified distinct *Bacteroides* compositions in the chicken cecal microbiota and suggested that the *Bacteroides* colonization may be of great importance to the gut homeostasis. However, what caused the distinct *Bacteroides* levels was not investigated in the thesis. Previously, it was reported that early exposure to chicken-derived microbes increased the cecal colonization of *Bacteroides* (5, 39, 40). Thus, we speculated that the chicks with elevated *Bacteroides* observed in the current study may be a consequence of being in contact with these microbes fortuitously in the hatchery and/or during transportation. Thus, in the future, using

germ-free chickens to mimic chicken-derived microbial exposure may be able to answer this question. In addition, the causal effect of *Bacteroides* in observed SCFA and host immunity remains unclear. To further address this, defined amount of known *Bacteroides* species could be introduced to broiler chicks at different timepoints using a germ-free chicken model. In addition, a pathogen challenge and/or a subclinical necrotic enteritis disease model could be used with defined *Bacteroides* as a treatment to investigate if *Bacteroides* confer anti-inflammatory effects.

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APPENDICES: SUPPLEMENTARY TABLES AND FIGURES

Table S1: Genetic pathways harbored by the gut microbial community associated with chicken age. – log2 fold-change and + log2 fold-change values indicate associations with D7 and D30 microbial functional capacities, respectively.

Age-related differentiate abundant pathways	log2 fold-change	*P*-value	FDR *P* value
GALACTARDEG-PWY: D-galactarate degradation I	-2.2687	1.77E-28	3.07E-27
GLUCARGALACTSUPER-PWY: super-pathway of D-glucarate and D-galactarate degradation	-2.2687	1.77E-28	3.07E-27
PWY0-1477: ethanolamine utilization	-2.2306	7.78E-57	2.43E-54
PWY-6588: pyruvate fermentation to acetone	-2.227	3.61E-33	1.03E-31
PWY66-409: super-pathway of purine nucleotide salvage	-2.0384	1.78E-22	2.22E-21
GLUCARDEG-PWY: D-glucarate degradation I	-2.0347	2.28E-28	3.74E-27
PWY-6961: L-ascorbate degradation II (bacterial, aerobic)	-1.9453	1.30E-19	1.45E-18
PWY0-301: L-ascorbate degradation I (bacterial, anaerobic)	-1.833	9.25E-18	8.74E-17
PWY0-1297: super-pathway of purine deoxyribonucleosides degradation	-1.7944	1.41E-29	3.14E-28
P161-PWY: acetylene degradation (anaerobic)	-1.792	5.50E-39	3.43E-37
GALACTITOLCAT-PWY: galactitol degradation	-1.7755	3.57E-20	4.28E-19
PWY0-1277: 3-phenylpropanoate and 3-(3-hydroxyphenyl) propanoate degradation	-1.7474	4.10E-16	3.12E-15
FAO-PWY: fatty acid β-oxidation I (generic)	-1.7348	3.13E-14	1.88E-13
PWY-7013: (S)-propane-1,2-diol degradation	-1.709	1.40E-10	6.31E-10
PWY-5723: Rubisco shunt	-1.7071	2.08E-14	1.27E-13
SALVADEHYPOX-PWY: adenosine nucleotides degradation II	-1.7038	6.61E-34	2.58E-32

