EFFECTS OF EARLY-LIFE CECAL MICROBIOTA TRANSPLANTATON ON AGGRESSIVE BEHAVIOR AND HEALTH IN ROOSTERS

by

Yuechi Fu

A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Animal Sciences

West Lafayette, Indiana

December 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Timothy Johnson, Chair

Department of Animal Sciences

Dr. Heng-wei Cheng

Department of Animal Sciences

Dr. Marisa Erasmus

Department of Animal Sciences

Approved by:

Dr. Zoltan Machaty

Dedicated to my parents for their unconditional love and support.

ACKNOWLEDGMENTS

Over the past two years, many great people made this dissertation possible. First and foremost, I would like to express my deepest gratitude for my major advisor, Dr. Heng-wei Cheng, the first supervisor who led me to the world of scientific research. His patience, guidance, support, and encouragement contributed to the accomplishment of my experiment and dissertation, making my first graduate school journey very positive and interesting.

Next, I would like to thank my committee members, Dr. Marisa Erasmus and Dr. Timothy Johnson, for taking the time to discuss my project with me and giving me valuable suggestions. Also, I would like to thank Dr. Jiaying Hu. She is not only the mentor who taught me volumes of knowledge and techniques in the laboratory but also a friend outside the research. Many thanks to the staff of Purdue Animal Sciences Poultry Research Center, Jason S Fields, Kordell Synesael, and Barry Little, for their daily care of animals and assistance with my experiment. Thanks to the technicians, staff, and scientists in the USDA-ARS-LBRU, particularly a big thank to Amanda Long for helping me to send the samples and order the various experimental kits. I really appreciate the encouragement and help received from Dr. Kolapo Ajuwon and Dr. Cindy Nakatsu for taking the time to answer my questions and offer valuable suggestions.

Last but not least, thanks must go to my parents, Ren, Manqiong and Fu, Bi, for their unconditional love, encouragement, and great understanding. They are the reason why I can grow up healthily and happily. They always support my decision and encourage me through countless video calls. I am grateful to my boyfriend, Jiansen Wang, for his encouragement and understanding. Although he has no idea about animal sciences, he is always the first audience who patiently listens to each of my presentations. Thank my friends at Purdue University, Xu Gao, Ziming Cao, Jing Yuan, Yufei Guo, Saud Ur Rehman, Opeadura Osunbami, and Yiru Dong, for their support, inspiration, and suggestions during my study and daily life at Purdue University.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HT	serotonin
5-HTergic	serotonergic
5-HIAA	5-hydroxuindoleacetic acid
5-HTT	serotonin transporter
6_3-CMT	cecal bacterial solution of 6_3 strain
7_2-CMT	cecal bacterial solution of 7_2 strain
ASV	amplicon sequence variant
BBB	blood-brain barrier
BT	beak trimming
BW	body weight
CA	catecholamine
CD	crypt depth
CMT	cecal microbiota transplantation
CNS	central nervous system
CTRL	control
DXL	dekalb XL
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
ELISA	enzyme-linked immunosorbent assay
EP	epinephrine
FP	feather pecking
FCR	feed conversion rate
GI	gastrointestinal
H/L	heterophils to lymphocytes
HPLC	high performance liquid chromatography
HPA	hypothalamic-pituitary-adrenal
Htr1a	5-HT 1a receptor
Htr1b	5-HT 1b receptor
IgG	immunoglobulin G

IL	interleukin
LPS	lipopolysaccharides
MDD	major depressive disorder
MAO	monoamine oxidase
NE	norepinephrine
PHA	phytohemagglutinin
RIA	radioimmunoassay
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SCFAs	short chain fatty acids
sIgA	secretory immunoglobulin A
SPF	specific pathogen free
TNF-α	tumor necrosis factor alpha
TPH	tryptophan hydroxylase
VH	villus height

ABSTRACT

Recent studies have revealed that fecal microbiota transplantation exerts beneficial effects on modulating stress-related inflammation and behavioral disorders in mammals. The aim of this study was to examine if cecal microbiota transplantation (CMT) presents similar efficiency in improving the health status and reducing aggression in egg-laying strain roosters. Cecal contents were collected from the divergently selected 6_3 (gentle) and 7_2 (aggressive) chicken lines based on resistance or susceptibility to Marek's disease, resulting in line's unique physiological and behavioral characteristics. Eighty-four 1-d-old male chicks of Dekalb XL strain were randomly assigned into 3 treatments with 7 replicates per treatment of 4 birds per replicate: CTRL (0.1 ml of saline), 6_3-CMT (0.1 ml cecal solution of line 6_3), and 7_2-CMT (0.1 ml cecal solution of line 7_2) for a 16-wk trial. Cecal microbiota transplantation was conducted once daily from d 1 to d 10 and then boosted once weekly from wk 3 to wk 5. At wk 5, 11, and 16, body weight and blood samples were collected for detecting CMT-induced physiological changes of recipient birds. Samples of the spleen, ileum, hypothalamus, and cecal contents were collected at wk 5 and 16. Behavioral data was analyzed at the same time points. The results indicated that transplantation altered the structures and diversity of the gut microbial community in recipient birds, which led to differences in performance traits, neuroendocrine and immune systems, and exhibition of aggression. Compared to CTRL birds, 7_2-CMT birds had better nutrient digestion and absorption but lower stress adaptive capacity at wk 5, while 6_3-CMT birds had reduced systemic inflammation resulting from the up-regulation of anti-inflammatory cytokine IL-10, together with down-regulation of pro-inflammatory cytokines TNF-α and IL-6 at wk 16. In addition, CMT induced a lower frequency of aggression with a higher hypothalamic serotonergic activity in 6_3-CMT birds at wk 5. Meanwhile, genus *Ruminococcaceae UCG-005* was found to be positively correlated with brain serotonin levels ($P < 0.05$) in 6_3-CMT birds, while genus *GCA-900066225* was negatively correlated with 5-HIAA ($P < 0.05$) in 7_2-CMT birds at wk 5. Taken together, early postnatal CMT

in recipient birds induces donor's line-related effects on performance traits, stress adaptive capability, aggressive behavior, immune and neuroendocrine functions through regulating the gut-microbiota-brain and gut-microbiota-immune axes. The findings may provide new insights into developing management methods for controlling aggressive behavior in poultry.

CHAPTER 1. LITERATURE REVIEW

1.1 Management and housing systems: Implications for health and welfare of egg-laying chickens

In modern poultry farming systems, hens are confronted with many management stressors, including high living density, transportation, handling, or improper lighting programs (Nordquist et al., 2017). Social stress caused by these potential problems appears to be at the forefront of welfare concerns, leading to pathophysiological disorders and stress-related injurious behaviors (aggression, severe feather pecking, and cannibalism) in chickens. To address these critical issues, egg producers have tried to develop more effective housing systems to prevent the adverse effects of these injurious behaviors; however, such behaviors exist under all current housing systems (Widowski et al., 2016). In cage systems such as conventional and enriched cages, restricted living space causes excessive social stress, which prevents birds from exhibiting species-specific behavior and increases the incidence of injurious behaviors (Hartcher and Jone, 2017). With increased public awareness regarding the health and welfare of farm animals, more than 200 corporate customers in the United States have pledged to buy cage-free eggs before or by 2025 (Xin and Liu, 2017). However, in cage-free systems, including the floor pen, aviary, and free-range systems, the unstable social structure within the large groups contributes to social stress and compromises hen welfare (Sherwin et al., 2010).

1.1.1 Selection and breeding programs

In commercial breeding programs, genetic selection has gained great progress in the improvement of productivity. To meet human needs, traditional selection based on production traits such as table-egg (layers) and meat (broilers) has been initially utilized (Cheng, 2010). However, in laying hens, the high production line developed based on selection for individual performance is often accompanied by undesired behavioral patterns such as aggression. In animals,

productivity is correlated with competitive capability, selection of birds with high production increases competition among individuals (Cheng, 2003; Edwards, 2006; Cheng, 2007). In Dekalb XL, a former commercial line characterized by high egg production was accompanied by significantly increased mortality (around 10-fold) due to elevated aggression and social dominance during adolescence (Muir, 1996). Additionally, several selected high productivity lines have been reported to experience dysfunction of the immune system and selection inadvertently introduced feather pecking behavior (Korte et al., 1997; Su et al., 2006; Yang et al., 2017).

1.1.2 Rearing environments and social stress

Conventional and enriched cages

In the 1940s, conventional cages (also known as battery cages) became a popular rearing method on account of their effectiveness and cost-savings. Increased hygiene in conventional cages prevents the spread of infectious diseases by preventing feces from dropping onto birds (Duncan, 2001). Compared with the traditional floor housing method, the group size of hens in cages is more stable, leading to decreased social stress and cannibalism (Craig, 1982; Tauson, 2002). However, criticism of battery cages includes limited space allowance that deprives the ability of birds to express a wide range of natural behaviors such as foraging, dustbathing, and nesting. In this barren environment, the lack of enrichment also redirects the birds' attention to other individuals, which results in aggression and severe feather pecking (Wood-Gush et al., 1975).

To better improve the behavioral repertoires, cages with several enrichments, including nests, litter boxes, and perches have been gradually developed (Bareham, 1976). In the early research, it has been reported that animals are prone to enter a state of frustration or experience emotional distress if they cannot perform strongly motivated behavioral patterns (Duncan and Wood-Gush, 1971; Fraser et al., 2013). The presence of enriched cages provides birds with more flexibility in movement and expression of natural behavior, which may effectively decrease the incidence of negative emotional states such as fearfulness, frustration, and stress (Newberry, 1995).

In agreement with this finding, studies found that hens in group-housed enriched cages exhibit less aggressive and feather pecking behaviors compared with those housed in conventional cages (Johannson et al., 2016). Nonetheless, it has been argued that birds in small and enriched cages may not have equal access to feeders or enrichments, which potentially increases the chance of competition and aggression among birds (Albentosa et al., 2007; Widowski et al., 2017). Also, birds housed in large groups within the colony cages may increase social instability as well as the likelihood of harmful social behaviors (Al-Rawi et al., 1976). Hence, the extent to which the behavioral needs of birds can be satisfied in cage systems is still questionable.

Cage-free systems

To better fulfill the welfare, especially the behavioral needs of birds, cage systems have been replaced by cage-free systems in most European Union (EU) countries. In 1965, the UK Farm Animal Welfare Council outlined five aspects of animal welfare under human control. "Freedom to express normal behavior", as one of the five requirements, indicates cage-free systems appear to be more animal-friendly in terms of behavioral freedom. Cage-free systems enable birds to roam vertically and horizontally, thereby allowing birds to move freely and exhibit natural behaviors (Weeks and Nicol, 2006). Correspondingly, the ability of birds to perform highly motivated behaviors leads to less emotional problems such as frustration, together with more locomotion activities compared with cage systems, which largely reduces the presence of negative affective states and further fulfills welfare in chickens (María et al., 2004). Moreover, hens prefer enough personal space, as such, enough space allowance in most non-cage systems may better meet their behavioral needs (Cooper and Albentosa, 2003).

However, non-cage systems elicit growing welfare concerns as well, including social stress, bacterial infections, or thermal discomfort (Relić et al., 2019). An early preference test found that hens prefer smaller rather than larger groups and familiar birds instead of strangers, suggesting that birds housed in the non-cage system may experience a high risk of social stress due to the

unstable social structure (Hughes, 1977). Hughes and Gentle (1995) showed that keeping birds in large groups may contribute to the prevalence of severe feather pecking. Based on chickens' learning capability, the exhibition of certain behavior, such as feather pecking and cannibalism, can be easier to spread throughout the whole flock (Bessei and Kjaer, 2015). In addition, further studies indicate that hens reared in cage-free systems experience a higher ratio of dirty eggs, poorer feed conversion, and more severe foot lesions in comparison with cage systems (Hartcher and Jones, 2017). Therefore, the risks to layer welfare in non-cage systems still need to be addressed by management practices and scientific research.

1.2 Aggression

1.2.1 Causes of aggression

The origin of aggression in layers can be traced back to their wild ancestors (red junglefowl *Gallus gallus*), who naturally live within a small and stable group that maintains the dominance hierarchy by showing aggressive fights towards individuals (Craig, 1986; D'Eath et al., 2003). Regardless of the domestication and confinement in the modern rearing system, most natural behaviors of the species remain. Nowadays, aggression among layers can be frequently seen in commercial flocks. In poultry, aggression has been defined as "*one bird directs forceful downward pecks at the head or neck of other birds*", but it is prone to transfer to the body or other areas if the peckers cannot reach certain areas (Dalton et al., 2013). From the standpoint of the evolutionary process, aggression serves an adaptive purpose since it promotes the survival and reproduction of animals in their living environment (Nelson, 2005). However, in modern intensive rearing systems, aggression is a critical welfare concern, leading to feather damage, severe injuries, and even cannibalism (Cheng and Muir, 2007; Tablante et al., 2000; Hartcher et al., 2015).

Aggression is a complicated behavioral pattern that can be caused by multiple factors such as inappropriate rearing conditions or management practices and dysfunctional physiological homeostasis (Edward and Kravitz, 1997; Lischinsky and Lin, 2020). On commercial farms, layers

are usually mixed with unfamiliar conspecifics when they are transferred from growing facilities to laying facilities, which interrupts the original social relationship and causes excessive social stress (Dennis et al., 2009). As social stress and aggression reciprocally work together, increased social stress will promote the incidence of aggression (Dennis et al., 2011; Marchewka et al., 2013). Moreover, the incidence of social disruption will induce aggressive behavior towards unfamiliar birds (Cloutier and Newberry, 2002). Fraser (1997) recognized the ability of animals to exhibit species-specific behaviors as one of the key components of welfare assessment. However, under restrictive environments such as battery cages, birds only have limited space to perform their natural behaviors, which largely increases the possibility of entering a state of frustration together with increased aggression (Gallucci et al., 2020).

1.2.2 Strategies used to control aggression

To address this issue, several methods including beak trimming (BT), genetic selection, and some preventative management practices have been conducted to control aggression (Marchant-Forde et al., 2008; Jendral and Robinson, 2004). Beak trimming, as a routine husbandry procedure, aims to reduce beak-related behavioral problems including aggressive pecking (Cheng, 2006). Currently, there are two major types of BT: the hot blade (HB) and infrared laser (IR) (Jendral and Robinson, 2004; Glatz, 2005). However, as the beak is a sensory organ containing important sensory feedback, the removal of part of the beak elicits numerous behavioral and physiological issues (Dennis and Cheng, 2012). Furthermore, implementing either HB or IR method causes acute or chronic pain and decreased duration of normal behaviors such as eating and drinking (Dennis and Cheng, 2012; Petrolli et al., 2017).

Other than beak trimming, producers have tried light manipulation programs such as altering the light wavelengths and reducing light intensity to ameliorate injurious behaviors including feather pecking (FP) and aggression (Mohammed et al., 2010). Although low light intensity reduces severe FP to some extent, it leads to impaired vision and exacerbates abnormal

development of eyes (Prescott and Wathes, 2003; Tauson, 2005). Impaired vision prevents birds from interacting with environmental cues which increases the incidence of stereotyped FP, a pattern of nonsensical behavior (Kjaer and Vestergaard, 1999; Sedlačková et al., 2004). On the other hand, decreased light intensity restricts the ability to perceive the environment, which potentially inhibits the movement of birds.

To further solve this issue, researchers developed a more promising method termed genetic selection. For instance, Guhl et al. (1960) conducted individual selection against aggression, which separates lines with high and low aggressiveness. Nonetheless, the selection method based on individual performance fails to be widely used in the commercial egg industry on account of the co-selection of unexpected behavioral traits (Rodenburg et al., 2008). In addition, due to genetic linkage, manipulating one trait may induce a negative impact on another by a connected response. It has also been claimed that direct selection based on behavioral traits may result in decreased productivity (Craig and Adams, 1984). To overcome this issue, a selection program has been conducted based on group performance (Muir, 1996). Under group selection, the annual percentage of mortality of the selected kinder and gentler birds decreased with increased production as compared to control birds (Muir and Cheng, 2004). However, due to high cost, ethical issues, and heritability, so far, selected kind strains of laying hens are not commercially available in the layer industry. Thus, developing a reliable alternative to control such behavior in layers is necessary to improve welfare as well as benefit the agricultural industry. In recent decades, the gut microbiota has become an intervention target in treating patients with neuropsychiatric disorders and emotional dysfunction, suggesting modulation of the gut microbiota may be a potential approach to modulate aggression in chickens.

1.3 Gut microbiota

1.3.1 The function of gut microbiota

The various microorganisms that inhabit the gastrointestinal tract are termed gut microbiota (Lederberg and McCray, 2001). It has been increasingly recognized for its profound influence on many aspects of the host, including neurophysiology, development of the immune system, and behavioral expression (Belkaid and Hand, 2014; Krishnan et al., 2015). In healthy individuals, the interactions between the gastrointestinal (GI) tract and microbiota are steady, while the occurrence of a disturbance can result in several diseases including inflammatory bowel diseases, metabolic diseases as well as mental disorders (Mayer, 2011; Ferreira et al., 2014; Karlsson et al., 2013). From the standpoint of immunology, the intestinal microbiota has a collaborative relationship with the epithelial cells to protect against invasive pathogenic microorganisms (Jandhyala et al., 2015). Additionally, the development of the innate and adaptive immune systems in the host is guided by the gut microbiota (Wang et al., 2016). In newborns, the susceptibility to environmental incursions is increased on account of the instability of gut microbiota makeup as well as the immaturity of the immune system, leading to an increased risk of suffering from infectious diseases, eventually death (Zheng et al., 2020).

In recent decades, there has been a growing emphasis on the exploration of the interaction between gut microbiota and brain function, together with its implications for mental disorders, including major depressive disorder (MDD), bipolar disorders, depression, and anxiety (Xu et al., 2021). Germ-free mice have been used as an ideal animal model to directly assess the effects of gut microbiota on host function. For instance, the transplantation of fecal content from MDD patients induces depressive-like behavior in GF recipient mice, which suggests that gut microbiota may have a causative role in the development of depression (Zheng et al., 2016). Emerging animal studies imply gut microbiota may have similar functions in the treatment of psychiatric disorders in humans. It has been reported that patients with neurological dysfunction present gastrointestinal infections (Stasi et al., 2019). For instance, children with autism have disordered commensal

bacteria in the gut, which confirms that the imbalanced composition of gut microbiota may be related to the neurodevelopmental disorder (de Theije et al., 2011). Compared with healthy individuals, children with autism have elevated concentrations of total short chain fatty acids in feces (Wang et al., 2012). Such microbial metabolites have been found to act on the central nervous system (CNS), suggesting a potential mechanism by which bacteria influence brain function (Wang et al., 2012). Correspondingly, clinical studies show patients with acute hepatic encephalopathy, a neuropsychiatric syndrome, can be treated by orally administered antibiotics (Amarapurkar, 2011). Taken as a whole, the essential role of gut microbiota in the regulation of mental and emotional disorders cannot be overlooked.

1.3.2 Multi-factorial effects on the gut microbiota composition

The gastrointestinal (GI) tract of chicken harbors numerous species of bacteria with the function of mediation of nutrition absorption, immunity, physiology, and promotion of local microstructural stability (Shakouri et al., 2009; Shang et al., 2018). The colonization of bacteria within the chicken intestines occurs immediately after hatch, bacteria received from the first inoculum with the eggshells and incubation environments (Schokker et al., 2015). However, microbiota composition is not stabilized because the colonization of gut bacteria occurs throughout the growth development, which is impacted by various factors, including the genetics or age of the host, gut locations, and rearing environment (Kers et al., 2018).

Segments of the gastrointestinal tract

In domestic birds, energy and nutrient extraction from feed intake are influenced by the microbiota within the GI tract. In addition, the gut microbiota composition and diversity spatially vary in different segments [i.e., the stomach; small intestine (duodenum, jejunum, and ileum); large intestine; and cecum] throughout the entire GI tract, which results from different biochemical functions in each segment (Rychlik et al., 2020). Compared to other gut sites, the cecum occupies

the greatest microbial population and commensal microbes, which constantly interact with host biological regulation systems as well as shape the host's immune and neuroendocrine systems (Polansky et al., 2016). For instance, in broilers, the microbial communities in the caeca were more diverse in comparison to the ilea (Shaufi et al., 2015). In adult chickens, the proximal parts of the GI tract are dominated by *Lactobacilli*; and the composition and complexity of gut microbiota increase in the distal parts of the intestinal tract including the cecum and colon (Rychlik et al., 2020). This location-specific shift has also been reported in other animal models including rodents (Lee et al., 2018), swine (Mu et al., 2017), and cattle (Taschuk and Griebel, 2012).

Age of birds

The gut microbiota composition changes from birth to adult life, mostly affected by diets and the ambient environment. In chickens, the colonization of intestinal microbiota starts immediately after hatching, and bacteria are received from the eggshells and the incubation environment (Schokker et al., 2015). Gut bacteria colonize in a successional manner throughout development. In young chickens, the gut microbiota composition is highly variable, and the dominant bacterial taxa of the gut change over time (Videvall et al., 2019; Rychlik et al., 2020). In the initial stage, most taxa at the genus level are *Bacteroides* and the changes of bacterial abundance become static at 8-12 week of age, mostly dominated by *Clostridia*. In broilers, the colonization of *Campylobacter* mainly occurs at around 2 week, which indicates the increased susceptibility of *Campylobacter* infection at an early age may be linked with the strong shift of gut microbiota as well as the immaturity of the immune system (Awad et al., 2016). Besides, Shaufi et al. (2015) showed that intestinal microbiota was age-dependent in broilers. For example, on day 7, *Clostridia* (62%) and *Gammaproteobacteria* (32%) were the most abundant in the ilea, while on day 21, the second dominant class in the ilea shifted from *Bacteriodia* (23%) to *Bacilli* (30%). In summary, the distinctive difference throughout the growth of chickens is associated with the early-age re-establishing of the gut microbiome.