HCAMHPDEG-PWY: 3-phenylpropanoate and 3-(3-hydroxyphenyl) propanoate degradation to 2-hydroxypentadienoate	-1.6991	3.57E-16	2.86E-15
PWY-6690: cinnamate and 3-hydroxycinnamate degradation to 2-hydroxypentadienoate	-1.6991	3.69E-16	2.88E-15
PRPP-PWY: super-pathway of histidine, purine, and pyrimidine biosynthesis	-1.6672	5.68E-10	2.33E-09
AST-PWY: L-arginine degradation II (AST pathway)	-1.6357	1.97E-16	1.62E-15
PWY-5676: acetyl-CoA fermentation to butanoate II	-1.6081	4.02E-40	3.13E-38
PWY-6606: guanosine nucleotides degradation II	-1.6023	8.56E-29	1.67E-27
PWY-5136: fatty acid β-oxidation II (plant peroxisome)	-1.5914	1.14E-25	1.61E-24
PWY0-1338: polymyxin resistance	-1.5642	1.21E-14	8.05E-14
PWY-7242: D-fructuronate degradation	-1.56	9.25E-23	1.20E-21
PWY-7858: (5Z)-dodecenoate biosynthesis II	-1.5501	3.57E-13	1.95E-12
PWY0-1298: super-pathway of pyrimidine deoxyribonucleosides degradation	-1.5497	1.58E-23	2.14E-22
ORNDEG-PWY: super-pathway of ornithine degradation	-1.546	7.28E-13	3.92E-12
PWY-5138: fatty acid β-oxidation IV (unsaturated, even number)	-1.5453	8.38E-16	6.22E-15
PWY-5367: petroselinate biosynthesis	-1.4997	6.20E-15	4.20E-14
HEXITOLDEGSUPER-PWY: super-pathway of hexitol degradation (bacteria)	-1.4995	4.86E-14	2.81E-13
PWY-7942: 5-oxo-L-proline metabolism	-1.4813	8.78E-13	4.64E-12
PWY4LZ-257: super-pathway of fermentation (Chlamydomonas reinhardtii)	-1.4774	1.09E-33	3.79E-32
PWY-5675: nitrate reduction V (assimilatory)	-1.4423	7.83E-10	3.13E-09
PWY-6507: 4-deoxy-L-threo-hex-4-enopyranuronate degradation	-1.4276	2.23E-17	2.05E-16
PWY-6608: guanosine nucleotides degradation III	-1.4213	3.07E-28	4.79E-27

PWY-7111: pyruvate fermentation to isobutanol (engineered)	-1.3935	5.58E-35	2.90E-33
PWY0-42: 2-methylcitrate cycle I	-1.3784	2.57E-11	1.23E-10
PWY0-1415: super-pathway of heme b biosynthesis from uroporphyrinogen-III	-1.372	8.12E-11	3.72E-10
PWY-6353: purine nucleotides degradation II (aerobic)	-1.3545	2.71E-27	4.02E-26
ECASYN-PWY: enterobacterial common antigen biosynthesis	-1.353	3.50E-14	2.06E-13
PWY-6284: super-pathway of unsaturated fatty acids biosynthesis (E. coli)	-1.3379	2.01E-09	7.74E-09
PWY-5860: super-pathway of demethylmenaquinol-6 biosynthesis I	-1.3299	4.62E-09	1.70E-08
PWY-5100: pyruvate fermentation to acetate and lactate II	-1.3127	2.39E-17	2.13E-16
PWY-6936: seleno-amino acid biosynthesis (plants)	-1.3024	3.64E-33	1.03E-31
PWY-7118: chitin deacetylation	-1.291	1.76E-09	6.87E-09
PWY-I9: L-cysteine biosynthesis VI (from L-methionine)	-1.289	1.15E-18	1.16E-17
PWY-7237: myo-, chiro- and scyllo-inositol degradation	-1.2788	4.93E-19	5.31E-18
PWY-5850: super-pathway of menaquinol-6 biosynthesis	-1.2775	2.13E-07	6.21E-07
PWY0-781: aspartate super-pathway	-1.2458	4.30E-09	1.60E-08
P41-PWY: pyruvate fermentation to acetate and (S)-lactate I	-1.2414	2.77E-18	2.70E-17
P4-PWY: super-pathway of L-lysine, L-threonine and L-methionine biosynthesis I	-1.2388	5.20E-09	1.84E-08
PWY-5384: sucrose degradation IV (sucrose phosphorylase)	-1.2329	1.08E-11	5.33E-11
GLYCOLYSIS-TCA-GLYOX-BYPASS: super-pathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass	-1.2268	3.14E-08	1.01E-07
GLYOXYLATE-BYPASS: glyoxylate cycle	-1.2197	5.20E-10	2.18E-09