Social stress

In livestock, environmental stress including management practices or diet changes disrupts the gut microbial community; as such, the gut microbiota diversity and composition could be considered as a biomarker for poultry health and welfare. Compared with optimal farm conditions (ventilation within the optimal parameters, ammonia ≤ 10 ppm), broilers kept under commercial farm conditions (non-optimal ventilation parameters; ammonia ≤ 25 ppm) showed a lower diversity level of gut microbiota at 42 days of age (Montoro-Dasi et al., 2021). This suggests that stress caused by environmental factors in commercial farms has a significant impact on the composition of the gut microbiota. Among the different sources of stress, one of the major problems in poultry production is that avian species are particularly sensitive to environmental challenges associated with stocking density, especially to social stress. Social stress damages the intestinal epithelial cells, accompanied by reduced intestinal barrier integrity and increased intestinal permeability (leaky gut). These changes stimulate toll-like receptors of various immune cells to produce proinflammatory cytokines, resulting in local or systemic inflammation or infection (Burkholder et al., 2008; Awad et al., 2015). “Leaky gut” syndrome has negative effects on feed consumption as well as growth performance in chickens because the damage of the intestinal barrier increases the passage of enteric pathogenic bacteria and releases toxins such as lipopolysaccharides (LPS), and reduces nutrient absorption (Ghareeb et al., 2016). In broilers, enteric pathogens such as *Escherichia coli*, *Salmonella enterica*, and *Clostridium perfringens* negatively affect growth performance, gut epithelium, and cellular functions, and consequently lead to reduced body weight gain and worsened feed conversion rate (FCR) (Awad et al., 2017). Subclinical necrotic enteritis caused by enterotoxigenic *C. perfringens* results in approximately a 12% reduction in production and a 10.9% increase in FCR compared with healthy birds, which imposes a heavy burden on the poultry industry (Immerseel et al., 2004; Awad et al., 2014).

Host genotype and phenotype

Host genetic makeup contributes to the gut microbiota composition, which directly influences gut microbiota diversity and population by regulating various chemical secretions through genetic-environmental interaction (Khachatryan et al., 2008, Belkaid et al., 2014). The role of host genotype in gut microbiota composition has been studied across many species including humans and various animals (i.e., mouse, cattle, and chicken).

In humans, the earliest support for the influence of host genotype on gut microbiome comes from the studies in monozygotic (MZ) and dizygotic (DZ) twins. Compared with the DZ twins, the fecal floras of the MZ twins are much more similar (Van de Merwe et al., 1983). Studies also found that the abundances and changes of bacterial taxa are associated with host genome single nucleotide polymorphisms, which contributes to the phenotype diversity (Bonder et al., 2016; Turpin et al., 2016). Host genetics and gut microbiota work together to maintain and stabilize gut homeostasis (Kovacs et al., 2011; Spor et al., 2011). The genetic variations and disruptions in gut microbiota modulate the susceptibility to metabolic dysfunctions and damage the physiological health of the host (Frazer et al., 2009, Zhang et al., 2010). For instance, there are remarkable variations in gut microbiota compositions in lean and obese individuals. Compared with lean individuals, people with obesity harbor greater bacteria from the phylum *Firmicutes* but fewer from *Bacteroidetes* (Le Chatelier et al., 2013; Furet et al., 2010). Interestingly, identified bacterial taxa have been found to be associated with metabolic features, such as body weight and insulin levels. Altogether, understanding how host genetics interact with gut microbiota is crucial for finding treatment for metabolic syndromes.

The understanding of human gut microbiota function is limited by technical issues such as high inter-individual microbiome variation, limited sample size, confounding effects of diet; consequently, animal models have become much popular in exploring the microbial-genetic association (Costello et al., 2009; Dave et al., 2012). A recent study in cattle showed the host genotype directly influences the colonization of certain bacteria and indirectly shapes the gut

microbiota structures through interaction with different bacteria (Fan et al., 2020). Similarly, genotypic traits have similar effects on birds: hens from high FP and low FP lines occupy distinct differences in their luminal microbial communities, suggesting FP genotype contributes to gut microbiota makeup (Van der eijk et al., 2019a). Compared with the low FP line, High FP birds were characterized by a higher abundance of genera of *Clostridiales* but a lower level of *Lactobacillus* (Van der eijk et al., 2019b; Videvall et al., 2019). However, the specific causal relationship between FP and intestinal microbiota is still unclear. In chickens, it has already been reported that genotype is correlated with increased susceptibility towards certain diseases and behavioral repertoires (Bacon et al., 2001). For example, chicken lines 6_3 and 7_2 were divergently selected for resistance or susceptibility to Marek's disease, which also differ in the exhibition of aggressive behavior, as line 6_3 is gentler than line 7_2 (Dennis et al., 2004). However, it is still unknown whether the unique behavioral expression of each line is correlated with the diversity and population of gut microbiota.

1.4 Fecal microbiota transplantation

1.4.1 History of fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is a procedure that entails transplanting a suspension of feces from a healthy donor into a patient's gut to directly alter the recipient's gut microbiota to gain therapeutic benefits (Wang et al., 2014). In humans, the first medical report of FMT for a therapeutic purpose was used for patients with pseudomembranous colitis (Eiseman et al., 1958). In the wake of the usage of FMT in humans, an Italian anatomist, Acquapendente (Fabricius, 1533–1619), further developed a broader concept called "transfaunation" in veterinary medicine, in which the FMT procedure has been extended from feces to gastrointestinal contents (Borody et al., 2004). The transfer of intestinal contents from healthy animals to sick ones is mainly used in ruminants (GS and AJ, 1958). Expectably, "transfaunation" enables the recipient animals to gain a greater diversity of microorganisms in their intestines with an enhanced digestion system

(De Groot et al., 2017). By now, the procedure has been considered as the most effective treatment for *Clostridium difficile* infection and potential therapy for gastrointestinal disorders as well as mental diseases in humans, including inflammatory bowel disease, irritable bowel syndrome, neurodegenerative and neurodevelopmental disorders (Borody and Khoruts, 2012; McDonald et al., 2018).

1.4.2 Superiority of fecal microbiota transplantation

Various approaches have been used for the reconstruction of gut microbiota to treat diseases as well as increase health and welfare in humans and farm animals, such as the administration of probiotics, antibiotics, and FMT. However, it has been reported that both antibiotics and probiotics have disadvantages. For example, bacterial resistance to antibiotics increased over time, especially in *Helicobacter pylori* infections (Megraud et al., 2012). In addition, antibiotics destroy the microbial flora, leading to antibiotic-associated intestinal inflammation and diarrhea (Cammarota et al., 2014a). The use of antibiotics has raised great concerns regarding drug residue-associated food safety and environmental pollution (Sofos, 2008). Although the benefits of probiotics have been reported in human subjects as well as animal models, a limited degree of success in treating *Clostridium difficile* infections has been gained using probiotics (Hopkin and Macfarlane, 2003). Furthermore, the efficiency of probiotics is affected by multiple factors, including bacterial species, applied methods, and the health status or living environment of the host (Delia and Männer, 2012). Compared with other methods, the application of FMT is more promising and reliable since FMT transfers the whole consortium of microorganisms instead of just several specified species (Chaitman et al., 2016). On the other hand, the administration of fecal flora builds up a durable change of the gut microbiota in the recipient (Grehan et al., 2010). Hence, FMT serves as a more viable and straightforward method to alter the gut microbiota in human trials.

In humans, cecal microbial ecology is poorly understood owing to sampling difficulties. In animal studies, especially in poultry, the cecum has been used as a representative sampling site in most research. Cecal microbiota is associated with the health and performance of birds, which defends against pathogens as well as regulates energy metabolism through degrading indigestible carbohydrates, or producing beneficial metabolites (Clench and Mathias, 1995; Pandit et al., 2018). Additionally, cecal content contains a high richness and complex microbial community compositions as compared to feces in chickens (Pauwels et al., 2015). Compared with other segments within the GI tract, the cecum is emptied twice per day and does not exhibit peristalsis constantly. The occurrence of anti-peristalsis reverses gut movement, as such, elements of the fecal microbiota must be directly derived from the cecum (Stanley et al., 2015; Feye et al., 2020). Hence, cecal sampling may be a reliable method to represent the changes of the gut microbial community.

1.4.3 The integrative role of postnatal transplantation

In humans, early-life microbial colonization plays a crucial role in shaping gut microbiota composition. Compared with vaginally delivered infants, the intestinal microbiota of neonates delivered by cesarean section appears to be less diverse in terms of bacterial species (Orrhage and Nord, 1999). During a vaginal delivery, direct contact with the vaginal and intestinal flora is a major source for the start of the infant's gut bacterial colonization. Immediately after birth, the diversity of microbial communities is increased as newborn babies contact with organisms from the environment (Rodríguez et al., 2015). Similarly, in chickens, bacteria are received from the first inoculum with the eggshell and incubation environment (Apajalahti et al., 2004). Pedroso et al. (2016) contended that in ovo inoculation of an adult-derived microbiota contributes to the development of gut microbiota and the resistance to pathogenic bacteria in chickens, which indicates the rapid establishment of the gut microbiota occurs at an early age. In agreement with this finding, studies found that administration of probiotics 2 hours post-hatch introduces beneficial effects to embryonic development in broilers (Baldwin et al., 2018). Thus, the early age

would be the optimal period to modulate the gut microbiota on account of its strong dynamics and long-term effects.

1.5 Cecal microbiota transplantation as a potential mode of acting the gut-brain axis

The impact of the gut microbiota on brain function has been widely recognized since the 19th century (Bradshaw, 1867). Increasing evidence points to bidirectional communication for linking the gut with brain cognitive and emotional function through the gut-brain axis. It encompasses the CNS, the enteric nervous systems (ENS), and the sympathetic and parasympathetic arms of the autonomic nervous system, as well as the neuroendocrine, enteroendocrine, and neuroimmune systems (Benarroch, 2019; de Jonge, 2013). The autonomic system drives afferent signals arising from the intestinal lumen via multiple pathways to the CNS including enteric, spinal, and vagal nerves, and projects efferent signals from the CNS to the intestinal epithelial cells (Forsythe and Kunze, 2013). The gut microbiota influences the CNS directly and indirectly by activating the vagal nerve and releasing various biochemical regulators, which potentially act on the immunity, neuroendocrine, and behavioral responses of the host through the gut-brain axis (Crumeyrolle-Arias et al., 2014).

1.5.1 The regulation of immunity

Microbial colonization in the intestines has an impact on the development of the immune system as intestinal bacteria serve as predominant regulators of immune responses, such as synthesizing and releasing biomarkers (i.e., interleukins, antibodies, and immune cells) in response to various stimulations and modulating inflammatory disorders in the CNS (Fung et al., 2017). Further emphasis is placed on the role of gut microbiota in the treatment of gastrointestinal diseases in humans. For instance, inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, result from activated mucosal innate immune response and pathogen-increased epithelial permeability (Chakaroun et al., 2020). Clinical studies have indicated that IBD can be

treated and even cured by restoring the gut microbiota using FMT (Sunkara et al., 2018). The mechanism underlying this microbiota-modulating therapy is that the heathy microbiota competes with pathogenic microorganisms and communicates with immune cells and the CNS via the vagus nerve within the GI tract. Correspondingly, the vagus nerve reduces inflammation by sending afferent signals to stimulate the function of immune tissues and to the brain, subsequently activating an efferent response by releasing mediators and cooperating with immune cells (Fung, 2020). Hence, the intimate association among the gut microbiota, intestinal epithelia, and the immune system maintains gut homeostasis.

Gut microbiota activates the immune system to protect the host from infections. Compared to the mucosa of germ-free (GF) rats, conventionally reared rats have more complex mucosa structures accompanied by a more activated immunological status (Čaja et al., 2021). This suggests the role of commensal microbiota in the formation of the intestinal structures and protecting the host against pathogens. Similarly, studies found the function of the gut microbiota in the maturation of the gut immune system in chickens. Different *Lactobacillus* species including *L. acidophilus*, *L. reuteri* and *L. salivarius* can promote cytokine expression, such as IL-10 and TNF-α, in T cells in the cecal tonsils to maintain gut homeostasis in chickens (Brisbin et al., 2012). Also, in broilers, certain dietary supplements promote intestinal health by modulating the dynamics of the gut microbiota as well as affecting host metabolism, which suggests alterations in microbiota not only inhibits the immune response, but also regulates the mRNA expression of pro-inflammatory cytokines such as IL-4 and IL-5 in the immune organ (the spleen) (Wu and Wu, 2012). Therefore, gut microbiota is a critical actor in the regulation of the immune response of the host.

1.5.2 The regulation of the neuroendocrine system

Function of the hypothalamic-pituitary-adrenal axis

The CNS influences the gut microbiota, mainly through the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is a core stress efferent center that coordinates the adaptive response of the organism to various stressors (Bhatnagar and Dallman, 1998). Activation of the HPA axis is often seen under external stimulations, releasing an ending compound, corticosterone, from the adrenal glands within a short period. There is also evidence that the gut microbiota can activate the HPA axis, mainly through mediators that cross the BBB such as prostaglandins or microbial antigens (Misiak et al., 2020). Patients with high mental stress are commonly occupied with gut inflammation and relative alterations in the composition of intestinal microbiota, leading to an exaggerated activation of the HPA axis accompanied by increased synthesis and release of corticosterone (Peirce and Alviña, 2019). This suggests mental stress may disrupt the gut homeostasis, and the disturbance of the gut microbiota induces neuroinflammation and consequently leads to mental disorders and abnormal behaviors. Similarly, compared with specific pathogen-free (SPF) mice under restraint stress conditions, GF mice are found to exhibit exaggerated activation of the HPA axis, as reflected by elevated levels of plasma corticosterone and adrenocorticotrophic hormone (Mättö et al., 2005; Sudo, 2014). Interestingly, the symptoms of hyperresponsiveness to stress in GF mice can be recovered by recolonization with SPF feces or by a bacterial strain, *Bifidobacterium infantis* (Sudo, 2014). Taken together, changes in gut microbiota composition may be associated with the activation of the HPA axis in response to stressors.

The pathway of serotonin

The gut-brain axis consists of a bidirectional interaction that links the gut microbiota to the brain and affects the metabolism or biosynthesis of neuroactive compounds and neurotransmitters, by which it regulates the exhibition of behaviors (Wang et al., 2016). It has been suggested that

disruption of the gut-brain axis may induce stress-related mental disorders due to the integration of neural signaling between the gut and brain (Dinan et al., 2015, 2017; Rogers et al., 2016). Serotonin (5-hydroxytryptamine, 5-HT) and its metabolites, 5-hydroxyindoleacetic acid (5-HIAA) as well as their receptors are functional in the regulation of neuroendocrine and certain behavior expressions including depression and anxiety (Twarog et al., 1953; Schinka et al., 2004; Pezawas et al., 2005; Goodman, 2011). In line with these findings, 5-HT has also been defined as the primary regulator of aggressive behaviors in humans and various species of animals (Berman et al., 1997; Dennis et al., 2008). For instance, in rodents, elevated levels of 5-HT as well as 5-HIAA have been found in "domesticated" rats with lower aggressive behavior (Popova et al., 1991). Also, 5-HT system dysfunction is associated with violent behavior in humans (Li et al., 2006). Similarly, the essential role of the brain 5-HTergic system in the modulation of aggression has been widely studied in laying hens (Cheng and Muir, 2007; Dennis et al., 2008). However, limited studies have been conducted to explore the effects of gut microbiota modulations via cecal microbiota transplantation on the serotonin pathway and its effects on preventing aggressive behaviors in laying hens.

It has been reported that 5-HT is produced in the gut as well as brain-stem neurons of the raphe nuclei (Hornung, 2003). Approximately 95% of 5-HT in the periphery is released from the enterochromaffin (EC) cells within the gut epithelia, while about 5% of the central 5-HT is synthesized in the raphe nuclei (Gershon, 2003). In the central nervous system, the levels of 5-HT are closely associated with aggression (Olivier, 2004), while multiple functions have been identified in peripheral 5-HT. Early studies suggested that the peripheral 5-HT participates in platelet aggregation as well as vasoconstriction (Rapport et al., 1949). In recent research, novel functions of gut-derived 5-HT have been determined, including modulations of gut motility, immune response, and host metabolism (Le Beyec et al., 2014; Herr et al., 2017; Keating and Spencer, 2019). Additionally, the gut microbiota plays a substantial role in the synthesis of the body's 5-HT. For example, GF mice have reduced levels of blood 5-HT in comparison with

conventional mice (Sjogren et al., 2012). Unlike tryptophan, the peripheral 5-HT cannot pass the blood-brain barrier (Yuwiler et al., 1977). Tryptophan, as a precursor of 5-HT, can be regulated by certain intestinal bacteria strains and is involved in the synthesis of central 5-HT. For example, studies found that *Bacteroides fragilis* harbors a tryptophanase enzyme, which has been linked to the degradation of tryptophan (Li and Young, 2013). As such, gut bacteria may affect the function of the brain 5-HT indirectly through releasing tryptophan, which further influences behavioral expressions of the host (Hsiao et al., 2013). In summary, gut microbiota has a profound influence on the activities of the serotonergic system.

The pathway of catecholamines

Catecholamines (CAs), such as norepinephrine (NE), epinephrine (EP), and dopamine (DA), interact with gut microbiota in the regulation of physiological status, cognition, and exhibition of behavior (Eisenhofer et al., 2004). Catecholamines, as stress-related neuroendocrine hormones, assist the host in adapting to acute or chronic stress. In response to stress, the noradrenergic system in the nervous system is often activated with increased levels of blood NE as well as elevated concentrations of hypothalamic DA (Mizrahi et al., 2012). Catecholamines, also known as mediators of the gut bacteria, establish the connection with the gut through the gut-brain axis (Bercik et al., 2012; De Palma et al., 2014). Correspondingly, gut microbiota represents an input to the brain and influences the neural pathways in stress responses. As the GI tract is sensitive to stress, recent studies hypothesized that gut bacteria may act on neuroactive compounds such as NE, leading to changes in the host physiology and behavioral expression (Lyte, 2011). Similarly, dopamine, a precursor of NE and EP, is associated with aggression and reward-motivated behavior (Borodovitsyna et al., 2017). The elevated level of DA has been reported to be related to aggressiveness (Cheng et al., 2003).

Lyte and Ernst (1992) found that some bacterial species such as E. *coli* could recognize exogenous CA in vitro, and such recognition increases the bacterial proliferative capacity (Lyte

and Ernst, 1992). Additionally, certain bacteria stimulate hormone production. In vitro, bacteria such as *E. coli*, *Bacillus subtilis*, *Serratia marcescens* can produce high concentrations of DA and NE (Tsavkelova et al., 2000). Compared with specific pathogen-free (SPF) mice, GF mice have decreased concentrations of NE in the cecal lumen. This observation further confirms that gut bacteria may contribute to the biosynthesis of hormones. Correspondingly, the research found that NE levels in the cecum could be restored through colonization with a mixture of 46 *Clostridia* species (Asano et al., 2012). In summary, these findings strongly indicate that there is a mutual communication between the gut bacteria and CAs metabolism.

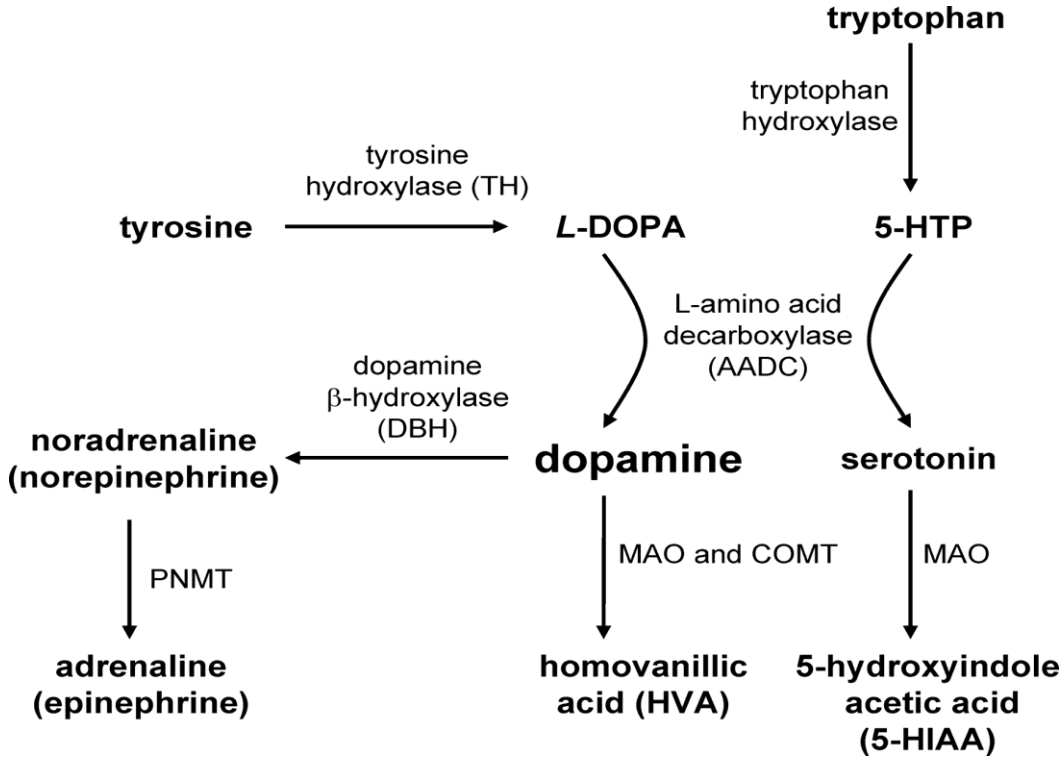

Figure 1.1 Pathways for the synthesis of dopamine, norepinephrine, epinephrine, and serotonin. (Rubí et al., 2010)

The regulation of behavior

The impact of the gut microbiota on brain function has been recognized since the 19th century (Bradshaw, 1867). Increasing evidence points to bidirectional communication for linking the gut with brain cognitive and behavioral exhibition through the gut-brain axis (Srikantha et al.,

2019). This view is supported by mounting studies in human trials and animal models exposed to various microbiota modulation methods including FMT, probiotics, and antibiotics (Cammarota et al., 2014b).

In humans, a recent study has found that schizophrenic patients and healthy individuals have different gut microbiota profiles. Compared with healthy individuals, anaerobes such as *L. fermentum, E. faecium* and *A. oremlandii* were more abundant in patients with schizophrenia (Zhu et al., 2020). To further understand the role of gut microbiota in the pathophysiology of schizophrenia, the schizophrenia-enriched bacterium *Streptococcus vestibularis* was transferred to mice with microbiota depletion. Interestingly, recipient mice appeared to exhibit schizophrenia-like behaviors such as impaired social behaviors, as those seen in patients (Zhu et al., 2020). Similar to those findings, mice fed with a beef-enriched diet and a normal diet have completely different gut microbiota compositions and behavioral exhibition; that is, mice fed with a beef-enriched diet exhibit less anxiety-like behaviors as compared to mice with a normal diet, which indicates the association between gut microbiota and anxiety-like behavior (Collins et al., 2012).

In poultry, gut microbiota plays multiple roles in production, physiology, nutrition, and behavior (Rubio, 2019; Shang et al., 2018). Abdel-Azeem (2013) suggested that the administration of the probiotic *Bacillus amyloliquefaciens* reduces distress and agonistic behavior in turkeys. In line with this study, in laying hens, birds received the luminal content (i.e., a mixed pool of content from ileum, caeca, and colon) from donors with different behavioral expressions (High FP vs. low FP) display divergent behavioral patterns (Van der Eijk et al., 2020). For instance, during the novel object test, recipients of the high FP lines tend to approach the novel object quicker than low FP lines (Van der Eijk et al., 2020). However, the specific causal relationship between FP and intestinal microbiota is still unclear. Research is needed to examine the potential mechanisms underlying the function of gut microbiota in the regulation of poultry behavior. Divergent genetic lines 6_3 and 7_2 of chickens are suitable candidates for examining the influence of the gut microbiota on aggressive behavior. In this project, the potential mechanisms underlying the function of gut

microbiota in the regulation of aggressive behavior were determined by the administration of cecal content from aggressive (line 7_2) and gentle (line 6_3) chicken lines, respectively.

1.6 The physiological and behavioral characteristics of chicken lines

1.6.1 Donors: 6_3 and 7_2 lines

Genetic selection programs have made tremendous progress in improving immunity and disease prevention in chickens. Highly inbred chicken lines 6_3 and 7_2 have been continuously selected for resistance or susceptibility to Marek's disease for more than 50 years at the Avian Disease and Oncology Laboratory (Bacon et al., 2000; Stone, 1975). Although the cellular mechanisms underlying the variation in disease resistance between these lines have not been clearly elucidated, previous studies have indicated that resistance may be related to line differences in gene expression that controls neuroendocrine, immunity, and gene-environmental interactions (Cheng et al., 2001a, b). Line difference in immune response has been investigated previously. Compared with line 7_2, line 6_3 has a low concentration of serum Immunoglobin G (Bacon, 2002; Yonash et al., 2002). In addition, genetic variability leads to the production of interferon-like (INF) activity by phytohemagglutinin (PHA)-stimulated peripheral white blood cells. When given 10 μg/mL PHA, line 7_2 constantly produces more interferon than line 6_3 (Bacon and Palmquist, 2002). Furthermore, the divergent behavioral pattern has been investigated between the lines. In response to social stress, line 6_3 is gentler while 7_2 is more aggressive (Dennis et al., 2004). Numerous experiments in different animal models showed aggressive behavior is associated with the activity of the serotonergic system. Consistent with these findings, roosters from line 7_2 have lower concentrations of central 5-HT in the brain than line 6_3 (Dennis and Cheng, 2014). Selection-induced stress may also be responsible for behavioral expression. Line 7_2 has a greater DA turnover rate than 6_3, suggesting line differences in the ability to cope with stressors (Dennis and Cheng, 2014). It has also been suggested that elevated activity of the dopaminergic system may link to increased aggressive behavior (Cheng et al., 2003).

1.6.2 Recipients: DeKalb XL lines

The Dekalb XL (DXL) line, a former commercial strain, was developed through individual selection for high egg production (Dennis and Cheng, 2011). It has been reported earlier that selection based on production characteristics may lead to the frequency of agonistic behavior and dominance (Mench, 1988). In agreement with the hypothesis, the behavioral analysis has shown that DXL exhibited great agonistic behaviors (Craig and Muir, 1996; Cheng and Muir, 2007). Apart from behavioral differences, several physiological differences have also been investigated in DXL birds. Compared with a group-selected line for high group productivity and survivability (HGPS), DA concentrations in the raphe nuclei as well as hypothalamic EP and NE levels were found to be lower in DXL hens (Dennis and Cheng, 2012). Low DA concentrations and stress hormones including EP or NE in DXL birds may indicate their poor ability to cope with stress. In chickens, a low ratio of CD4:CD8 could be a predictor for reduced immunocompetence (Bridle et al., 2006). Previous studies found that HGPS birds had a higher ratio of CD4:CD8 than DXL birds, indicating the poor cell-mediated immunity in DXL birds (Fahey and Cheng, 2008). Taken together, increased aggressive behavior in DXL birds may be attributed to the genetic background-related immunological status and hormone changes in response to stimulations.

1.7 The aims of this study

Accumulating evidence from human and animal trials has indicated that modulation of gut microbiota affects host physiology and behavioral expression, suggesting alterations in gut microbiota may be considered as a feasible strategy for preventing aggression and improving well-being in chickens. In this study, we hypothesized that early intervention with transplantation of cecal content from gentle (line 6_3) and aggressive birds (line 7_2) would differently affect recipient birds' physiological and behavioral expressions at adulthood based on the donors' biological characteristics. Furthermore, aggressive behavior would be reduced or inhibited by transplanting the microbiota from gentle chickens. Therefore, this project aimed to determine if 1) CMT from

different donors would change the biological characters and aggressive behavior of recipient chickens differently; 2) CMT from different donors would alter the recipient chickens' gut microbiota population and composition differently; 3) the biological and behavioral changes in recipients would correlate with the donors' biological and behavioral characteristics.

1.8 References

Abdel-Azeem, M. (2013). Do probiotics affect the behavior of turkey poults?. *Journal of Veterinary Medicine and Animal Health*, *5*(5), 144-148.

Albentosa, M. J., Cooper, J. J., Luddem, T., Redgate, S. E., Elson, H. A., and Walker, A. W. (2007). Evaluation of the effects of cage height and stocking density on the behaviour of laying hens in furnished cages. *British Poultry Science*, *48*(1), 1-11.

Al-Rawi, B., Craig, J. V., and Adams, A. W. (1976). Agonistic behavior and egg production of caged layers: genetic strain and group-size effects. *Poultry Science*, *55*(2), 796-807.

Amarapurkar, D. N. (2011). Prescribing medications in patients with decompensated liver cirrhosis. *International Journal of Hepatology*, *2011*, 519526.

Apajalahti, J., Kettunen, A., and Graham, H. (2004). Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal*, *60*(2), 223-232.

Asano, Y., Hiramoto, T., Nishino, R., Aiba, Y., Kimura, T., Yoshihara, K., Koga, Y. and Sudo, N. (2012). Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *303*(11), G1288-G1295.

Awad, W. A., Aschenbach, J. R., Ghareeb, K., Khayal, B., Hess, C., and Hess, M. (2014). Campylobacter jejuni influences the expression of nutrient transporter genes in the intestine of chickens. *Veterinary Microbiology*, *172*(1-2), 195-201.

Awad, W. A., Molnár, A., Aschenbach, J.R., Ghareeb, K., Khayal, B., Hess, C., Liebhart, D., Dublecz, K. and Hess, M. (2015). Campylobacter infection in chickens modulates the intestinal epithelial barrier function. *Innate Immunity*, *21*(2), 151-160.

Awad, W. A., Mann, E., Dzieciol, M., Hess, C., Schmitz-Esser, S., Wagner, M., and Hess, M. (2016). Age-related differences in the luminal and mucosa-associated gut microbiome of broiler chickens and shifts associated with Campylobacter jejuni infection. *Frontiers in Cellular and Infection Microbiology*, *6*, 154.

Awad, W. A., Hess, C., and Hess, M. (2017). Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. *Toxins*, *9*(2), 60.

Bacon, L. D., Hunt, H. D., and Cheng, H. H. (2000). A review of the development of chicken lines to resolve genes determining resistance to diseases. *Poultry Science*, *79*(8), 1082-1093.

Bacon, L. D., and Palmquist, D. (2002). Chicken lines differ in production of interferon-like activity by peripheral white blood cells stimulated with phytohemagglutinin. *Poultry Science*, *81*(11), 1629-1636.

Bacon, L. D., Hunt, H. D., and Cheng, H. H. (2001). Genetic resistance to Marek's disease. *Marek's Disease*, 121-141.

Baldwin, S., Hughes, R. J., Hao Van, T. T., Moore, R. J., and Stanley, D. (2018). At-hatch administration of probiotic to chickens can introduce beneficial changes in gut microbiota. *PLoS One*, *13*(3), e0194825.

Bareham, J. R. (1976). A comparison of the behaviour and production of laying hens in experimental and conventional battery cages. *Applied Animal Ethology*, *2*(4), 291-303.

Belkaid, Y., and Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell*, *157*(1), 121-141.

Benarroch, E. E. (2019). Autonomic nervous system and neuroimmune interactions: New insights and clinical implications. *Neurology*, *92*(8), 377-385.

Bercik, P., Collins, S. M., and Verdu, E. F. (2012). Microbes and the gut-brain axis. *Neurogastroenterology and Motility*, *24*(5), 405-413.

Berman, M. E., Tracy, J. I., and Coccaro, E. F. (1997). The serotonin hypothesis of aggression revisited. *Clinical Psychology Review*, *17*(6), 651-665.

Bessei, W. and Kjaer, J. (2015) Feather pecking in layers - State of research and implications, in: *26th Annual Australian Poultry Science Symposium 26*(9): 214-221.

Bhatnagar, S., and Dallman, M. (1998). Neuroanatomical basis for facilitation of hypothalamic-pituitary-adrenal responses to a novel stressor after chronic stress. *Neuroscience*, *84*(4), 1025-1039.

Bonder, M.J., Kurilshikov, A., Tigchelaar, E.F., Mujagic, Z., Imhann, F., Vila, A.V., Deelen, P., Vatanen, T., Schirmer, M., Smeekens, S.P. and Zhernakova, D.V. (2016). The effect of host genetics on the gut microbiome. *Nature Genetics*, *48*(11), 1407-1412.

Borodovitsyna, O., Flamini, M., and Chandler, D. (2017). Noradrenergic modulation of cognition in health and disease. *Neural Plasticity*, 6031478.

Borody, T. J., and Khoruts, A. (2012). Fecal microbiota transplantation and emerging applications. *Nature reviews Gastroenterology and Hepatology*, *9*(2), 88-96.

Borody, T. J., Warren, E. F., Leis, S. M., Surace, R., Ashman, O., and Siarakas, S. (2004). Bacteriotherapy using fecal flora: toying with human motions. *Journal of Clinical Gastroenterology*, *38*(6), 475-483.

Bradshaw, W. (1867). Brain and Stomach or Mind and Matter. London: W. Philip.

Bridle, B. W., Julian, R., Shewen, P. E., Vaillancourt, J. P., and Kaushik, A. K. (2006). T lymphocyte subpopulations diverge in commercially raised chickens. *Canadian Journal of Veterinary Research*, *70*(3), 183.

Brisbin, J. T., Parvizi, P., and Sharif, S. (2012). Differential cytokine expression in T-cell subsets of chicken caecal tonsils co-cultured with three species of Lactobacillus. *Beneficial Microbes*, *3*(3), 205-210.

Burkholder, K. M., Thompson, K. L., Einstein, M. E., Applegate, T. J., and Patterson, J. A. (2008). Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to Salmonella enteritidis colonization in broilers. *Poultry Science*, *87*(9), 1734-1741.

Čaja, F., Stakheev, D., Chernyavskiy, O., Křížan, J., Dvořák, J., Rossmann, P., Štěpánková, R., Makovický, P., Makovický, P., Kozáková, H. and Vannucci, L. (2021). Immune activation by microbiome shapes the colon mucosa: Comparison between healthy rat mucosa under conventional and germ-free conditions. *Journal of Immunotoxicology*, *18*(1), 37-49.

Cammarota, G., Ianiro, G., and Gasbarrini, A. (2014a). Fecal microbiota transplantation for the treatment of Clostridium difficile infection: a systematic review. *Journal of Clinical Gastroenterology*, *48*(8), 693-702.

Cammarota, G., Ianiro, G., Bibbo, S., and Gasbarrini, A. (2014b). Gut microbiota modulation: probiotics, antibiotics or fecal microbiota transplantation?. *Internal and Emergency Medicine*, *9*(4), 365-373.

Chaitman, J., Jergens, A. E., Gaschen, F., Garcia-Mazcorro, J. F., Marks, S. L., Marroquin-Cardona, A. G., Richter, K., Rossi, G., Suchodolski, J. S. and Weese, J. S. (2016). Commentary on key aspects of fecal microbiota transplantation in small animal practice. *Veterinary Medicine: Research and Reports*, *7*, 71.

Chakaroun, R. M., Massier, L., and Kovacs, P. (2020). Gut microbiome, intestinal permeability, and tissue bacteria in metabolic disease: perpetrators or bystanders?. *Nutrients*, *12*(4), 1082.

Cheng, H. (2006). Morphopathological changes and pain in beak trimmed laying hens. *World's Poultry Science Journal*, *62*(1), 41-52.

Cheng, H. W. (2010). Breeding of tomorrow's chickens to improve well-being. *Poultry Science*, *89*(4), 805-813.

Cheng, H. W., Dillworth, G., Singleton, P., Chen, Y., and Muirt, W. M. (2001a). Effects of group selection for productivity and longevity on blood concentrations of serotonin, catecholamines, and corticosterone of laying hens. *Poultry Science, 80*(9), 1278-1285.

Cheng, H. W., Eicher, S. D., Chen, Y., Singleton, P., and Muirt, W. M. (2001b). Effect of genetic selection for group productivity and longevity on immunological and hematological parameters of chickens. *Poultry Science*, *80*(8), 1079-1086.

Cheng, H. W., and Muir, W. M. (2007). Mechanisms of aggression and production in chickens: genetic variations in the functions of serotonin, catecholamine, and corticosterone. *World's Poultry Science Journal*, *63*(2), 233-254.

Cheng, H. W., Singleton, P., and Muir, W. M. (2003). Social stress differentially regulates neuroendocrine responses in laying hens: I. Genetic basis of dopamine responses under three different social conditions. *Psychoneuroendocrinology*, *28*(5), 597-611.

Clench, M. H., and Mathias, J. R. (1995). The avian cecum: a review. *The Wilson Bulletin*, *107*, 93-121.

Cloutier, S., and Newberry, R. C. (2002). A note on aggression and cannibalism in laying hens following re-housing and re-grouping. *Applied Animal Behaviour Science*, *76*(2), 157-163.

Collins, S. M., Surette, M., and Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. *Nature Reviews Microbiology*, *10*(11), 735-742.

Cooper, J. J., and Albentosa, M. J. (2003). Behavioural priorities of laying hens. *Avian and Poultry Biology Reviews*, *14*(3), 127-149.

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., and Knight, R. (2009). Bacterial community variation in human body habitats across space and time. *Science*, *326*(5960), 1694-1697.

Craig, J. V. (1982). Behavioral and genetic adaptation of laying hens to high-density environments. *Bioscience*, *32*(1), 33-37.

Craig, J. V. (1986). Measuring social behavior: social dominance. *Journal of Animal Science*, *62*(4), 1120-1129.

Craig, J. V., and Adams, A. W. (1984). Behaviour and well-being of hens (Gallus domesticus) in alternative housing environments. *World's Poultry Science Journal*, *40*(3), 221-240.

Craig, J. V., and Muir, W. M. (1996). Group selection for adaptation to multiple-hen cages: beak-related mortality, feathering, and body weight responses. *Poultry Science*, *75*(3), 294-302.

Crumeyrolle-Arias, M., Jaglin, M., Bruneau, A., Vancassel, S., Cardona, A., Daugé, V., Naudon, L. and Rabot, S. (2014). Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. *Psychoneuroendocrinology*, *42*, 207-217.

D'Eath, R. B., and Keeling, L. J. (2003). Social discrimination and aggression by laying hens in large groups: from peck orders to social tolerance. *Applied Animal Behaviour Science*, *84*(3), 197-212.

Dalton, H. A., Wood, B. J., and Torrey, S. (2013). Injurious pecking in domestic turkeys: development, causes, and potential solutions. *World's Poultry Science Journal*, *69*(4), 865-876.

Dave, M., Higgins, P. D., Middha, S., and Rioux, K. P. (2012). The human gut microbiome: current knowledge, challenges, and future directions. *Translational Research*, *160*(4), 246-257.

De Groot, P. F., Frissen, M. N., De Clercq, N. C., and Nieuwdorp, M. (2017). Fecal microbiota transplantation in metabolic syndrome: history, present and future. *Gut Microbes*, *8*(3), 253-267.

de Jonge, W. J. (2013). The gut's little brain in control of intestinal immunity. *International Scholarly Research Notices*, *2013*, 630159.

De Palma, G., Collins, S. M., Bercik, P., and Verdu, E. F. (2014). The microbiota–gut–brain axis in gastrointestinal disorders: stressed bugs, stressed brain or both?. *The Journal of Physiology*, *592*(14), 2989-2997.

de Theije, C. G., Wu, J., Da Silva, S. L., Kamphuis, P. J., Garssen, J., Korte, S. M., and Kraneveld, A. D. (2011). Pathways underlying the gut-to-brain connection in autism spectrum disorders as future targets for disease management. *European Journal of Pharmacology*, *668*(S1), S70-S80.

Delia, E., Tafaj, M., and Männer, K. (2012). Efficiency of probiotics in farm animals. *Probiotic in Animals*, 247-272.

Dennis, R. L., and Cheng, H. W. (2011). The dopaminergic system and aggression in laying hens. *Poultry Science*, *90*(11), 2440-2448.

Dennis, R. L., and Cheng, H. W. (2012). Effects of different infrared beak treatment protocols on chicken welfare and physiology. *Poultry Science*, *91*(7), 1499-1505.

Dennis, R. L., and Cheng, H. W. (2014). Differential serotonergic mediation of aggression in roosters bred for resistance and susceptibility to Marek's disease. *British Poultry Science*, *55*(1), 13-20.

Dennis, R. L., Chen, Z. Q., and Cheng, H. W. (2008). Serotonergic mediation of aggression in high and low aggressive chicken strains. *Poultry Science*, *87*(4), 612-620.

Dennis, R. L., Fahey, A. G., and Cheng, H. W. (2009). Infrared beak treatment method compared with conventional hot-blade trimming in laying hens. *Poultry Science*, *88*(1), 38-43.

Dennis, R., Zhang, H. M., Bacon, L. D., Estevez, I., and Cheng, H. W. (2004). Behavioral and physiological features of chickens diversely selected for resistance to avian disease. 1. Selected inbred lines differ in behavioral and physical responses to social stress. *Poultry Science*, *83*(9), 1489-1496.

Dinan, T. G., and Cryan, J. F. (2015). The impact of gut microbiota on brain and behaviour: implications for psychiatry. *Current Opinion in Clinical Nutrition and Metabolic Care*, *18*(6), 552-558.

Dinan, T. G., and Cryan, J. F. (2017). Gut instincts: microbiota as a key regulator of brain development, ageing and neurodegeneration. *The Journal of physiology*, *595*(2), 489-503.

Duncan, I. J. (2001). The pros and cons of cages. *World's Poultry Science Journal*, *57*(4), 381-390.

Duncan, I. J. H., and Wood-Gush, D. G. M. (1971). Frustration and aggression in the domestic fowl. *Animal Behaviour*, *19*(3), 500-504.