HEME-BIOSYNTHESIS-II-1: heme b biosynthesis V (aerobic)	-1.218	4.96E-09	1.79E-08
KETOGLUCONMET-PWY: ketogluconate metabolism	-1.2137	4.41E-08	1.39E-07
PWY-6285: super-pathway of fatty acids biosynthesis (E. coli)	-1.14	7.36E-06	1.74E-05
PWY-561: super-pathway of glyoxylate cycle and fatty acid degradation	-1.1355	1.40E-07	4.24E-07
TCA-GLYOX-BYPASS: super-pathway of glyoxylate bypass and TCA	-1.0869	4.48E-07	1.24E-06
PWY-7385: 1,3-propanediol biosynthesis (engineered)	-1.0835	2.85E-07	8.17E-07
PWY-5920: super-pathway of heme b biosynthesis from glycine	-1.062	1.85E-08	6.19E-08
PWY-8004: Entner-Doudoroff pathway I	-1.0498	1.65E-13	9.18E-13
PWY-7211: super-pathway of pyrimidine deoxyribonucleotides de novo biosynthesis	-1.0478	3.45E-12	1.79E-11
HOMOSER-METSYN-PWY: L-methionine biosynthesis I	-1.0376	1.64E-07	4.88E-07
P108-PWY: pyruvate fermentation to propanoate I	-1.0221	1.14E-05	2.59E-05
PWY66-389: phytol degradation	-1.0002	3.90E-05	8.51E-05
PPGPPMET-PWY: ppGpp metabolism	1.1234	4.56E-06	1.12E-05
PWY-8073: lipid IVA biosynthesis (P. putida)	1.1533	1.32E-14	8.55E-14
NAGLIPASYN-PWY: lipid IVA biosynthesis (E. coli)	1.1533	1.34E-14	8.55E-14
P42-PWY: incomplete reductive TCA cycle	1.1623	7.09E-10	2.87E-09
PWY-1269: CMP-3-deoxy-D-manno-octulosonate biosynthesis	1.1729	1.60E-16	1.35E-15
PWY-5121: super-pathway of geranylgeranyl diphosphate biosynthesis II (via MEP)	1.2348	5.25E-10	2.18E-09
PWY-6969: TCA cycle V (2-oxoglutarate synthase)	1.3108	3.84E-15	2.66E-14
PWY-6902: chitin degradation II (Vibrio)	1.3415	4.09E-08	1.30E-07

PWY-7388: octanoyl-[acyl-carrier protein] biosynthesis (mitochondria, yeast)	1.4459	5.00E-09	1.79E-08
CITRULBIO-PWY: L-citrulline biosynthesis	1.4899	9.41E-19	9.79E-18
PWY-7254: TCA cycle VII (acetate-producers)	1.579	1.43E-14	8.92E-14
PWY0-1241: ADP-L-glycero-β-D-manno-heptose biosynthesis	1.6134	6.43E-12	3.24E-11
PWY-6834: spermidine biosynthesis III	1.6637	6.04E-09	2.12E-08
PWY-7371: 1,4-dihydroxy-6-naphthoate biosynthesis II	2.4597	5.28E-12	2.70E-11
PWY-7392: taxadiene biosynthesis (engineered)	2.4911	1.14E-30	2.97E-29
PWY-6922: L-Nδ-acetylornithine biosynthesis	2.5988	2.77E-29	5.77E-28
PWY-4984: urea cycle	2.6001	4.86E-17	4.21E-16
PWY-7992: super-pathway of menaquinol-8 biosynthesis III	2.7342	5.23E-14	2.97E-13
PWY-5030: L-histidine degradation III	2.9661	7.08E-30	1.70E-28
POLYAMINSYN3-PWY: super-pathway of polyamine biosynthesis II	2.9832	8.84E-49	9.19E-47
PWY-5005: biotin biosynthesis II	3.8625	2.64E-34	1.18E-32
PWY-6906: chitin derivatives degradation	4.0059	1.29E-49	2.01E-47