Edwards, A. C., Rollmann, S. M., Morgan, T. J., and Mackay, T. F. (2006). Quantitative genomics of aggressive behavior in Drosophila melanogaster. *PLoS Genetics*, *2*(9), e154.

Edwards, D. H., and Kravitz, E. A. (1997). Serotonin, social status and aggression. *Current Opinion in Neurobiology*, *7*(6), 812-819.

Eiseman, B., Silen, W., Bascom, G. and Kauvar, A. (1958) Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. *Surgery*, *44*(5), 854-859.

Eisenhofer, G., Kopin, I. J., and Goldstein, D. S. (2004). Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacological Reviews*, *56*(3), 331-349.

Fahey, A. G., and Cheng, H. W. (2008). Group size and density effects on physical indices and cell-mediated immunity in two genetic lines of white leghorn layers. *Poultry Science*, *87*(12), 2500-2504.

Fan, P., Bian, B., Teng, L., Nelson, C. D., Driver, J., Elzo, M. A., and Jeong, K. C. (2020). Host genetic effects upon the early gut microbiota in a bovine model with graduated spectrum of genetic variation. *The ISME Journal*, *14*(1), 302-317.

Ferreira, C. M., Vieira, A. T., Vinolo, M. A. R., Oliveira, F. A., Curi, R., and Martins, F. D. S. (2014). The central role of the gut microbiota in chronic inflammatory diseases. *Journal of Immunology Research*, *2014*, 689492.

Feye, K. M., Baxter, M. F. A., Tellez-Isaias, G., Kogut, M. H., and Ricke, S. C. (2020). Influential factors on the composition of the conventionally raised broiler gastrointestinal microbiomes. *Poultry Science*, *99*(2), 653-659.

Forsythe, P., and Kunze, W. A. (2013). Voices from within: gut microbes and the CNS. *Cellular and Molecular Life Sciences*, *70*(1), 55-69.

Fraser, D., Duncan, I. J., Edwards, S. A., Grandin, T., Gregory, N. G., Guyonnet, V., Hemsworth, P. H., Huertas, S. M., Huzzey, J. M., Mellor, D. J. and Mench, J. A. (2013). General principles for the welfare of animals in production systems: the underlying science and its application. *The Veterinary Journal*, *198*(1), 19-27.

Fraser, D., Weary, D. M., Pajor, E. A., and Milligan, B. N. (1997). A scientific conception of animal welfare that reflects ethical concerns. *Animal Welfare*, *6*, 187-205.

Frazer, K. A., Murray, S. S., Schork, N. J., and Topol, E. J. (2009). Human genetic variation and its contribution to complex traits. *Nature Reviews Genetics*, *10*(4), 241-251.

Fung, T. C. (2020). The microbiota-immune axis as a central mediator of gut-brain communication. *Neurobiology of Disease*, *136*, 104714.

Fung, T. C., Olson, C. A., and Hsiao, E. Y. (2017). Interactions between the microbiota, immune and nervous systems in health and disease. *Nature Neuroscience*, *20*(2), 145.

Furet, J. P., Kong, L. C., Tap, J., Poitou, C., Basdevant, A., Bouillot, J. L, et al. (2010). Differential adaptation of human gut microbiota to bariatric surgery–induced weight loss: links with metabolic and low-grade inflammation markers. *Diabetes*, *59*(12), 3049-3057.

Gallucci, A., Riva, P., Lauro, L. J. R., and Bushman, B. J. (2020). Stimulating the ventrolateral prefrontal cortex (VLPFC) modulates frustration-induced aggression: A tDCS experiment. *Brain Stimulation*, *13*(2), 302-309.

Gershon, M. D. (2003). Serotonin and its implication for the management of irritable bowel syndrome. *Reviews in Gastroenterological Disorders*, *3*, S25-S34.

Ghareeb, K., Awad, W. A., Böhm, J., and Zebeli, Q. (2016). Impact of luminal and systemic endotoxin exposure on gut function, immune response and performance of chickens. *World's Poultry Science Journal*, *72*(2), 367-380.

Glatz, P. C. (2005). *Poultry Welfare Issues: Beak Trimming*. Nottingham University Press, Nottingham, United Kingdom.

Goodman, S. H., Rouse, M. H., Connell, A. M., Broth, M. R., Hall, C. M., and Heyward, D. (2011). Maternal depression and child psychopathology: A meta-analytic review. *Clinical Child and Family Psychology Review*, *14*(1), 1-27.

Grehan, M. J., Borody, T. J., Leis, S. M., Campbell, J., Mitchell, H., and Wettstein, A. (2010). Durable alteration of the colonic microbiota by the administration of donor fecal flora. *Journal of Clinical Gastroenterology*, *44*(8), 551-561.

GS, B., and AJ, K. (1958). Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. *Surgery*, *44*(5), 854-859.

Guhl, A. M., Craig, J. V., and Mueller, C. D. (1960). Selective breeding for aggressiveness in chickens. *Poultry Science*, *39*(4), 970-980.

Hartcher, K. M., and Jones, B. (2017). The welfare of layer hens in cage and cage-free housing systems. *World's Poultry Science Journal*, *73*(4), 767-782.

Hartcher, K. M., Tran, K. T. N., Wilkinson, S. J., Hemsworth, P. H., Thomson, P. C., and Cronin, G. M. (2015). The effects of environmental enrichment and beak-trimming during the rearing period on subsequent feather damage due to feather-pecking in laying hens. *Poultry Science*, *94*(5), 852-859.

Herr, N., Bode, C., and Duerschmied, D. (2017). The effects of serotonin in immune cells. *Frontiers in Cardiovascular Medicine*, *4*, 48.

Hopkins, M. J., and Macfarlane, G. T. (2003). Nondigestible oligosaccharides enhance bacterial colonization resistance against Clostridium difficile in vitro. *Applied and Environmental Microbiology*, *69*(4), 1920.

Hornung, J. P. (2003). The human raphe nuclei and the serotonergic system. *Journal of Chemical Neuroanatomy*, *26*(4), 331-343.

Hsiao, E. Y., McBride, S. W., Hsien, S., Sharon, G., Hyde, E. R., McCue, T., Codelli, J.A., Chow, J., Reisman, S. E., Petrosino, J. F. and Patterson, P. H. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell*, *155*(7), 1451-1463.

Hughes, B. O. (1977). Selection of group size by individual laying hens. *British Poultry Science*, *18*(1), 9-18.

Hughes, B. O., and Gentle, M. J. (1995). Beak trimming of poultry: its implications for welfare. *World's Poultry Science Journal*, *51*(1), 51-61.

Immerseel, F. V., Buck, J. D., Pasmans, F., Huyghebaert, G., Haesebrouck, F., and Ducatelle, R. (2004). Clostridium perfringens in poultry: an emerging threat for animal and public health. *Avian Pathology*, *33*(6), 537-549.

Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., and Reddy, D. N. (2015). Role of the normal gut microbiota. *World journal of gastroenterology: WJG*, *21*(29), 8787.

Jendral, M. J., and Robinson, F. E. (2004). Beak trimming in chickens: Historical, economical, physiological and welfare implications, and alternatives for preventing feather pecking and cannibalistic activity. *Avian and Poultry Biology Reviews*. *15*, 9-23.

Johannson, S. G., Raginski, C., Schwean-Lardner, K., and Classen, H. L. (2016). Providing laying hens in group-housed enriched cages with access to barley silage reduces aggressive and feather-pecking behaviour. *Canadian Journal of Animal Science*, *96*(2), 161-171.

Karlsson, F., Tremaroli, V., Nielsen, J., and Bäckhed, F. (2013). Assessing the human gut microbiota in metabolic diseases. *Diabetes*, *62*(10), 3341-3349.

Keating, D. J., and Spencer, N. J. (2019). What is the role of endogenous gut serotonin in the control of gastrointestinal motility?. *Pharmacological research*, *140*, 50-55.

Kers, J. G., Velkers, F. C., Fischer, E. A., Hermes, G. D., Stegeman, J. A., and Smidt, H. (2018). Host and environmental factors affecting the intestinal microbiota in chickens. *Frontiers in Microbiology, 9*, 235.

Khachatryan, Z. A., Ktsoyan, Z. A., Manukyan, G. P., Kelly, D., Ghazaryan, K. A., and Aminov, R. I. (2008). Predominant role of host genetics in controlling the composition of gut microbiota. *PloS One*, *3*(8), e3064.

Kjaer, J. B., and Vestergaard, K. S. (1999). Development of feather pecking in relation to light intensity. *Applied Animal Behaviour Science*, *62*, 243-254.

Korte, S. M., Beuving, G., Ruesink, W. I. M., and Blokhuis, H. J. (1997). Plasma catecholamine and corticosterone levels during manual restraint in chicks from a high and low feather pecking line of laying hens. *Physiology and Behavior*, *62*(3), 437-441.

Kovacs, A., Ben-Jacob, N., Tayem, H., Halperin, E., Iraqi, F.A. and Gophna, U. (2011). Genotype is a stronger determinant than sex of the mouse gut microbiota. *Microbial Ecology*, *61*(2), 423-428.

Krishnan, S., Alden, N., and Lee, K. (2015). Pathways and functions of gut microbiota metabolism impacting host physiology. *Current Opinion in Biotechnology*, *36*, 137-145.

Le Beyec, J., Pelletier, A. L., Arapis, K., Hourseau, M., Cluzeaud, F., Descatoire, V., Ducroc, R., Aparicio, T., Joly, F., Couvelar, A., Marmuse, J-P., Gall L. M., and and Bado, A. (2014). Overexpression of gastric leptin precedes adipocyte leptin during high-fat diet and is linked to 5HT-containing enterochromaffin cells. *International Journal of Obesity*, *38*(10), 1357-1364.

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., ... and Pedersen, O. (2013). Richness of human gut microbiome correlates with metabolic markers. *Nature*, *500*(7464), 541-546.

Lederberg, J., and McCray, A. T. (2001). Ome SweetOmics--A genealogical treasury of words. *The Scientist*, *15*(7), 8-8.

Lee, S. M., Kim, N., Park, J. H., Nam, R. H., Yoon, K., and Lee, D. H. (2018). Comparative analysis of ileal and cecal microbiota in aged rats. *Journal of Cancer Prevention*, *23*(2), 70.

Li, D., Duan, Y., and He, L. (2006). Association study of serotonin 2A receptor (5-HT2A) gene with schizophrenia and suicidal behavior using systematic meta-analysis. *Biochemical and biophysical research communications*, *340*(3), 1006-1015.

Li, G., and Young, K. D. (2013). Indole production by the tryptophanase TnaA in Escherichia coli is determined by the amount of exogenous tryptophan. *Microbiology*, *159*, 402-410.

Lischinsky, J. E., and Lin, D. (2020). Neural mechanisms of aggression across species. *Nature neuroscience*, *23*(11), 1317-1328.

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. *Bioessays*, *33*(8), 574-581.

Lyte, M., and Ernst, S. (1992). Catecholamine induced growth of gram negative bacteria. *Life sciences*, *50*(3), 203-212.

Marchant-Forde, R. M., Fahey, A. G., and Cheng, H. W. (2008). Comparative effects of infrared and one-third hot-blade trimming on beak topography, behavior, and growth. *Poultry Science*, *87*(8), 1474-1483.

Marchewka, J., Watanabe, T.T.N., Ferrante, V. and Estevez, I., 2013. Review of the social and environmental factors affecting the behavior and welfare of turkeys (Meleagris gallopavo). *Poultry Science*, *92*(6), 1467-1473.

Maria, G. A., Escós, J., and Alados, C. L. (2004). Complexity of behavioural sequences and their relation to stress conditions in chickens (Gallus gallus domesticus): a non-invasive technique to evaluate animal welfare. *Applied Animal Behaviour Science*, *86*(1-2), 93-104.

Mättö, J., Maunuksela, L., Kajander, K., Palva, A., Korpela, R., Kassinen, A., and Saarela, M. (2005). Composition and temporal stability of gastrointestinal microbiota in irritable bowel syndrome—a longitudinal study in IBS and control subjects. *FEMS Immunology and Medical Microbiology*, *43*(2), 213-222.

Mayer, E. A. (2011). Gut feelings: the emerging biology of gut–brain communication. *Nature Reviews Neuroscience*, *12*(8), 453-466.

McDonald, L. C., Gerding, D. N., Johnson, S., Bakken, J. S., Carroll, K. C., Coffin, S. E., ... and Wilcox, M. H. (2018). Clinical practice guidelines for Clostridium difficile infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clinical Infectious Diseases*, *66*(7), e1-e48.

Mégraud, F. (2012). The challenge of Helicobacter pylori resistance to antibiotics: the comeback of bismuth-based quadruple therapy. *Therapeutic advances in gastroenterology*, *5*(2), 103-109.

Mench, J. A. (1988). The development of aggressive behavior in male broiler chicks: a comparison with laying-type males and the effects of feed restriction. *Applied Animal Behaviour Science*, *21*(3), 233-242.

Misiak, B., Łoniewski, I., Marlicz, W., Frydecka, D., Szulc, A., Rudzki, L., and Samochowiec, J. (2020). The HPA axis dysregulation in severe mental illness: Can we shift the blame to gut microbiota?. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *102*, 109951.

Mizrahi, R., Addington, J., Rusjan, P. M., Suridjan, I., Ng, A., Boileau, I., Pruessner, C. J., Remington, G., Houle, S., and Wilson, A. A. (2012). Increased stress-induced dopamine release in psychosis. *Biological Psychiatry*, *71*(6), 561-567.

Mohammed, H. H., Grashorn, M. A., and Bessei, W. (2010). The effects of lighting conditions on the behaviour of laying hens. *Archiv für Geflügelkunde*, *74*(3), 197-202.

Montoro-Dasi, L., Villagra, A., de Toro, M., Pérez-Gracia, M. T., Vega, S., and Marin, C. (2021). Assessment of Microbiota Modulation in Poultry to Combat Infectious Diseases. *Animals*, *11*(3), 615.

Mu, C., Yang, Y., Su, Y., Zoetendal, E. G., and Zhu, W. (2017). Differences in microbiota membership along the gastrointestinal tract of piglets and their differential alterations following an early-life antibiotic intervention. *Frontiers in Microbiology*, *8*, 797.

Muir, W. M. (1996). Group selection for adaptation to multiple-hen cages: selection program and direct responses. *Poultry Science*, *75*(4), 447-458.

Muir, W. M., and Cheng, H. W. (2004). Breeding for productivity and welfare of laying hens. *Welfare of the Laying Hen*, *27*, 123-138.

Nelson, R. J. (Ed.). (2005). Biology of aggression. Oxford University, New York.

Newberry, R. C. (1995). Environmental enrichment: increasing the biological relevance of captive environments. *Applied Animal Behaviour Science*, *44*(2-4), 229-243.

Nordquist, R. E., Van der Staay, F. J., Van Eerdenburg, F. J., Velkers, F. C., Fijn, L., and Arndt, S. S. (2017). Mutilating procedures, management practices, and housing conditions that may affect the welfare of farm animals: implications for welfare research. *Animals*, *7*(2), 12.

Olivier, B. (2004). Serotonin and aggression. *Annals of the New York Academy of Sciences*, *1036*(1), 382-392.

Orrhage, K., and Nord, C. E. (1999). Factors controlling the bacterial colonization of the intestine in breastfed infants. *Acta Paediatrica*, *88*, 47-57.

Pandit, R.J., Hinsu, A.T., Patel, N.V., Koringa, P.G., Jakhesara, S.J., Thakkar, J.R., Shah, T.M., Limon, G., Psifidi, A., Guitian, J. and Hume, D.A. (2018). Microbial diversity and community composition of caecal microbiota in commercial and indigenous Indian chickens determined using 16s rDNA amplicon sequencing. *Microbiome*, *6*(1), 1-13.

Pauwels, J., Taminiau, B., Janssens, G. P. J., De Beenhouwer, M., Delhalle, L., Daube, G., and Coopman, F. (2015). Cecal drop reflects the chickens' cecal microbiome, fecal drop does not. *Journal of microbiological methods*, *117*, 164-170.

Pedroso, A. A., Batal, A. B., and Lee, M. D. (2016). Effect of in ovo administration of an adult-derived microbiota on establishment of the intestinal microbiome in chickens. *American Journal of Veterinary Research*, *77*(5), 514-526.

Peirce, J. M., and Alviña, K. (2019). The role of inflammation and the gut microbiome in depression and anxiety. *Journal of Neuroscience Research*, *97*(10), 1223-1241.

Petrolli, T. G., Petrolli, O. J., Girardini, L. K., Zotti, M. L. A. N., Baggio, R. A., and Junqueira, O. M. (2017). Effects of laser beak trimming on the development of brown layer pullets. *Brazilian Journal of Poultry Science*, *19*(1), 123-128.

Pezawas, L., Meyer-Lindenberg, A., Drabant, E. M., Verchinski, B. A., Munoz, K.E., Kolachana, B. S., Egan, M. F., Mattay, V. S., Hariri, A. R. and Weinberger, D. R. (2005). 5-HTTLPR polymorphism impacts human cingulate-amygdala interactions: a genetic susceptibility mechanism for depression. *Nature Neuroscience*, *8*(6), 828-834.

Polansky, O., Sekelova, Z., Faldynova, M., Sebkova, A., Sisak, F., and Rychlik, I. (2016). Important metabolic pathways and biological processes expressed by chicken cecal microbiota. *Applied and Environmental Microbiology*, *82*(5), 1569.

Popova, N. K., Kulikov, A. V., Nikulina, E. M., Kozlachkova, E. Y., and Maslova, G. B. (1991). Serotonin metabolism and serotonergic receptors in Norway rats selected for low aggressiveness to man. *Aggressive Behavior*, *17*(4), 207-213.

Prescott, N. B., Wathes, C. M., and Jarvis, J. R. (2003). Light, vision and the welfare of poultry. *Animal Welfare*, *12*(2), 269-288.

Rapport, M. M., Green, A. A., and Page, I. H. (1949). Serum vasoconstrictor (serotonin). *Journal of Biological Chemistry*, *176*, 1243-1251.

Relić, R., Sossidou, E., Dedousi, A., Perić, L., Božičković, I., and Đukić-Stojčić, M. (2019). Behavioral and health problems of poultry related to rearing systems. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, *66*(4), 423-428.

Rodenburg, T. B., Tuyttens, F. A. M., De Reu, K., Herman, L., Zoons, J., and Sonck, B. (2008). Welfare assessment of laying hens in furnished cages and non-cage systems: an on-farm comparison. *Animal Welfare*, *17*(4), 363-373.

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., Avershina, E., Rudi, K., Narbad, A., Jenmalm, M. C. and Marchesi, J. R. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health and Disease*, *26*(1), 26050.

Rogers, G. B., Keating, D. J., Young, R. L., Wong, M. L., Licinio, J., and Wesselingh, S. (2016). From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. *Molecular Psychiatry*, *21*(6), 738-748.

Rubí, B., and Maechler, P. (2010). Minireview: new roles for peripheral dopamine on metabolic control and tumor growth: let's seek the balance. *Endocrinology*, *151*(12), 5570-5581.

Rubio, L. A. (2019). Possibilities of early life programming in broiler chickens via intestinal microbiota modulation. *Poultry Science*, *98*(2), 695-706.

Rychlik, I. (2020). Composition and function of chicken gut microbiota. *Animals*, *10*(1), 103.

Schinka, J. A., Busch, R. M., and Robichaux-Keene, N. (2004). A meta-analysis of the association between the serotonin transporter gene polymorphism (5-HTTLPR) and trait anxiety. *Molecular Psychiatry*, *9*(2), 197-202.

Schokker, D., Veninga, G., Vastenhouw, S.A., Bossers, A., de Bree, F.M., Kaal-Lansbergen, L.M., Rebel, J.M. and Smits, M.A. (2015). Early life microbial colonization of the gut and intestinal development differs between genetically divergent broiler lines. *BMC Genomics*, *16*(1), 1-13.

Sedlačková, M., Bilčik, B., and Košťál, Ľ. (2004). Feather pecking in laying hens: Environmental and endogenous factors. *Acta Veterinaria Brno, 73*(4), 521-531.

Shakouri, M. D., Iji, P. A., Mikkelsen, L. L., and Cowieson, A. J. (2009). Intestinal function and gut microflora of broiler chickens as influenced by cereal grains and microbial enzyme supplementation. *Journal of Animal Physiology and Animal Nutrition, 93*(5), 647-658.

Shang, Y., Kumar, S., Oakley, B., and Kim, W. K. (2018). Chicken gut microbiota: importance and detection technology. *Frontiers in Veterinary Science, 5*, 254.

Shaufi, M. A. M., Sieo, C. C., Chong, C. W., Gan, H. M., and Ho, Y. W. (2015). Deciphering chicken gut microbial dynamics based on high throughput 16S rRNA metagenomic analyses. *Gut Pathogens, 7*(1), 4.

Sherwin, C. M., Richards, G. J., and Nicol, C. J. (2010). Comparison of the welfare of layer hens in 4 housing systems in the UK. *British Poultry Science, 51*(4), 488-499.

Sjögren, K., Engdahl, C., Henning, P., Lerner, U.H., Tremaroli, V., Lagerquist, M.K., Bäckhed, F. and Ohlsson, C. (2012). The gut microbiota regulates bone mass in mice. *Journal of Bone and Mineral Research, 27*(6), 1357-1367.