Table S2: Functional network properties*.

a) Cluster at HB

Name	Frequency
0	1
1	8
2	8
3	7
4	11
5	5
6	4
7	3
8	3

b) Cluster at LB

Name	Frequency
0	1
1	8
2	10
3	9
4	8
5	8
6	4
7	2

c) Degree (Normalized)

Gene	HB	LB
Highest values in the HB group		
3.4.16.4: Serine-type D-Ala-D-Ala carboxypeptidase	0.12245	0.04082
3.1.6.1: Arylsulfatase (type I)	0.08163	0
7.2.1.1: NADH:ubiquinone reductase (Na(+)-transporting)	0.08163	0
3.2.1.185: Non-reducing end beta-L-arabinofuranosidase	0.08163	0.04082
5.1.3.13: dTDP-4-dehydrorhamnose 3,5-epimerase	0.08163	0.06122
Highest values in the LB group		
2.7.13.3: Histidine kinase	0	0.12245
5.2.1.8: Peptidylprolyl isomerase	0.06122	0.10204
2.1.1.37: DNA (cytosine-5-)-methyltransferase	0.02041	0.10204
6.3.5.5: Carbamoyl-phosphate synthase (glutamine-hydrolyzing	0	0.10204

3.6.4.12: DNA helicase	0	0.08163

d) Betweenness centrality

Gene	HB	LB
Highest values in the HB group		
3.4.16.4: Serine-type D-Ala-D-Ala carboxypeptidase	0.36538	0.06117
3.1.6.1: Arylsulfatase (type I)	0.35897	0
1.1.1.133: dTDP-4-dehydrorhamnose reductase	0.30256	0.0256
1.4.1.13: Glutamate synthase (NADPH	0.25128	0.1138
3.1.1.72: Acetylxylan esterase	0.23462	0.11095
Highest values in the LB group		
6.3.5.5: Carbamoyl-phosphate synthase (glutamine-hydrolyzing)	0	0.3101
3.2.1.23: Beta-galactosidase	0	0.23471
2.7.13.3: Histidine kinase	0	0.23186
3.4.14.12: Xaa-Xaa-Pro tripeptidyl-peptidase	40	0.17212
2.7.7.7: DNA-directed DNA polymerase	0	0.1707

e) Closeness centrality

Gene	HB	LB
Highest values in the HB group		
3.4.16.4: Serine-type D-Ala-D-Ala carboxypeptidase	0.6044	0.42686
3.1.6.1: Arylsulfatase (type I)	0.55322	0
1.4.1.13: Glutamate synthase (NADPH)	0.54411	0.52069
1.1.1.133: dTDP-4-dehydrorhamnose reductase	0.52886	0.43419
5.1.3.3: Aldose 1-epimerase	0.52224	0.46572
Highest values in the LB group		

2.7.13.3 histidine kinase	0	0.67076
6.3.5.5: Carbamoyl-phosphate synthase (glutamine-hydrolyzing)	0	0.64418
2.7.7.7: DNA-directed DNA polymerase	0	0.62537
3.6.4.12: DNA helicase	0	0.61005
5.2.1.8: Peptidylprolyl isomerase	0.44302	0.60121

* a) Frequency table of clusters in the HB group network. b) Frequency table of clusters in the LB group network. c-e) Centrality values of the encoded enzyme with the highest centrality in decreasing order. The upper part of the table contains the 5 genes with the highest centrality in HB and the lower part those with the highest centrality in LB, respectively