Sofos, J. N. (2008). Challenges to meat safety in the 21st century. *Meat Science, 78*, 3-13.

Spor, A., Koren, O., and Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. *Nature Reviews Microbiology, 9*(4), 279-290.

Srikantha, P., and Mohajeri, M. H. (2019). The possible role of the microbiota-gut-brain-axis in autism spectrum disorder. *International Journal of Molecular Sciences, 20*(9), 2115.

Stanley, D., Geier, M. S., Chen, H., Hughes, R. J., and Moore, R. J. (2015). Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. *BMC Microbiology, 15*(1), 1-11.

Stasi, C., Caserta, A., Nisita, C., Cortopassi, S., Fani, B., Salvadori, S., Pancetti, A., Bertani, L., Gambaccini, D., de Bortoli, N. and Dell'Osso, L. (2019). The complex interplay between gastrointestinal and psychiatric symptoms in irritable bowel syndrome: A longitudinal assessment. *Journal of Gastroenterology and Hepatology*, *34*(4), 713-719.

Stone, H. A. (1975). *Use of highly inbred chickens in research*. USDA Agricultural Research Service Technical Bulletin, 1514.

Su, G., Kjaer, J. B., and Sørensen, P. (2006). Divergent selection on feather pecking behavior in laying hens has caused differences between lines in egg production, egg quality, and feed efficiency. *Poultry Science*, *85*(2), 191-197.

Sudo, N. (2014). Microbiome, HPA axis and production of endocrine hormones in the gut. *Advances in Experimental Medicine and Biology*, *817*, 177-194.

Sunkara, T., Rawla, P., Ofosu, A., and Gaduputi, V. (2018). Fecal microbiota transplant–a new frontier in inflammatory bowel disease. *Journal of Inflammation Research*, *11*, 321.

Tablante, N. L., Vaillancourt, J. P., Martin, S. W., Shoukri, M., and Estevez, I. (2000). Spatial distribution of cannibalism mortalities in commercial laying hens. *Poultry Science*, *79*(5), 705-708.

Taschuk, R., and Griebel, P. J. (2012). Commensal microbiome effects on mucosal immune system development in the ruminant gastrointestinal tract. *Animal Health Research Reviews*, *13*(1), 129.

Tauson, R. (2002). Furnished cages and aviaries: production and health. *World's Poultry Science Journal*, *58*(1), 49-63.

Tauson, R. (2005). Management and housing systems for layers–effects on welfare and production. *World's Poultry Science Journal*, *61*(3), 477-490.

Turpin, W., Espin-Garcia, O., Xu, W., Silverberg, M.S., Kevans, D., Smith, M.I., Guttman, D.S., Griffiths, A., Panaccione, R., Otley, A. and Xu, L. (2016). Association of host genome with intestinal microbial composition in a large healthy cohort. *Nature Genetics*, *48*(11), 1413-1417.

Twarog, B. M., and Page, I. H. (1953). Serotonin content of some mammalian tissues and urine and a method for its determination. *American Journal of Physiology-Legacy Content*, *175*(1), 157-161.

Van de Merwe, J. P., Stegeman, J. H., and Hazenberg, M. P. (1983). The resident faecal flora is determined by genetic characteristics of the host. Implications for Crohn's disease?. *Antonie Van Leeuwenhoek*, *49*(2), 119-124.

van der Eijk, J.A., de Vries, H., Kjaer, J.B., Naguib, M., Kemp, B., Smidt, H., Rodenburg, T.B. and Lammers, A. (2019a). Differences in gut microbiota composition of laying hen lines divergently selected on feather pecking. *Poultry Science*, *98*(12), 7009-7021.

van der Eijk, J. A., Lammers, A., Kjaer, J. B., and Rodenburg, T. B. (2019b). Stress response, peripheral serotonin and natural antibodies in feather pecking genotypes and phenotypes and their relation with coping style. *Physiology and Behavior*, *199*, 1-10.

Videvall, E., Song, S.J., Bensch, H.M., Strandh, M., Engelbrecht, A., Serfontein, N., Hellgren, O., Olivier, A., Cloete, S., Knight, R. and Cornwallis, C.K. (2019). Major shifts in gut microbiota during development and its relationship to growth in ostriches. *Molecular Ecology*, *28*(10), 2653-2667.

Wang, L., Christophersen, C. T., Sorich, M. J., Gerber, J. P., Angley, M. T., and Conlon, M. A. (2012). Elevated fecal short chain fatty acid and ammonia concentrations in children with autism spectrum disorder. *Digestive Diseases and Sciences*, *57*(8), 2096-2102.

Wang, H. X., and Wang, Y. P. (2016). Gut microbiota-brain axis. *Chinese Medical Journal*, *129*(19), 2373.

Wang, Z. K., Yang, Y. S., Chen, Y., Yuan, J., Sun, G., and Peng, L. H. (2014). Intestinal microbiota pathogenesis and fecal microbiota transplantation for inflammatory bowel disease. *World Journal of Gastroenterology*, *20*(40), 14805-14820.

Weeks, C. A., and Nicol, C. J. (2006). Behavioural needs, priorities and preferences of laying hens. *World's Poultry Science Journal*, *62*(2), 296-307.

Widowski, T. M., Caston, L. J., Casey-Trott, T. M., and Hunniford, M. E. (2017). The effect of space allowance and cage size on laying hens housed in furnished cages, Part II: Behavior at the feeder. *Poultry Science*, *96*(11), 3816-3823.

Widowski, T. M., Hemsworth, P. H., Barnett, J. L., and Rault, J. L. (2016). Laying hen welfare I. Social environment and space. *World's Poultry Science Journal*, *72*(2), 333-342.

Wood-Gush, D. G. M., Duncan, I. J. H., and Fraser, D. (1975). Social stress and welfare problems in agricultural animals. *The Behaviour of Domestic Animals*. 182-200.

Wu, H. J., and Wu, E. (2012). The role of gut microbiota in immune homeostasis and autoimmunity. *Gut Microbes*, *3*(1), 4-14.

Xin, H., and Liu, K. (2017). Precision livestock farming in egg production. *Animal Frontiers*, *7*(1), 24-31.

Xu, H. M., Huang, H. L., Zhou, Y. L., Zhao, H. L., Xu, J., Shou, D. W., Liu, Y. D., Zhou, Y. J. and Nie, Y. Q. (2021). Fecal microbiota transplantation: a new therapeutic attempt from the gut to the brain. *Gastroenterology Research and Practice*, 2021, 6699268.

Yang, L., Liu, S., Ding, J., Dai, R., He, C., Xu, K., Honaker, C.F., Zhang, Y., Siegel, P. and Meng, H. (2017). Gut microbiota co-microevolution with selection for host humoral immunity. *Frontiers in Microbiology*, *8*, 1243.

Yonash, N., Bacon, L. D., and Smith, E. J. (2002). Concentration of immunoglobulin G in plasma varies among 6C. 7 recombinant congenic strains of chickens. *Poultry Science*, *81*(8), 1104-1108.

Yuwiler, A., Oldendorf, W. H., Geller, E., and Braun, L. (1977). Effect of albumin binding and amino acid competition on tryptophan uptake into brain. *Journal of Neurochemistry, 28*(5), 1015-1023.

Zhang, C., Zhang, M., Wang, S., Han, R., Cao, Y., Hua, W., Mao, Y., Zhang, X., Pang, X., Wei, C. and Zhao, G. (2010). Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. *The ISME Journal*, *4*(2), 232-241.

Zheng, D., Liwinski, T., and Elinav, E. (2020). Interaction between microbiota and immunity in health and disease. *Cell Research, 30*(6), 492-506.

Zheng, P., Zeng, B., Zhou, C., Liu, M., Fang, Z., Xu, X., Zeng, L., Chen, J., Fan, S., Du, X. and Zhang, X. (2016). Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. *Molecular Psychiatry, 21*(6), 786-796.

CHAPTER 2. EFFECTS OF EARLY-LIFE CECAL MICROBIOTA TRANSPLANTATION ON GROWTH, GUT SEROTONIN RELEASE, AND INNMUNE PARAMETERS IN ROOSTERS

2.1 Abstract

Increasing evidence points to the bidirectional communication between the gut microbiota and the host's biological functions, including absorption and metabolism of nutrients, immune and neuroendocrine responses, and stress reactions through the gut-brain axis. Fecal microbiota transplantation has become a promising therapeutic method for treating patients with gastrointestinal disorders. The aim of this study was to examine if cecal microbiota transplantation (CMT) presents similar efficiency in improving the health status of egg-laying chickens. Cecal contents were collected from the divergently selected 6_3 and 7_2 chicken lines based on resistance or susceptibility to Marek's disease, resulting in each line's unique physiological and behavioral characteristics. Cecal microbiota transplantation was conducted through oral gavage daily during the first ten days and then boosted once a week from week 3 to 5. Eighty-four d-old male chicks of the Dekalb XL strain were randomly assigned into 3 treatments with 7 replicates per treatment and 4 birds per replicate: CTRL (saline, control), 6_3-CMT (cecal solution of line 6_3), and 7_2-CMT (cecal solution of line 7_2). The results indicated that 7_2-CMT birds had the highest body weight and ileal villus/crypt ratios among treatments at week 5 (P = 0.050 and 0.014); and higher heterophil/lymphocyte ratios than that of 6_3-CMT birds at week 16 (P = 0.024). In addition, 7_2-CMT birds tended to have heavier relative adrenal glands than 6_3-CMT birds (P = 0.090). However, there were no treatment effects on corticosterone and testosterone concentrations at week 16 (P = 0.789 and 0.345). 7_2-CMT birds also had higher levels of plasma Interleukin (IL)-6 at week 11 and immunoglobulin (Ig)G at week 16 (P = 0.046), with a trend towards higher levels of TNF-α at week 11 and IL-6 at week 16 (P = 0.091 and 0.070), while 6_3-CMT birds had higher concentrations of ileal mucosal secretory IgA at week 5 and plasma IL-10 at week 16 (P = 0.045). In line with these findings, there was a trend for 7_2-CMT birds to exhibit higher TNF-α and IL-6 splenic mRNA

expression (P = 0.065 and 0.080) than 6_3-CMT birds. Among the treatments, 7_2-CMT birds tended to have the lowest serotonin concentrations (P = 0.074) with the highest turnover rate of serotonin in the ileum at week 5 (P = 0.028). In conclusion, early postnatal CMT shows line-dependent effects on the growth and health status of recipients via the regulation of ileal morphological structures, gut-derived serotonergic activities, peripheral cytokines, and antibody production in chickens.

2.2 Introduction

In large-scale commercial poultry production systems, chickens may experience various stressors, such as overcrowding, unstable social structure, transportation, and nutrient deprivation (Cheng et al., 2004; Matur et al., 2015). These problems are important risk factors that drive pathophysiological changes in the gastrointestinal (GI) tract (Konturek et al., 2011) and disrupt systemic endocrine and immune functions (Gensollen et al., 2016), resulting in decreased feed efficiency, poor health status, and economic losses in poultry (Li et al., 2017). To better fulfill the nutritional and health needs, the gut microbiota has emerged as a common intervention target for improving the production and welfare of farm animals (O'Callaghan et al., 2016). In humans, fecal microbiota transplantation (FMT) is an effective bacteriotherapy for treating recurrent *Clostridium difficile* infections and other gastrointestinal infectious diseases (Ianiro et al., 2020), with a potential for treating neuropsychiatric disorders (Settanni et al., 2021). Similarly, FMT has been gradually applied to treat various conditions of farm animals, such as digestive disorders (inappetence and hypomotility) in ruminants (Mandal et al., 2017), resistance to African swine fever virus in pigs (Zhang et al., 2020), and post-weaning diarrhea in piglets (Ma et al., 2021). Hence, microbiota transplantation may have similar beneficial effects on the health and welfare of chickens.

In recent years, research on gut microbiota has gained great attention due to the essential contributions of microorganisms to host health across the host's lifespan (Rooks et al., 2016).

Intestinal microbiota can influence the functioning of a variety of biological systems including the immune and neuroendocrine systems via the gut-brain and gut-immune axes, by which it impacts host physiological homeostasis (Marchesi et al., 2016). Under normal circumstances, the intestinal epithelial cells comprise a single layer of tight junctions, providing a physical barrier to actively defend against invasions of pathogenic bacteria (Zhang et al., 2015). However, various sources of stress from current intensive production systems may damage the mucosa epithelial microstructures and increase gut permeability to toxins and pathogens, resulting in a pathophysiological syndrome, "leaky gut" (Buffie and Pamer, 2013). Consequently, the damaged intestinal barrier increases the synthesis and release of cytokines into the blood circulation, causing systemic inflammation with the activation of the hypothalamus-pituitary-adrenocortical (HPA) axis (Dinan and Cryan, 2012; Polansky et al., 2016) and increased susceptibility to various diseases (Rychlik, 2020). An early study has suggested that the HPA axis interacts with serotonin (5-hydroxytryptamine, 5-HT) to regulate pathophysiological homeostasis in humans and other animals (López et al., 1998). Serotonin, as a neurotransmitter, is involved in mediating nutrient absorption, mental health, stress, and immune responses (Ahern, 2011; Hestermann et al., 2014; Herr et al., 2017). However, the specific relationship between gut-derived 5-HT and stress-induced intestinal dysfunction is still under ongoing debate (Dong et al., 2017).

Early life has been increasingly recognized as a critical "window of opportunity" to modulate the gut microbiota due to its long-lasting effects on the host's biological homeostasis (Torow and Hornef, 2017; Sprockett et al., 2018). At an early age, there are fluctuating changes in the gut microbial composition and structure since gut colonization begins immediately after birth (Rodríguez et al., 2015). In newborns, the first microbial encounters with maternal bacteria happen during passage through the birth canal, together with the bacteria in the local environment, contributing to the development of the baby's gut microbiota composition (Khoruts, 2016). Alteration in neonatal gut microbiota, such as early exposure to antibiotics before six months of age, contributes to an increased incidence of obesity in infancy and childhood (Trasande et al.,

2013). Similarly, administration of probiotics 2 hours after initiated incubation introduces beneficial effects to the embryonic development of broiler chickens (Baldwin et al., 2018). Therefore, priority effects of ecological theory (early arrival of microbe) play an important role in gut microbial development (assembly of the gut microbiota). On commercial poultry farms, chicks are hatched in controlled environments without contact with hens. It provides an opportunity to modify recipient chick physiological and behavioral characteristics based on adult donors' biological status through microbiota transplantation as a strategy for improving bird health and welfare. We hypothesized that similar to FMT in humans, early-life microbiota transplantation may potentially improve immune and stress responses in chickens. Cecal content was collected from two chicken strains, 6_3 and 7_2, divergently selected for resistance or susceptibility to Marek's disease, respectively, creating the line's unique physiological and behavioral characteristics. The birds of line 6_3 are much gentler with higher egg production and lower social stress responses than those of line 7_2 (Bacon and Palmquist, 2002; Dennis and Cheng, 2014). Cecal microbiota transplantation (CMT) was conducted to establish the intestinal microbiota of the recipient birds, with the objective to investigate the effects of early-life CMT on performance traits, stress status, and immune characteristics.

2.3 Materials and methods

All procedures were approved by the Purdue University Animal Care and Use Committee (PACUC# 1712001657) and the study was conducted in accordance with the guidelines set by the Federation of Animal Science Societies (2010).

2.3.1 Birds and experimental design

Inbred chickens of the 6_3 and 7_2 lines developed at Avian Disease and Oncology Laboratory (East Lansing, MI) were used as donors (Bacon et al., 2001). At 60 week of age, the cecal content samples were randomly collected from 10 hens per line and then evenly pooled (within lines). Five

grams of pooled cecal contents were diluted with gut microbiome media (adopted from Goodman et al., 2011) at a ratio of 1:10, and then kept at -20°C freezer until oral gavage.

A total of 84 1-day-old male chicks (Dekalb XL, a commercial strain) were used as recipients and randomly allocated to 1 of 3 treatments with 7 cages per treatment and 4 birds per cage (n=7): CTRL (0.1 ml saline, control), 6_3-CMT (0.1 ml cecal solution of line 6_3), and 7_2-CMT (0.1 ml cecal solution of line 7_2) for a 16-week trial. Cecal microbiota transplantation was conducted through oral gavage once daily from day 1 to day 10, and then boosted once weekly from week 3 to week 5.

Water and feed were provided *ad libitum*. The general management, including vaccination, dietary formulation and nutrient contents, ambient temperature, and lighting program, followed the Hy-line guidelines (2019).

2.3.2 Sample collection

At week 5, 11, and 16, one bird per replicate was randomly selected to measure body weight and for blood sampling (n=7). A 5 mL blood sample was collected from the brachial vein of each sampled bird using a heparinized tube. After collection, samples were centrifuged at 700 × g for 15 min at 4 °C. Plasma was separated and stored at -80°C until analysis.

At week 5 and 16, one bird per cage was euthanized through cervical dislocation after blood sampling (n=7). The liver, spleen, left adrenal gland, and heart were collected from each bird and weighed, and then the spleen tissues were frozen at -80°C for further analysis. In addition, approximately 7 cm of the ileum (near the diverticulum) was collected from each bird and flushed with sterile PBS to remove the contents, then separated into two parts: One part (approximately 4 cm) was immediately fixed in 10% buffered formalin. The mucosal samples were scraped and collected from the other part of the sampled ileum (3 cm) using sterile glass slices and then frozen in liquid nitrogen and stored at -80 °C.

2.3.3 Blood smear test

At week 16, one bird per replicate (n=7) was randomly selected to measure the ratio of heterophils to lymphocytes (H/L) from blood smears following previously published procedures (Cheng et al., 2001b). One hundred heterophils and lymphocytes were counted from each slide (total 200 cells from 2 slides per bird) under a light microscope to determine the H/L ratio for each bird.

2.3.4 Ileal histomorphology

A 1-cm ileal specimen per bird was prepared as described by Jiang et al. (2020). Briefly, the fixed samples were dehydrated in graded ethanol, cleared in xylene, and then embedded in paraffin. Thereafter, 5.0 µm thick sections were sliced using a Leica RM 2145 microtome (Leica, Nussloch, Germany). The sections were stained with hematoxylin and eosin (Thermo, Waltham, MA) and then examined using an Olympus BX40F-3 microscope (Olympus Cooperation, Tokyo, Japan). Three tissue sections containing intact lamina propria were selected from each bird, and an average of six readings (of each of villus height, VH and crypt depth, CD, both measured in µm) were made from each section. Image J software (NIH, Bethesda, MD) was used to measure VH and CD on each image. The VH and CD per tissue sample were averaged, and the VH/CD ratio was calculated.

2.3.5 High-performance liquid chromatography (HPLC)

To determine the gut serotonergic activity, the ileal samples were analyzed in triplicate using HPLC (UltiMateTM 3000 RSLCnano System, Thermo Fisher Scientific Inc., Waltham, MA) as per Yan et al. (2020). Briefly, the ileal samples were weighed and homogenized in 4 M perchloric acid at 1: 5, and then vortexed for 1 minute. Afterward, the mixture was centrifuged at 15,000 × g for 10 min at 4°C. The supernatants were added and absorbed onto an alumina column and set on a rocker to link catecholamines with the alumina. The mobile phase (Sigma, USA) flow

rate was 0.8 m/min. The ileal concentrations of 5-hydroxuindoleacetic acid (5-HIAA); serotonin (5-HT), and tryptophan were calculated as nanograms per gram of wet tissue (ng/g) using a reference curve generated from each of the corresponding calibrators.

2.3.6 Enzyme-linked immunosorbent assay (ELISA)

Cecal microbiota transplantation (CMT)-induced changes of plasma concentrations of Interleukin (IL)-6, IL-10, Tumor necrosis factor (TNF)-α, and Immunoglobulin (Ig)G were detected following each respective company's instructions. The levels of total IL-6 (MBS037319, My BioSource, San Diego, CA), IL-10 (Catalog #: MBS007312, My BioSource, San Diego, CA), TNF-α (Catalog #: MBS260419, My BioSource, San Diego, CA), and IgG (Catalog #: E33-104, Bethyl Laboratories, Inc.) were measured using the respective ELISA kits. Duplicate samples were taken with CV ≤ 15%.

Total protein in the ileal mucosal homogenates was measured by a Sigma Protein Assay kit (Sigma Chemical Co., St. Louis, MO) using bovine serum albumin as the standard (Dahlqvist, 1964). Mucosal secretory (s)IgA concentrations were determined using a commercial ELISA kit (Catalog #: E33-103, Bethyl Laboratories, Inc.) following the manufacturer's guidelines. Concentrations of sIgA were expressed as micrograms of sIgA per gram protein (mg/g).

2.3.7 Radioimmunoassay (RIA)

Total plasma concentrations of corticosterone and testosterone were determined in duplicate using commercially available I^{125} RIA kits (Catalog #: 07120103 and Catalog #: 07189102, MP Biomedicals, Solon, OH) as previously described (Cheng et al., 2001a). Briefly, 20 µl plasma to 80 ul diluents and incubated in a water bath at room temperature for 120 min. After the incubation, the tubes were vacated and the radioactivity was counted with a gamma counter (1470 Wizard Gamma Counter, PerkinElmer, Waltham, MA). The sensitivity of the assay was

0.02 ng/ ml. All samples were assayed at the same time and duplicate samples were taken with CV ≤ 15%.

2.3.8 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the frozen spleen samples using RNeasy Mini Kit (Catalog #: 74804, Qiagen, Valencia, CA) following the instructions provided by the company. The purity and concentration of total RNA were checked using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Reverse transcription was conducted using the Reverse Transcription Reagent Pack (Catalog #: N8080234, Applied Biosystems, Foster City, CA). A mixture of reverse transcription reagents consists of 2 µl RNase inhibitor, 2.5 µl multi-scribe reverse transcriptase, 5 µl random hexamers, 10 µl of TaqMan reverse transcription buffer, 20 µl deoxynucleotides, and 22 µl of 25 mM magnesium chloride. A total master mixture of each sample consists of 61.5 µl with the quantified RNA sample and RNase-free water for a final 100 µl. The RNA samples were reverse transcribed to cDNA using a Techne TC-3000G PCR Thermal Cycler (Bibby Scientific Limited, Stone, UK). Splenic mRNA expressions of IL-6 (Assay ID #: Gg03337980_m1), TNF-α (Assay ID #: Gg03364359_m1), and IL-10 (Assay ID #: Gg03358689_m1) were detected by RT-qPCR using the provided primers and probes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Catalog #: 4448489, Applied Biosystems, Foster City, CA, Assay ID #: Gg03346982_m1) was used as a reference gene. The PCR mixture contained 1.625 µL of TaqMan probe, 2.25 µL of gene-specific TaqMan forward and reverse primers each, 12.5 µL of PCR Master mix (Catalog #: 4304437, Applied Biosystems, Foster City, CA), 3.875 µL RNase-free water, and 2.5 µL of sample cDNA. The cycling conditions were 50°C for 2 min and 95°C for 10 min of the holding stage, followed by 40 cycles of 95°C for 15 s, then 60°C for 1 min. Results were quantitated by the standard curve method. Standards were measured in triplicates with a standard deviation of less than 2.0 and a coefficient of variation less than 2.0%.

2.3.9 Statistical analysis

The data was analyzed using R studio (version 3.6.2). The fixed effects were treatment and age. The response variables were measured levels of physiological parameters. The Shapiro-Wilk test was used to analyze the normality of the data and non-normal data were logarithmically transformed. Statistical analysis was conducted using R studio one-way ANOVA for normalized data. The Tukey-Kramer test was used to partition any significant differences among least-square means due to treatment main effects (Steel et al., 1997). Significance was set at $P \leq 0.05$ and a trend was defined as $0.05 < P \leq 0.1$.

2.4 Results

2.4.1 Performance traits

Transplantation of cecal content from the different chicken lines affected the recipient birds' physical and physiological measurements. At week 5, 7_2-CMT birds had the highest body weights ($P = 0.050$), but the differences in BW disappeared by 16 week of age ($P = 0.426$, Figure 2.1). In addition, 7_2-CMT birds tended to have heavier relative adrenal glands ($P = 0.090$, Table 2.1) than 6_3-CMT birds but not CTRL birds (Table 2.1), while there was no treatment effect on relative spleen, liver, and heart weights Table 2.1). In line with this finding, 7_2-CMT birds exhibited higher H/L ratios than 6_3-CMT birds ($P = 0.024$, Table 2.2) but not CTRL birds, while no treatment effect was found on the concentrations of corticosterone and testosterone at 16 week of age (Table 2.2). At 5 week of age, the ratio of VH:CD was significantly higher in 7_2-CMT birds compared with both 6_3-CMT and CTRL birds ($P = 0.014$, Figure 2.2A) due to a higher VH, especially between 7_2-CMT and 6_3-CMT birds. The differences were no longer present at week 16 (Figure 2B).

2.4.2 Immune response

Among CMT recipient birds, 7_2-CMT birds had greater concentrations of natural IgG at week 16 ($P = 0.046$, Figure 2.3C), while 6_3-CMT birds had higher concentrations of ileal mucosa

sIgA at week 5 (P = 0.045, Figure 2.4A). In addition, treatment effects on the measured cytokines were found at week 11 and 16 (Figure 2.5). 7_2-CMT birds had higher levels of plasma IL-6 than CTRL birds (P = 0.002) and a tendency for higher plasma TNF-α levels compared to 6_3-CMT birds (P = 0.091, Figure 2.5B) at week 11. These changes were detected at week 16 (Figure 2.5C). 7_2-CMT birds tended to have higher plasma concentrations of IL-6 (P = 0.070), while 6_3-CMT birds had higher levels of plasma IL-10 at week 16 (P = 0.045). Consistent with this finding, 6_3-CMT birds had a tendency of lower splenic IL-6 (P = 0.080) and TNF-α (P = 0.065) mRNA expressions than 7_2-CMT birds at week 16 (Figure 2.6B).

2.4.3 Ileal serotonergic activities

At week 5, 6_3-CMT birds tended to have the highest concentrations of 5-HT (P = 0.074) and 5-HIAA (a metabolite of 5-HT) in the ileum compared to both CTRL and 7_2-CMT birds (P = 0.015, Figure 7A), without treatment effects on the concentrations of tryptophan (P = 0.467). In addition, 5-HIAA/5-HT ratios differed among treatments (Figure 7B). Compared to CTRL birds, 5-HT turnover was higher in 7_2-CMT birds at week 5 (P = 0.028). These treatment differences disappeared by week 16 (Figure 2.7C and D).

2.5 Discussion

2.5.1 Cecal microbiota transplantation alters body weight and ileal morphology in roosters in the early-life stage

One function of the gut microbiota is food digestion and nutrient absorption (Angelakis, 2017). In humans, patients with acute malnutrition can be treated with probiotic supplements, which in turn results in weight gain (Kerac et al., 2009). In our study, microbiota transplantation led to BW changes in the recipient birds; that is, 7_2-CMT had the heaviest body weight among treatments at week 5 and 11, while BW became similar in all treatment groups at week 16. The weight gain during the growing phase may be associated with a high abundance of several

bacteria, such as bacteria belonging to *Firmicutes* in chickens (Videnska et al., 2014). Additionally, Joat et al. (2021) reported that the gut microbiota composition in caged laying hens changed significantly from the rearing stage (pullets) to the production stage (layers) and varied mostly due to the different management systems. In our study, birds were maintained in the same growing facilities for the whole trial, as such, the unchanged BW in adult birds may be partially attributed to the evidence that functional core gut microbiota involved in the feed utilization has stabilized in adulthood. In addition, weight gain requires sufficient nutrient absorption at an early age. Changes in VH and CD have been commonly considered as key measurements for the assessment of gut maturation and nutritional effects. In line with the BW changes, we observed that 7_2-CMT birds had the highest ileal VH as well as the greatest ratios of VH/CD among treatments. The ileum is the major absorption location for several nutrients such as vitamin B12 and fat in chickens (Mantle, 2020; Rupprecht and Bohórquez, 2021). Increased VH may suggest that 7_2-CMT birds have improved feeding digestion and nutrient absorption due to the enlarged epithelial surface area in early life (Caspary, 1992), while the treatment effects were reduced as the birds matured. Collectively, early-life microbiota transplantation plays a critical role in the growth performance through altering BW and ileal microstructures.

2.5.2 Gut microbiota differently influences basic stress reactive capability of recipient birds

Hyperactivation of the HPA axis is commonly seen under stress conditions, and corticosterone, as the final compound, is released from the adrenal glands within a short period of time (Peirce and Alviña, 2019). However, the HPA axis is less developed in newly hatched chicks (Frankiensztajn et al., 2020). Generally, roosters become sexually mature at around 16 week of age, which is a critical time point to assess hormonal responses. Testosterone, as one of the sexual hormones in roosters, is synthesized by the testis under the regulation of gonadotrophin and gonadotrophin-releasing hormone released from the pituitary and the hypothalamus, respectively

(Ulloa-Aguirre and Timossi, 2000). The activation of the HPA axis often causes the inhibitory response of the hypothalamus-pituitary-gonad axis, resulting in a decreased level of testosterone (Tsutsui et al., 2012). In chickens, changes in the stress-related hormone, corticosterone, and stress indicators, such as H/L ratio, have been considered as acute and chronic stress markers, respectively (Cheng et al., 2001a; Gross and Siegel, 1988; Kunz-Ebrecht et al., 2003). In the current study, we did not observe measurable changes in the basic levels of plasma corticosterone and testosterone among the treated birds at week 16. In agreement with our findings, van der Eijk et al. (2020) also reported that transplanting luminal content from high FP and low FP selected chicken lines did not affect the levels of corticosterone in recipient birds. Similar research conducted by Zhu et al. (2020) indicated that transplanting fecal content from either schizophrenic or healthy individuals did not alter the basic levels of corticosterone in recipient mice (Zhu et al., 2020). Another potential explanation for the unchanged corticosterone may be attributed to the strain variations. For instance, mouse strain differently contributes to the functional variations of the HPA axis, and thereby strain-related differences in reactivity and associated corticosterone levels in response to stimulations (Neufeld et al., 2011; Shanks et al., 1990, Sudo et al., 2004). Future elucidation of the mechanisms on how the microbiota regulate hormonal changes in response to stress is needed.

Recent advances in genetic technologies have unraveled the critical contributions of host genetics to the regulation of stress reactivity. For instance, the differences in stress adaptability are apparent in the donor lines used in this study (Dennis et al., 2004). Line 7_2 exhibits more aggressive behavior than line 6_3 in response to social stress, which may be associated with their variations in coping styles (reactive vs. proactive). Notably, we found that the recipient birds' basic stress response is associated with that of the donor, reflected by a significantly lower H/L ratio together with a tendency of lighter adrenal gland weight in 6_3-CMT birds as compared to 7_2-CMT birds. Behavior was not directly observed here, and behavioral differences in aggression will be needed to further examine similarities among recipients and donors. In avian species, adrenal gland weight

has been considered as a chronic stress indicator (Harvey et al., 1984; Cheng et al., 2003). Taken together, the results may suggest that 6_3-CMT birds have a better capability to adapt to stress. These results suggest that cecal contents from different donors differently influence the recipients' stress response via the donor-host genetic-microbiota interactions in chickens.

2.5.3 Cecal microbiota transplantation modulates basic immune response and gut health of recipient birds

Extensive evidence has indicated that the crosstalk between the gut microbiota and the immune system plays a vital role in maintaining the health status of the host. For example, newly hatched birds are more susceptible to infections and diseases due to the absence of microbiota as well as an immature avian immune system (Beal et al., 2005). In young birds, the innate immune system constitutes the first line of defense system against pathogenic infections or inflammation induced by environmental stressors (Bar-Shira and Friedman, 2006). In adulthood, the intestinal microbiota affects the recruitment of immune cells, activating antibody-dominated and cellular immune responses (differentiation and proliferation) (Broom and Kogut, 2018; Dempsey et al., 2003). To better understand how microbiota transplantation impacts the immune system, we examined the changes of plasma concentrations of circulating natural antibody (IgG), pro-inflammatory cytokines (IL-6 and TNF-α), and anti-inflammatory cytokine (IL-10) at 5, 11, and 16 week of age. It is interesting to note that there were significant differences in plasma IgG concentrations among treatments during sexual maturity of birds (around 16 week); 7_2-CMT birds had higher levels of natural IgG in the plasma compared with 6_3-CMT birds. Natural IgG, as one of the most abundant antibodies, presents in the circulation after birth even in the absence of prior exposure to a cognate antigen (Casali and Schettino, 1996). IgG may reflect a pathological process as autoantibodies in the blood, which is manifested in disease-induced tissue, cell damage or a breakdown in the host's self-tolerance (Nagele et al., 2013). In human patients, organ-specific or systemic autoimmune diseases could be aggravated by the increased binding of self-reactive IgG

with the targeted tissues, organs, and free molecules including phospholipids (Elkon and Casali, 2008; Nimmerjahn and Ravetch, 2021). In our study, recipient birds were exposed to similar environmental conditions and under the same management practices; higher concentrations of natural antibody (IgG) in 7_2-CMT birds may be explained by exaggerated immunological responsiveness to social-environmental influences and related chronic inflammation in comparison with both CTRL and 6_3-CMT. This view is supported by the susceptibility of donor line 7_2 to Marek's disease and increased aggressiveness (Dennis et al., 2014). Together with the host genetic-microbiota interaction, cecal content from donor line 7_2 may induce a greater production of autoantibody IgG in 7_2-CMT birds. Meanwhile, high immune responses require large amounts of resources; birds with a higher antibody synthesis may have poorer feed efficiencies and decreased effectiveness of macrophages (Gross and Siegel, 1988). These results may reveal that the host's genotype and gut microbiota work together to influence the immunity of recipient birds.

Maintenance of intestinal homeostasis requires appropriate discrimination between beneficial and pathogenic bacteria (Yoo et al., 2020). Mucosal secretory(s) IgA, as one of the most abundant antibodies within the intestinal lumen, protects the gut epithelium from the invasion of pathogenic bacteria and related infections (Mantis et al., 2011). Mucosal sIgA has also been used as a biomarker for the evaluation of intestinal homeostasis and stress reactions (de Santis et al., 2015). As expected, our results showed sIgA concentrations were significantly affected by microbiota transplantation at week 5. The higher levels of sIgA in 6_3-CMT birds may help them to maintain gut health as well as improve nutrient absorption. Considering the protective roles that mucosal sIgA plays in the intestinal barrier, sIgA may directly and indirectly mediate cytokine production. Brzozowski et al. (2016) reported that the breakdown of the intestinal barrier is associated with changes in mRNA abundance and the production of inflammatory cytokines. In our study, results suggested that 7_2-CMT had attenuated levels of an anti-inflammatory cytokine, IL-10, but greater concentrations of pro-inflammatory cytokines, IL-6 and TNF-α among treatment.

In agreement with our findings, CMT from different donors tended to decrease mRNA abundance of pro-inflammatory splenic cytokines such as IL-6 and TNF-α in 6_3-CMT birds, which may suggest that transferred microbiota improve the immune system of recipients. These results further revealed the critical role of the gut microbiota in regulating gut health and immune response in chickens via CMT.

2.5.4 Potential physiological implications of the gut-derived serotonin

The gastrointestinal (GI) tract is the major location of peripheral 5-HT. Approximately 95% of the body's 5-HT is synthesized by the gut mucosal enterochromaffin cells (ECs) (Racké et al., 1989). Subsequently, investigations have uncovered a range of functions of gut-derived 5-HT including regulating gut motility, secretion of bioactive factors (Mawe and Hoffman, 2013), metabolic processes (Jones et al., 2020), and bone formation (Sjögren et al., 2012). Further, gut-derived 5-HT may act on the activation of immune cells via signaling a variety of 5-HT receptors, which in turn regulates cytokine production and gut homeostasis (Fukudo, 2013; Liu et al., 2021; Spohn and Mawe, 2017). In addition, it has been reported that the microbiome regulates gastrointestinal 5-HTergic response and consequently interacts with released hormones, cytokines, and short-chain fatty acids (de Haas and van der Eijk, 2018). Given its multiple roles within the GI tract, changes of gastrointestinal 5-HT may have implications for inflammatory signaling and the stress response of the host. In the current study, 6_3-CMT birds had higher 5-HIAA concentrations, with a tendency for higher concentrations of 5-HT in the ileum than 7_2-CMT birds at week 5. These results may suggest that 6_3-CMT birds have a more activated serotonergic system than 7_2-CMT birds. Imbalanced serotonin synthesis promotes the pathological process of stress-related diarrhea in mice (Dong et al., 2017). Also, Margolis et al. (2014) suggested that 5-HT can modulate gut physiology by facilitating gut inflammation. In our study, the high activated gut serotonergic systems in 6_3-CMT birds (higher 5-HT biosynthesis) were paralleled by higher concentrations of mucosal sIgA as well as IL-10, which may indicate the transferred microbiota

induces anti-inflammatory effects in recipients. Therefore, these changes may indicate that 6_3-CMT birds have a greater basic gut immunity protecting them from inflammation and infection, but future work will be needed to verify such changes.

Previous studies have reported that chronic stress resulted in decreased mRNA abundance of TPH1 in the intestines, an enzyme for synthesizing peripheral 5-HT (Yue et al., 2017). In agreement with this finding, Wang et al. (2007) and Babskota et al. (2019) suggested that stress alters gut-derived 5-HT signaling, thereby downregulating 5-HT concentrations in the jejunum and colon, leading to various gut disorders such as inflammatory bowel disease. This hypothesis is supported by the fact that administration of 5-HT or its precursor 5-hydroxytryptophan (5-HTP) promotes the production of tight junction proteins and reduces mucosal permeability (Haub et al., 2010; Nylander and Pihl, 2006). Tight junction proteins are prominent players in maintaining intestinal barriers under both normal and stressful circumstances. Taken together, high levels of gut-derived 5-HT in 6_3-CMT birds may indicate that they have better stress adaptive capability.

2.6 Conclusion

The results of this study demonstrate that early postnatal cecal microbiota transplantation influences growth, gut morphological development, immunity, and stress adaptive capability of recipient chickens, dependent on microbiota-donor-host interactions. The results indicate that microbiota transplantation, especially at an early age, could be a novel strategy for ameliorating stress responses and improving the health and welfare status of chickens. Future studies are needed to investigate the potential associations between specific beneficial bacterial taxa and physiological characteristics in the donor-recipient relationship, which could provide a novel management strategy for poultry production.

2.7 References

Ahern, G. P. (2011). 5-HT and the immune system. *Current opinion in pharmacology*, *11*(1), 29-33.

Angelakis, E. (2017). Weight gain by gut microbiota manipulation in productive animals. *Microbial Pathogenesis*, *106*, 162-170.

Bacon, L. D., and Palmquist, D. (2002). Chicken lines differ in production of interferon-like activity by peripheral white blood cells stimulated with phytohemagglutinin. *Poultry science*, *81*(11), 1629-1636.

Bacon, L. D., Hunt, H. D., and Cheng, H. H. (2001). Genetic resistance to Marek's disease. *Marek's Disease*, *255*, 121-141.

Baldwin, S., Hughes, R. J., Hao Van, T. T., Moore, R. J., and Stanley, D. (2018). At-hatch administration of probiotic to chickens can introduce beneficial changes in gut microbiota. *PLoS One*, *13*(3), e0194825.

Banskota, S., Ghia, J. E., and Khan, W. I. (2019). Serotonin in the gut: Blessing or a curse. *Biochimie*, *161*, 56-64.

Bar-Shira, E., and Friedman, A. (2006). Development and adaptations of innate immunity in the gastrointestinal tract of the newly hatched chick. *Developmental and Comparative Immunology*, *30*(10), 930-941.

Beal, R. K., Powers, C., Wigley, P., Barrow, P. A., Kaiser, P., and Smith, A. L. (2005). A strong antigen-specific T-cell response is associated with age and genetically dependent resistance to avian enteric salmonellosis. *Infection and Immunity*, *73*(11), 7509-7516.

Broom, L. J., and Kogut, M. H. (2018). The role of the gut microbiome in shaping the immune system of chickens. *Veterinary Immunology and Immunopathology*, *204*, 44-51.

Brzozowski, B., A. Mazur-Bialy, R. Pajdo, S. Kwiecien, J. Bilski, M. Zwolinska-Wcislo, T. Mach, and T. Brzozowski. 2016. Mechanisms by which Stress Affects the Experimental and Clinical Inflammatory Bowel Disease (IBD): Role of Brain-Gut Axis. *Current Neuropharmacology*, *14*(8), 892-900.

Buffie, C. G., and Pamer, E. G. (2013). Microbiota-mediated colonization resistance against intestinal pathogens. *Nature Reviews Immunology*, *13*(11), 790-801.

Casali, P., and Schettino, E. W. (1996). Structure and function of natural antibodies. *Immunology of silicones*, *210*, 167-179.

Caspary, W. F. (1992). Physiology and pathophysiology of intestinal absorption. *The American Journal of Clinical Nutrition*, *55*(1), 299S-308S.

Cheng, H. W., Dillworth, G., Singleton, P., Chen, Y., and Muirt, W. M. (2001a). Effects of group selection for productivity and longevity on blood concentrations of serotonin, catecholamines, and corticosterone of laying hens. *Poultry Science*, *80*(9), 1278-1285.

Cheng, H. W., Eicher, S. D., Chen, Y., Singleton, P., and Muirt, W. M. (2001b). Effect of genetic selection for group productivity and longevity on immunological and hematological parameters of chickens. *Poultry Science*, *80*(8), 1079-1086.

Cheng, H. W., Freire, R., and Pajor, E. A. (2004). Endotoxin stress responses in chickens from different genetic lines. 1. Sickness, behavioral, and physical responses. *Poultry Science*, *83*(5), 707-715.

Cheng, H. W., Singleton, P., and Muir, W. M. (2003). Social stress in laying hens: differential effect of stress on plasma dopamine concentrations and adrenal function in genetically selected chickens. *Poultry Science*, *82*(2), 192-198.

Dahlqvist, A. (1964). Method for assay of intestinal disaccharidases. *Analytical biochemistry*, *7*, 18-25.

de Haas, E. N., and van der Eijk, J. A. (2018). Where in the serotonergic system does it go wrong? Unravelling the route by which the serotonergic system affects feather pecking in chickens. *Neuroscience and Biobehavioral Reviews*, *95*, 170-188.

De Santis, S., Cavalcanti, E., Mastronardi, M., Jirillo, E., and Chieppa, M. (2015). Nutritional keys for intestinal barrier modulation. *Frontiers in Immunology*, *6*, 612.