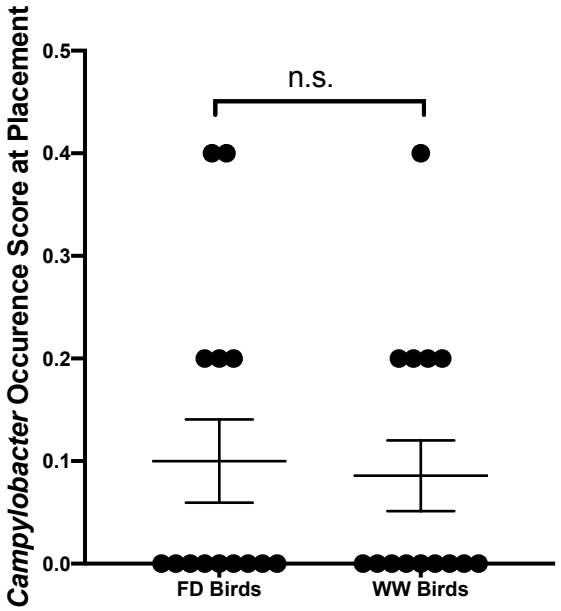

Figure S1. Broiler chicken cecal Campylobacter occurrence score at placement. Results showed the mean flock score ± SEM (n = 14/treatment, n.s. : $p > 0.05$). *Campylobacter* occurrence score = number of pathogen positive broilers/total number of broilers sampled per barn. WW, water-wash; FD, full disinfection

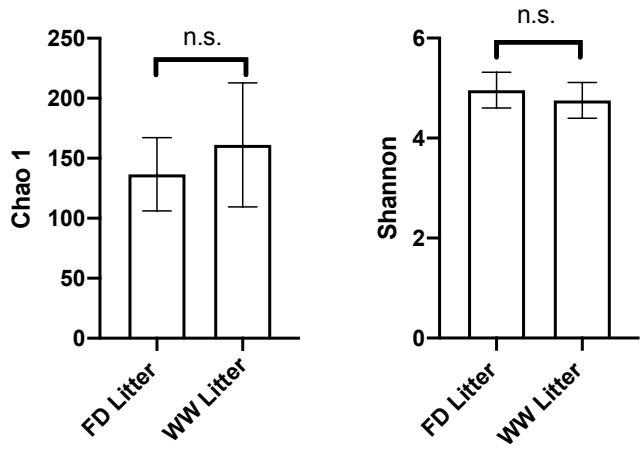

Figure S2. Alpha diversity of the microbiome of the litter samples collected before the broiler placement. Box-plots showing alpha diversity in samples using Chao1 index and Shannon index (n=12/treatment, n.s., $p > 0.05$). WW, water-wash; FD, full disinfection.