Dempsey, P. W., Vaidya, S. A., and Cheng, G. (2003). The art of war: Innate and adaptive immune responses. *Cellular and Molecular Life Sciences*, *60*(12), 2604-2621.

Dennis, R. L., and Cheng, H. W. (2014). Differential serotonergic mediation of aggression in roosters bred for resistance and susceptibility to Marek's disease. *British Poultry Science*, *55*(1), 13-20.

Dennis, R., H. M. Zhang, L. D. Bacon, I. Estevez, and H. W. Cheng. (2004). Behavioral and physiological features of chickens diversely selected for resistance to avian disease. 1. Selected inbred lines differ in behavioral and physical responses to social stress. *Poultry Science. 83*, 1489-1496.

Dinan, T. G., and Cryan, J. F. (2012). Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. *Psychoneuroendocrinology*, *37*(9), 1369-1378.

Dong, Y., Han, Y., Wang, Z., Qin, Z., Yang, C., Cao, J., and Chen, Y. (2017). Role of serotonin on the intestinal mucosal immune response to stress-induced diarrhea in weaning mice. *BMC Gastroenterology*, *17*(1), 1-10.

Elkon K and Casali P. (2008). Autoantibodies: nature and functions. *Nature Clinic Practice Rheumatology*, *4*, 491-498.

Frankiensztajn, L. M., Elliott, E., and Koren, O. (2020). The microbiota and the hypothalamus-pituitary-adrenocortical (HPA) axis, implications for anxiety and stress disorders. *Current Opinion in Neurobiology*, *62*, 76-82.

Fukudo, S. (2013). Stress and visceral pain: focusing on irritable bowel syndrome. *Pain*, *154*, S63-S70.

Gensollen, T., Iyer, S. S., Kasper, D. L., and Blumberg, R. S. (2016). How colonization by microbiota in early life shapes the immune system. *Science*, *352*(6285), 539-544.

Goodman, A. L., Kallstrom, G., Faith, J. J., Reyes, A., Moore, A., Dantas, G., and Gordon, J. I. (2011). Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proceedings of the National Academy of Sciences*, *108*(15), 6252-6257.

Gross, W. B., and Siegel, H. S. (1983). Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian Diseases*, 972-979.

Gross, W. B., and Siegel, P. B. (1988). Environment-genetic influences on immunocompetence. *Journal of Animal Science*, *66*(8), 2091-2094.

Harvey, S., Phillips, J. G., Rees, A., and Hall, T. R. (1984). Stress and adrenal function. *Journal of Experimental Zoology*, *232*(3), 633-645.

Haub, S., Kanuri, G., Volynets, V., Brune, T., Bischoff, S. C., and Bergheim, I. (2010). Serotonin reuptake transporter (SERT) plays a critical role in the onset of fructose-induced hepatic steatosis in mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *298*(3), G335-G344.

Herr, N., Bode, C., and Duerschmied, D. (2017). The effects of serotonin in immune cells. *Frontiers in Cardiovascular Medicine*, *4*, 48.

Hestermann, D., Temel, Y., Blokland, A., and Lim, L. W. (2014). Acute serotonergic treatment changes the relation between anxiety and HPA-axis functioning and periaqueductal gray activation. *Behavioural Brain Research*, *273*, 155-165.

Ianiro, G., Segal, J.P., Mullish, B.H., Quraishi, M.N., Porcari, S., Fabiani, G., Gasbarrini, A. and Cammarota, G., (2020). Fecal microbiota transplantation in gastrointestinal and extraintestinal disorders. *Future Microbiology*, *15*(12),1173-1183.

Jiang, S., Mohammed, A. A., Jacobs, J. A., Cramer, T. A., and Cheng, H. W. 2020. Effect of synbiotics on thyroid hormones, intestinal histomorphology, and heat shock protein 70 expression in broiler chickens reared under cyclic heat stress. *Poultry Science*, *99*, 142-150.

Joat, N., Van, T. T. H., Stanley, D., Moore, R. J., and Chousalkar, K. (2021). Temporal dynamics of gut microbiota in caged laying hens: a field observation from hatching to end of lay. *Applied Microbiology and Biotechnology*, *105*(11), 4719-4730.

Jones, L. A., Sun, E. W., Martin, A. M., and Keating, D. J. (2020). The ever-changing roles of serotonin. *The International Journal of Biochemistry and Cell Biology*, *125*, 105776.

Kerac, M., Bunn, J., Seal, A., Thindwa, M., Tomkins, A., Sadler, K., Bahwere, P. and Collins, S. (2009). Probiotics and prebiotics for severe acute malnutrition (PRONUT study): a double-blind efficacy randomised controlled trial in Malawi. *The Lancet*, *374*(9684), 136-144.

Khoruts, A. (2016). First microbial encounters. *Nature Medicine*, *22*(3), 231-232.

Konturek, P. C., Brzozowski, T., and Konturek, S. J. (2011). Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. *Journal of Physiology and Pharmacology, 62*(6), 591-599.

Kunz-Ebrecht, S. R., Mohamed-Ali, V., Feldman, P. J., Kirschbaum, C., and Steptoe, A. (2003). Cortisol responses to mild psychological stress are inversely associated with proinflammatory cytokines. *Brain, Behavior, and Immunity, 17*(5), 373-383.

Li, Y., Song, Z., Kerr, K. A., and Moeser, A. J. (2017). Chronic social stress in pigs impairs intestinal barrier and nutrient transporter function and alters neuro-immune mediator and receptor expression. *PloS One*, *12*(2), e0171617.

Liu, N., Sun, S., Wang, P., Sun, Y., Hu, Q., and Wang, X. (2021). The Mechanism of Secretion and Metabolism of Gut-Derived 5-Hydroxytryptamine. *International Journal of Molecular Sciences, 22*(15), 7931.

López, J. F., Chalmers, D. T., Little, K. Y., and Watson, S. J. (1998). Regulation of serotonin1A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. *Biological Psychiatry*, *43*(8), 547-573.

Ma, X., Zhang, Y., Xu, T., Qian, M., Yang, Z., Zhan, X., and Han, X. (2021). Early-life intervention using exogenous fecal microbiota alleviates gut injury and reduce inflammation caused by weaning stress in piglets. *Frontiers in Microbiology*, *12*, 671683.

Mandal, R. S. K., Joshi, V., Balamurugan, B., Gautam, D., Chethan, G. E., and Lekshman, A. (2017). Rumen transfaunation an effective method for treating simple indigestion in ruminants. *North-East Veterinarian*, *17*(1), 31-33.

Mantis, N. J., Rol, N., and Corthésy, B. (2011). Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunology*, *4*(6), 603-611.

Mantle, D. (2020). Nutritional supplementation for vitamin B12 and vitamin K2 deficiency following ileostomy or colostomy formation. *Gastrointestinal Nursing*, *18*, S12-S16.

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D., Hirschfield, G. M., Hold, G., ... and Hart, A. (2016). The gut microbiota and host health: a new clinical frontier. *Gut*, *65*(2), 330-339.

Margolis, K. G., Stevanovic, K., Li, Z., Yang, Q. M., Oravecz, T., Zambrowicz, B., ... and Gershon, M. D. (2014). Pharmacological reduction of mucosal but not neuronal serotonin opposes inflammation in mouse intestine. *Gut*, *63*(6), 928-937.

Matur, E., Eraslan, E., Akyazi, I., Ekiz, E. E., Eseceli, H., Keten, M., ... and Bala, D. A. (2015). The effect of furnished cages on the immune response of laying hens under social stress. *Poultry Science*, *94*(12), 2853-2862.

Mawe, G. M., and Hoffman, J. M. (2013). Serotonin signalling in the gut—functions, dysfunctions and therapeutic targets. *Nature Reviews Gastroenterology and Hepatology*, *10*(8), 473-486.

Nagele, E. P., Han, M., Acharya, N. K., DeMarshall, C., Kosciuk, M. C., and Nagele, R. G. (2013). Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PloS One*, *8*(4), e60726.

Neufeld, K. M., Kang, N., Bienenstock, J., and Foster, J. A. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology and Motility*, *23*(3), 255-e119.

Nimmerjahn, F., and Ravetch, J. V. (2021). Four keys to unlock IgG. *Journal of Experimental Medicine*, *218*(3).

Nylander, O., and Pihl, L. (2006). Luminal hypotonicity increases duodenal mucosal permeability by a mechanism involving 5-hydroxytryptamine. *Acta Physiologica*, *186*(1), 45-58.

O'Callaghan, T. F., Ross, R. P., Stanton, C., and Clarke, G. (2016). The gut microbiome as a virtual endocrine organ with implications for farm and domestic animal endocrinology. *Domestic Animal Endocrinology*, *56*, S44-S55.

Peirce, J. M., and Alviña, K. (2019). The role of inflammation and the gut microbiome in depression and anxiety. *Journal of Neuroscience Research*, *97*(10), 1223-1241.

Polansky, O., Sekelova, Z., Faldynova, M., Sebkova, A., Sisak, F., and Rychlik, I. (2016). Important metabolic pathways and biological processes expressed by chicken cecal microbiota. *Applied and Environmental Microbiology*, *82*(5), 1569-1576.

Racké, K., Schwörer, H., and Kilbinger, H. (1989). Complex regulation of 5-hydroxytryptamine (5-HT) release from enterochromaffin cells (ECs). *European Journal of Endocrinology*, *120*(3S), S200-S201.

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., ... and Collado, M. C. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health and Disease*, *26*(1), 26050.

Rooks, M. G., and Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. *Nature Reviews Immunology*, *16*(6), 341-352.

Rupprecht, L. E., and D. v. Bohórquez. (2021). The nerve not taken. *Cell Metabolism*, *33*, 466-467.

Rychlik, I. (2020). Composition and function of chicken gut microbiota. *Animals*, *10*(1), 103.

Settanni, C. R., Ianiro, G., Bibbò, S., Cammarota, G., and Gasbarrini, A. (2021). Gut microbiota alteration and modulation in psychiatric disorders: Current evidence on fecal microbiota transplantation. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *109*, 110258.

Shanks, N., Griffiths, J., Zalcman, S., Zacharko, R. M., and Anisman, H. (1990). Mouse strain differences in plasma corticosterone following uncontrollable footshock. *Pharmacology Biochemistry and Behavior*, *36*(3), 515-519.

Sjögren, K., Engdahl, C., Henning, P., Lerner, U. H., Tremaroli, V., Lagerquist, M. K., ... and Ohlsson, C. (2012). The gut microbiota regulates bone mass in mice. *Journal of Bone and Mineral Research*, *27*(6), 1357-1367.

Spohn, S. N., and Mawe, G. M. (2017). Non-conventional features of peripheral serotonin signalling—the gut and beyond. *Nature reviews Gastroenterology and Hepatology*, *14*(7), 412-420.

Sprockett, D., Fukami, T., and Relman, D. A. (2018). Role of priority effects in the early-life assembly of the gut microbiota. *Nature Reviews Gastroenterology and Hepatology*, *15*(4), 197-205.

Steel, R. G. D., J. H. Torrie, and D. J. Dickey. (1997). Principles and procedures of statistics: A biometrical approach. 3rd ed. McGraw Hill Book Co., New York, NY.

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.N., Kubo, C. and Koga, Y. (2004). Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *The Journal of Physiology*, *558*(1), 263-275.

Torow, N., and Hornef, M. W. (2017). The neonatal window of opportunity: setting the stage for life-long host-microbial interaction and immune homeostasis. *The Journal of Immunology*, *198*(2), 557-563.

Trasande, L., Blustein, J., Liu, M., Corwin, E., Cox, L. M., and Blaser, M. J. (2013). Infant antibiotic exposures and early-life body mass. *International Journal of Obesity*, *37*(1), 16-23.

Tsavkelova, E. A., Botvinko, I. V., Kudrin, V. S., and Oleskin, A. V. (2000). Detection of neurotransmitter amines in microorganisms using of high performance liquid chromatography. *Doklady Biochemistry and Biophysics*. *372*, 115-117.

Tsutsui, K., Ubuka, T., Bentley, G. E., and Kriegsfeld, L. J. (2012). Gonadotropin-inhibitory hormone (GnIH): discovery, progress and prospect. *General and Comparative Endocrinology*, *177*(3), 305-314.

Ulloa-Aguirre, A., and Timossi, C. (2000). Biochemical and functional aspects of gonadotrophin-releasing hormone and gonadotrophins. *Reproductive BioMedicine Online*, *1*(2), 48-62.

van der Eijk, J.A., Rodenburg, T.B., de Vries, H., Kjaer, J.B., Smidt, H., Naguib, M., Kemp, B. and Lammers, A. (2020). Early-life microbiota transplantation affects behavioural responses, serotonin and immune characteristics in chicken lines divergently selected on feather pecking. *Scientific Reports*, *10*(1), 1-13.

Videnska, P., K. Sedlar, M. Lukac, M. Faldynova, L. Gerzova, D. Cejkova, F. Sisak, and I. Rychlik. (20140. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. *PLoS One*, *9*, e115142.

Wang, S. H., Dong, L., Luo, J. Y., Gong, J., Li, L., Lu, X. L., and Han, S. P. (2007). Decreased expression of serotonin in the jejunum and increased numbers of mast cells in the terminal ileum in patients with irritable bowel syndrome. *World Journal of Gastroenterology*, *13*(45), 6041.

Yan, F. F., Wang, W. C., and Cheng, H. W. (2020). Bacillus subtilis-based probiotic promotes bone growth by inhibition of inflammation in broilers subjected to cyclic heating episodes. *Poultry Science*, *99*(11), 5252-5260.

Yoo, J. Y., M. Groer, S. V. O. Dutra, A. Sarkar, and D. I. McSkimming. (2020). Gut microbiota and immune system interactions. *Microorganisms*. *8*(12), 2046.

Yue, Y., Guo, Y., and Yang, Y. (2017). Effects of dietary L-tryptophan supplementation on intestinal response to chronic unpredictable stress in broilers. *Amino Acids*, *49*(7), 1227-1236.

Zhang, J., F. Rodríguez, M. J. Navas, M. Costa-Hurtado, V. Almagro, L. Bosch-Camós, E. López, R. Cuadrado, F. Accensi, S. Pina-Pedrero, J. Martínez, and F. Correa-Fiz. 2020. Fecal microbiota transplantation from warthog to pig confirms the influence of the gut microbiota on African swine fever susceptibility. *Scientific Reports*, *10*(1), 1-14.

Zhang, K., Hornef, M. W., and Dupont, A. (2015). The intestinal epithelium as guardian of gut barrier integrity. *Cellular Microbiology*, *17*(11), 1561-1569.

Zhu, F., Guo, R., Wang, W., Ju, Y., Wang, Q., Ma, Q., Sun, Q., Fan, Y., Xie, Y., Yang, Z. and Jie, Z., (2020). Transplantation of microbiota from drug-free patients with schizophrenia causes schizophrenia-like abnormal behaviors and dysregulated kynurenine metabolism in mice. *Molecular Psychiatry*, *25*(11), 2905-2918.

Table 2.1 Effects of cecal microbiota transplantation on relative organ weight of roosters at week 16.

Items	[1]Relative organ weight			
	spleen	liver	adrenal gland	heart
CTRL	1.849	17.991	0.042^{AB}	6.373
7_2-CMT	1.720	18.059	0.048^{A}	6.661
6_3-CMT	1.631	17.754	0.033^{B}	7.119
SEM	0.134	0.923	0.004	0.662
P- value	0.524	0.970	**0.090**	0.639

Values are least square means ± SEM, n=7. $^{A, B}$ indicates a trend difference ($0.05 < P \leq 0.1$).
[1]Relative organ weight = absolute organ weight (g)/ body weight (kg)

Table 2.2 Effects of cecal microbiota transplantation on stress parameters (H/L ratio, corticosterone) and sexual hormone (testosterone) of roosters at week 16.

Measures	Treatment			SEM	*P*-value
	CTRL	7_2-CMT	6_3-CMT		
H/L ratio	0.327^{ab}	0.367^{a}	0.243^{b}	0.029	**0.024**
Corticosterone (ng/ml)	4.235	4.678	3.697	0.900	0.789
Testosterone (ng/ml)	1.423	1.132	1.744	0.277	0.345

Values are least square means ± SEM, n= 7. ^{a,b} indicates a significant difference ($P \leq 0.05$).

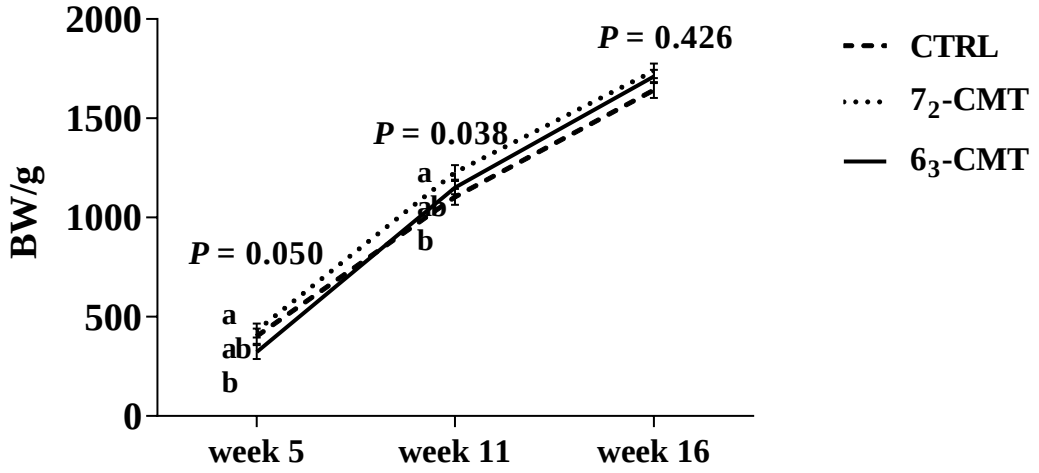

Figure 2.1 Effects of cecal microbiota transplantation on body weight of roosters at week 5, 11 and 16. Values are least square means ± SEM, n=7. [a, b] indicates a significant difference ($P \leq 0.05$).

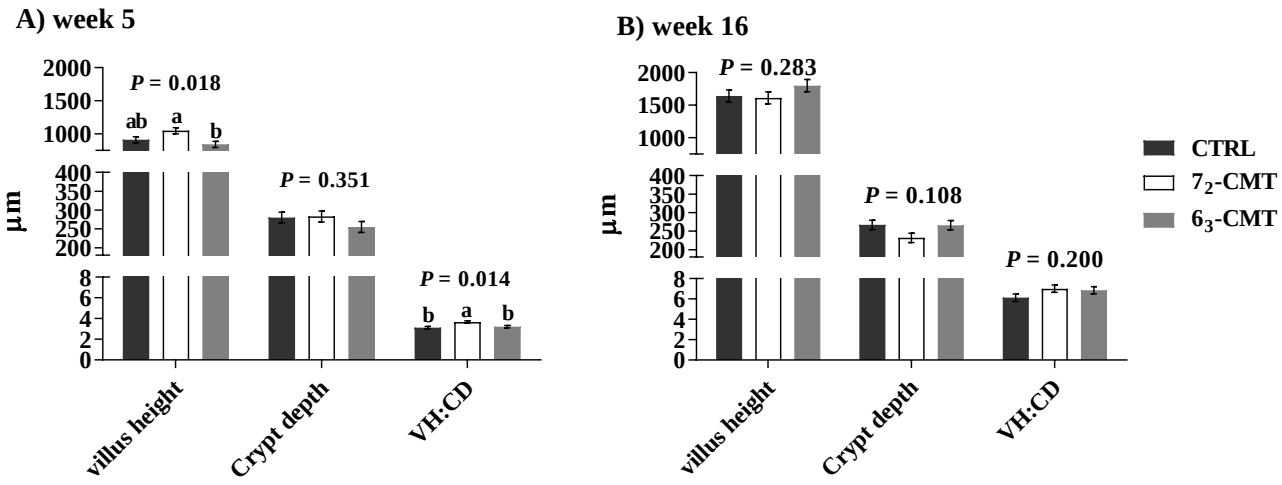

Figure 2.2 Effects of cecal microbiota transplantation on ileal morphology of roosters at week 5 and 16. Values are least square means ± SEM, n=7. [a, b] indicates a significant difference ($P \leq 0.05$).

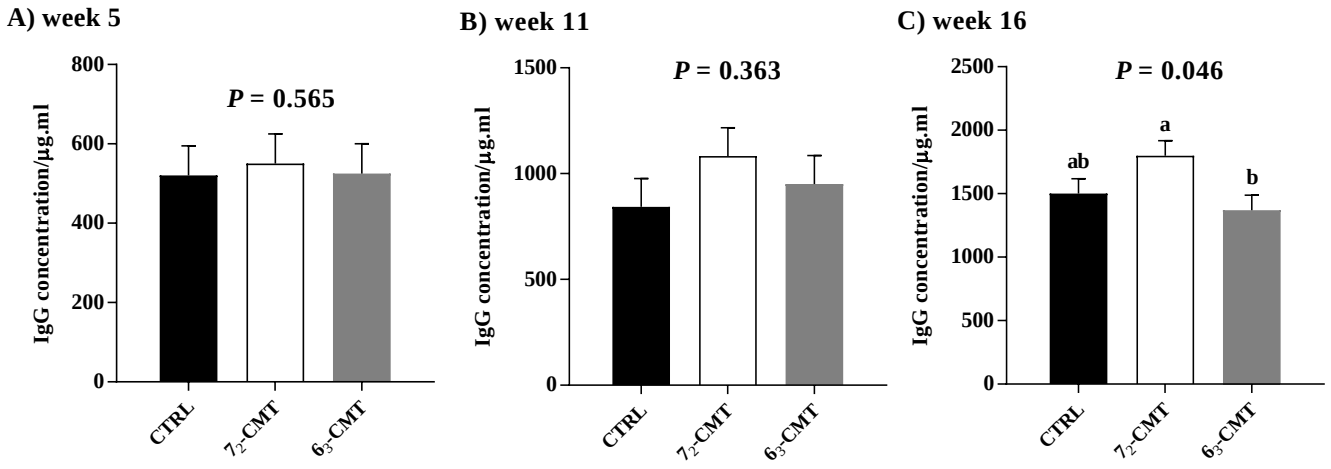

Figure 2.3 Effects of cecal microbiota transplantation on plasma IgG concentration of roosters at week 5, 11 and 16. Values are least square means ± SEM, n=7. [a, b] indicates a significant difference ($P \leq 0.05$).