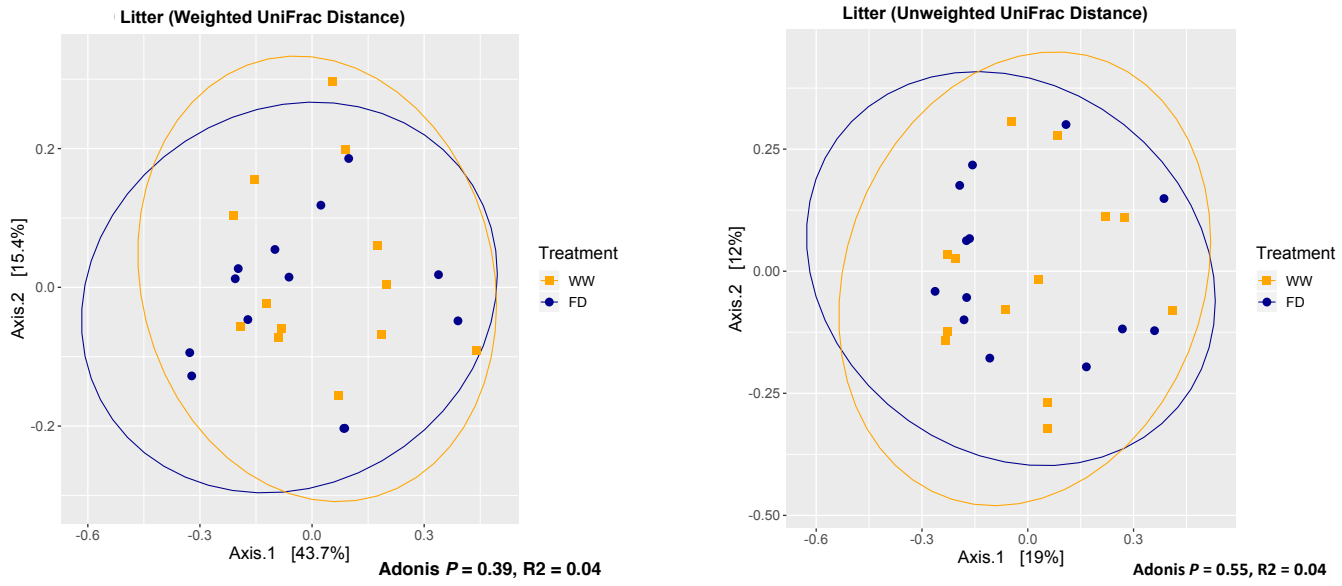

Figure S3. Principal coordinate analysis plots based on weighted- and unweighted- Unifrac distance metrics. Barn cleaning treatments did not influence the microbial community structure in the barn litter samples before broiler placement (n=12/treatment). WW, water-wash; FD, full disinfection.

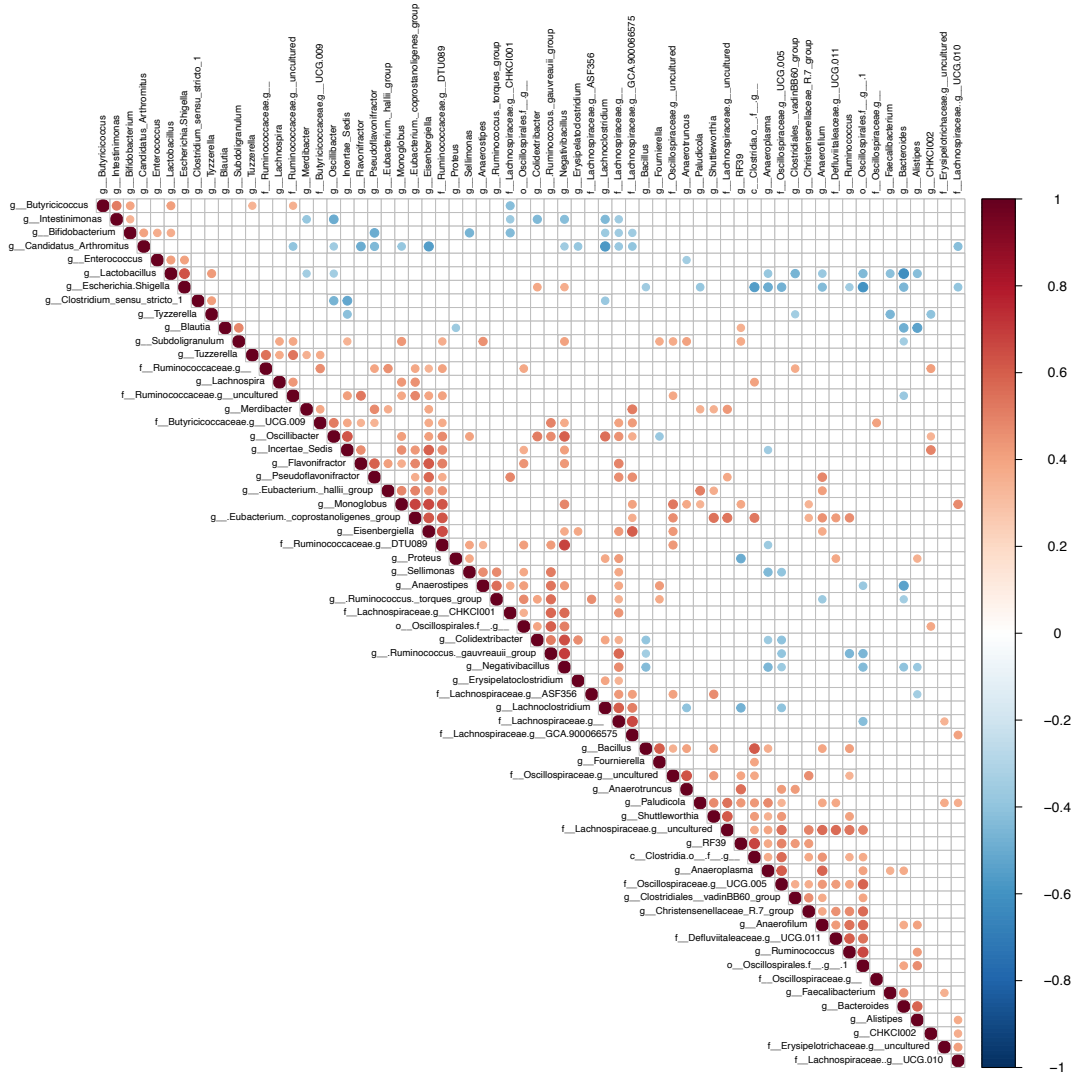

Figure S4. Spearman's correlation between cecal microbial taxa. Only significantly correlated taxa ($P < 0.05$) were shown. Spearman correlations between microbes revealed that Bacteroides was negatively correlated with the genera *Lactobacillus, Escherichia-Shigella, Blautia, Subdoligranulum, Anaerostipes, Negativibacillus*, the [*Ruminococcus*]-*torques*-group, and an uncultured genus belonging to the family Ruminococcaceaea. In addition, the genera *Faecalibacterium*, *Anaerofilum*, *Anaeroplasma*, *Alistpes*, as well as an undetermined genus from the order Oscillospirales showed positive correlations to *Bacteroides*.

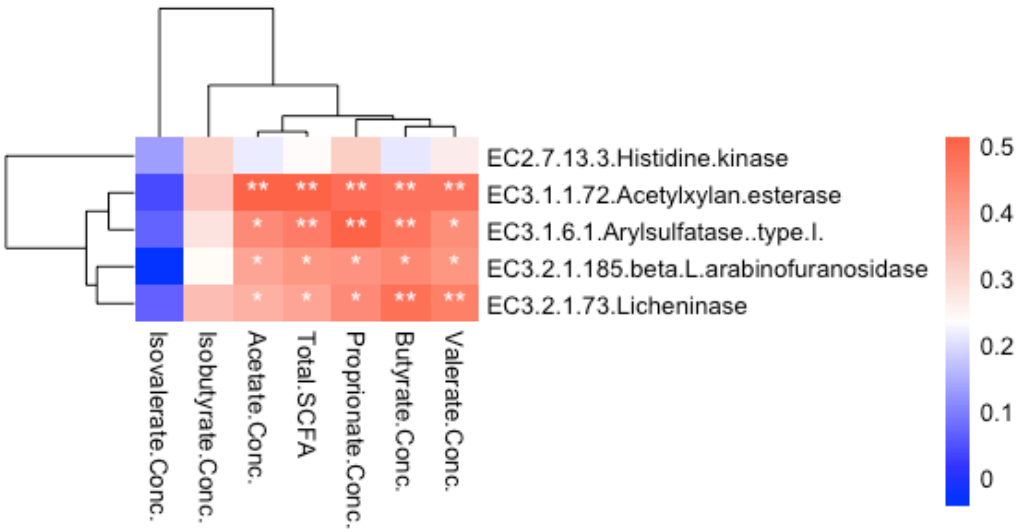

Figure S5. Spearman's correlation between the key enzymes of the chicken cecal microbial functional network and cecal SCFA concentrations. Key enzymes identified in the microbial functional network of the microbiome with elevated *Bacteroides* (i.e. acetylxylan esterase, arylsulfatase, β-L-arabinofuranosidase and licheninase) were significantly associated with the chicken cecal SCFA concentrations at day 7.