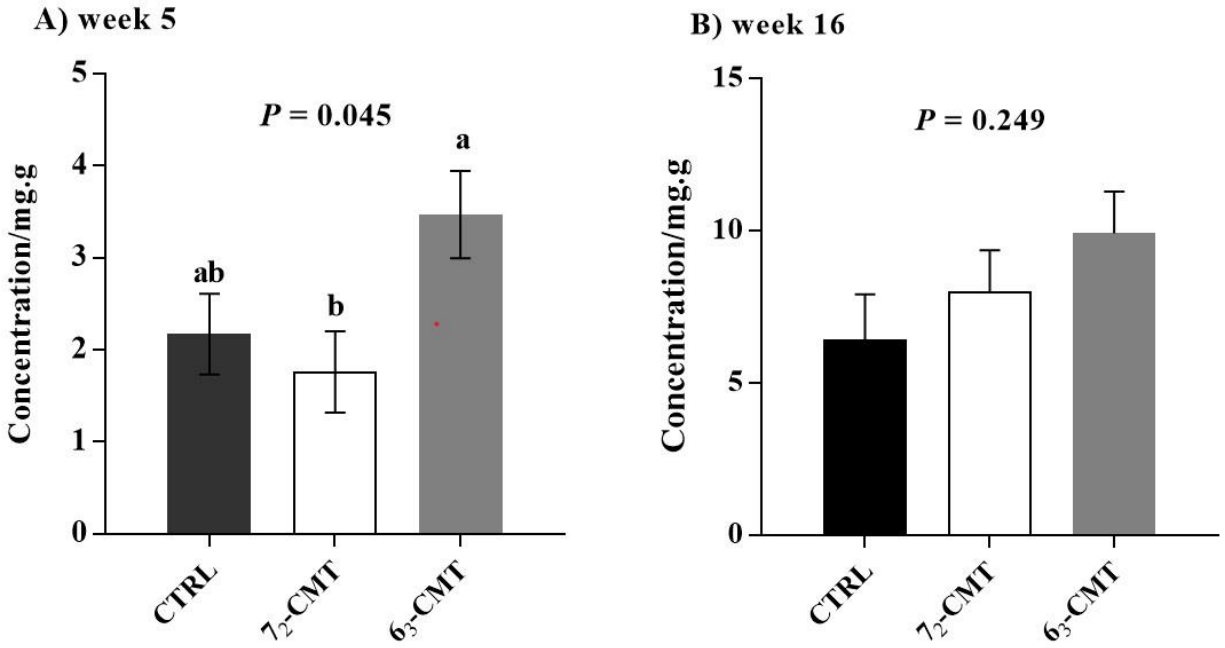

Figure 2.4 Effects of cecal microbiota transplantation on mucosa sIgA concentrations of roosters at week 5 and 16. Values are least square means ± SEM, n=7. [a, b] indicates a significant difference ($P \leq 0.05$).

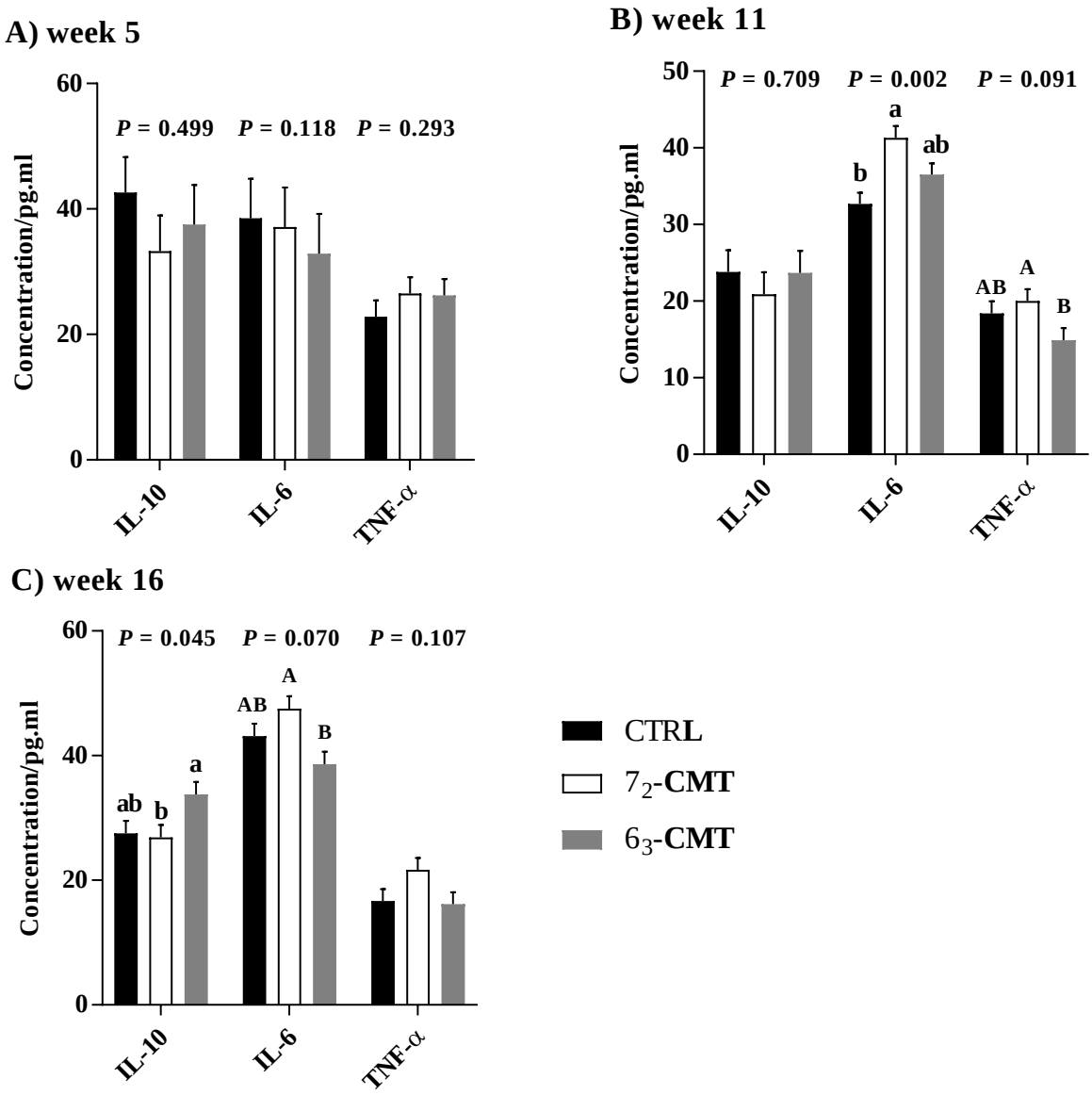

Figure 2.5 Effects of cecal microbiota transplantation on plasma inflammatory cytokines of roosters at week 5, 11 and 16. Values are least square means ± SEM, n=7. [a, b] indicates significant differences ($P \leq 0.05$), [A, B] shows trend differences ($0.05 < P \leq 0.1$).

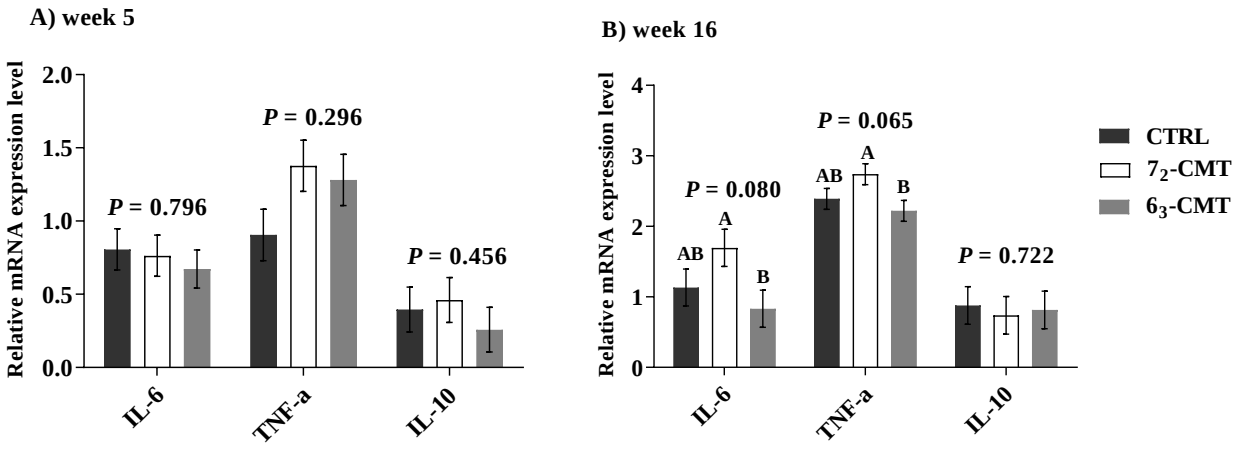

Figure 2.6 Effects of cecal microbiota transplantation on splenic mRNA abundance of inflammatory cytokines of roosters at week 5 and 16. Values are least square means ± SEM, n=7. $^{A, B}$ shows trend differences ($0.05 < P \leq 0.1$).

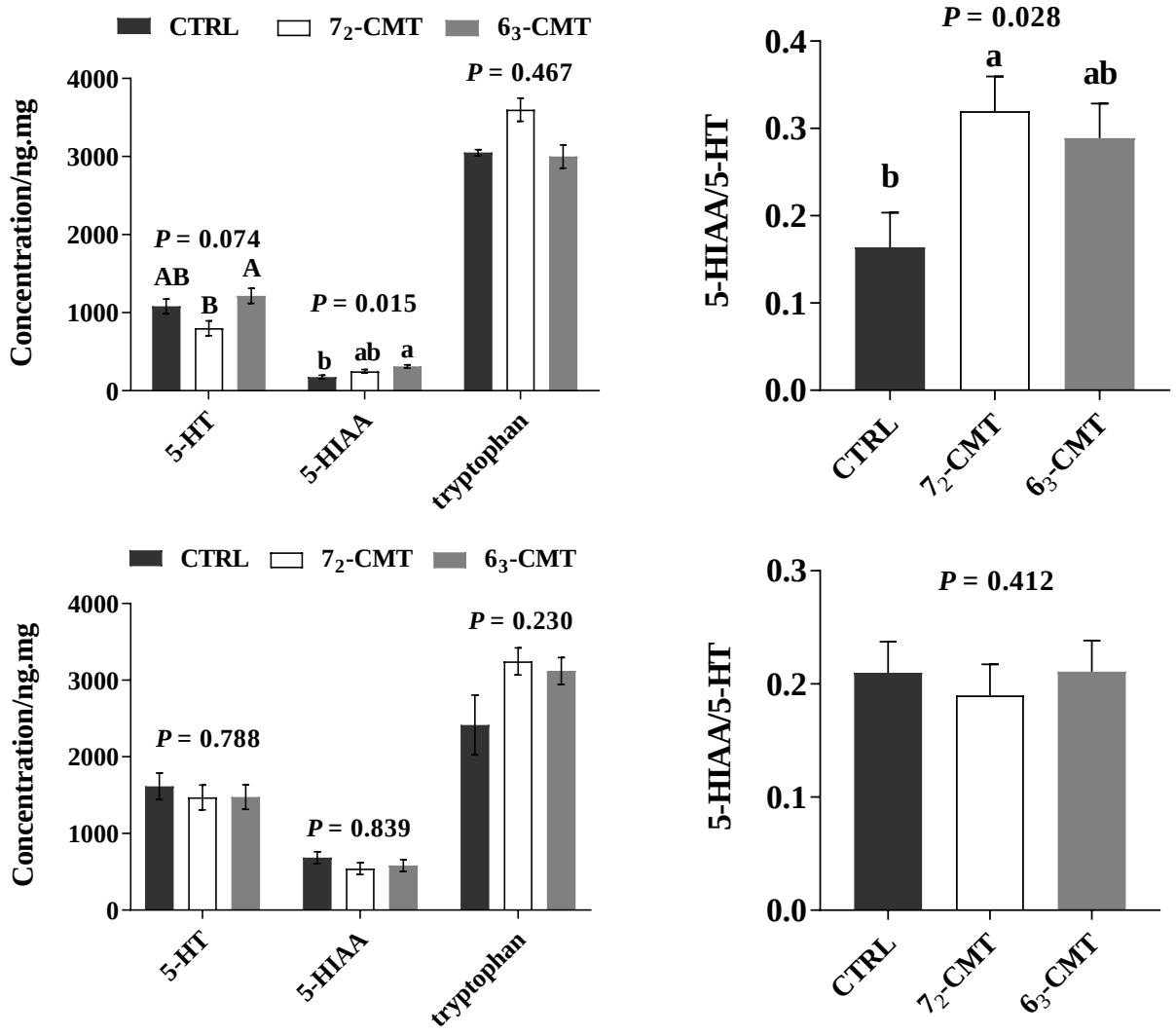

Figure 2.7 Effects of cecal microbiota transplantation on ileal serotonergic activities of roosters at week 5 and 16. Values are least square means ± SEM, n=7. $^{a, b}$ indicates significant differences ($P \leq 0.05$), $^{A, B}$ shows trend differences ($0.05 < P \leq 0.1$).

CHAPTER 3. THE GUT-BRAIN AXIS: TARGETING GUT MICROBIOTA TO REDUCE AGGRESSION AND IMPROVE WELFARE IN POULTRY

3.1 Abstract

Numerous studies have indicated that modulations of the gut microbiota affect host physiological homeostasis and behavior. Fecal microbiota transplantation is a clinical therapy for treating patients with psychiatric disorders. Similarly, modulation of gut microbiota could be a feasible strategy for reducing aggression and improving the welfare of chickens. The objective of this study was to determine the effects of early postnatal cecal microbiota transplantation (CMT) on aggression, brain serotonergic and catecholaminergic activity in roosters. Chicken lines 6_3 (gentle birds) and 7_2 (aggressive birds) were used as donors and chickens of a commercial strain (Dekalb XL) were used as recipients for the CMT. A total of 84 d-old male chicks were randomly assigned to 1 of 3 treatments with 7 cages per treatment and 4 birds per cage (n=7): Control (0.1 ml saline), 6_3-CMT (0.1 ml cecal solution of line 6_3), and 7_2-CMT (0.1 ml cecal solution of line 7_2). Cecal microbiota transplantation was conducted via oral gavage once daily from d 1 to d 10, and then once weekly from wk 3 to wk 5. At wk 5, 11, and 16, birds with similar BW were assigned to paired aggression tests between treatments. Samples of blood, brain and cecal content were collected from post-tested birds at each time point for detecting CMT-induced changes in the cecal microbial community and correlations with the alterations of serotonergic activity and aggressive behaviors. The results indicated that CMT altered the phylogenetic diversity of recipients at both wk 5 and 16 (P = 0.039 and P = 0.090). CMT also altered gut microbial community structures at wk 5, which was reflected in the changes of beta diversity metrics including Jaccard (P = 0.020) and Unweighted UniFrac (P = 0.005). Moreover, microbiota modulations alter the activities of central serotonergic and catecholaminergic systems at wk 5 and 16. At both time points, 6_3-CMT birds tended to have higher concentrations of hypothalamic tryptophan (P = 0.089 and 0.063). 6_3-CMT birds also had the highest serotonin turnover at wk 16 (P = 0.011), while 7_2-CMT birds had

the lowest norepinephrine (NE) and dopamine (DA) concentrations among treatments (P = 0.072 and 0.031). However, there were no treatment effects on dopamine turnover at wk 16 (P = 0.957). 7_2-CMT birds also had a lower mRNA abundance of monoamine oxidase A (MAOA) at wk 16 (P = 0.024). In line with this finding, 6_3-CMT birds exhibited less frequent aggression both in the home cages (P = 0.039) and paired tests at wk 5 (P = 0.040), with a trend of difference at wk 16 (P = 0.093) compared to 7_2-CMT birds. At wk 5, genus *Ruminococcaceae UCG-005* was positively correlated with brain serotonin levels in 6_3-CMT birds (P = 0.036), while genus *GCA-900066225* was negatively correlated with 5-HIAA in 7_2-CMT birds (P = 0.039). Overall, these results indicate that gut microbiota modulations may be a management strategy for reducing aggressive behavior in chickens through the gut-brain axis to regulate the activities of the central serotonergic and catecholaminergic systems.

3.2 Introduction

Aggression, as an evolutionarily conserved behavior, exists widely across the animal kingdom from humans to various animals including chickens. From the perspective of an evolutionary process, aggression serves as a crucial survival skill for animals to secure resources and adapt to the surrounding environment (Buss and Duntley, 2006). However, dysfunctional aggression within a group causes social stress, interfering with the social needs of safety damaging the health of both dominants and subordinates (Raine, 2002). In modern intensive production systems, mixing and regrouping unfamiliar individuals is a common management practice. This procedure, however, is often accompanied by the disruption of social structures and increased aggression among animals, which arouses great health and welfare concerns (Hubbard et al., 2021). For example, mixing aggression in sows causes skin lesions, elevates stress levels, and impairs reproductive performance (Turner et al., 2006; Lagoda et al., 2021). Similarly, in commercial chickens, aggression leads to excessive stress, injuries, and sometimes cannibalism and death (Cheng and Muir, 2007, Hartcher et al., 2015). In addition, the victim birds with feather loss require

more feed intake to maintain thermal comfort and energy storage, resulting in increased feed consumption and economic costs for the poultry industry (Drake et al., 2010). Although several preventative approaches such as beak trimming, genetic selection, and illumination control have been implemented in poultry production, these management practices cannot completely prevent the incidence of aggression and related injurious behaviors. Hence, it is pivotal to develop a new approach to control aggression, improve bird welfare, and increase the profits of the poultry industry.

In recent years, great research attention has been paid to the gut microbiota and its link with host physiological and psychological health via the gut-brain axis. The first evidence for the existence of the gut-brain axis was reported in the early 19th century, and researchers found that a person's mood could been governed by the gut state (Cannon, 1909; Wolf and Wolff, 1943). Correspondingly, the gut microbiota has been recognized as an endocrine organ influencing both mood stabilization and behavioral exhibition of the host via regulating biofunctions of neurotransmitters and neurotrophic factors (Sudo et al., 2004), inflammatory process (Hand et al., 2016), and energy metabolism (Zheng et al., 2021). In humans, disordered gut microbiota composition has been linked to the pathophysiology of mental disorders (Cryan and Dinan, 2012). For instance, patients with depression have decreased abundance of phyla *Firmicutes* and increased *Bacteroidetes* and *Proteobacteria* as compared to healthy individuals (Yu et al., 2017). Furthermore, several bacterial taxa such as the *Enterobacteriaceae* family have been used as a biomarker for evaluating the severity of depression (Jiang et al., 2015; Zheng et al., 2016). The causal link between the gut microbiota and brain function has been further revealed in germ-free (GF) mice. For example, fecal microbiota transplantation (FMT) from patients with depression could induce the specific depression-like behavior, together with a decrease in several brain neurotransmitters such as serotonin (5-HT), norepinephrine (NE), and epinephrine (EP) in GF recipients (Zheng et al., 2016). Meanwhile, a positive correlation between decreased levels of 5-HT and the bacterial taxa such as genus *Akkermansia* has been revealed in the same study. Similar

findings of FMT eliciting the phenotype of the donors in the recipients have been reported in several psychiatric disorders including autism (Hisao et al., 2013; Kang et al., 2019), schizophrenia (Zhu et al., 2020), and Alzheimer's disease (Sun et al., 2019a), which indicates the functional link between the gut bacteria and brain physiology. Based on these previous findings, we hypothesized gut microbiota may present similar efficiency in regulating aggressive behavior in chickens.

The regulation of behaviors is primarily mediated through altering central neuronal activities (Berman et al., 1997). Serotonin (5-HT) is a primary modulatory neurotransmitter involved in this process. Approximately 5% of the body's 5-HT is synthesized in the brain, while 95% is released from the enterochromaffin cells within the gut epithelia (Gershon, 2003). In humans, the changes of 5-HT within the central nervous system (CNS) have been linked with mood and behavioral disorders including interpersonal aggression and impulsive behavior (Pourhamzeh et al., 2021). The 5-HT deficiency theory of aggression is driven by the negative correlation between the central 5-HT and aggressiveness in humans (Klasen et al., 2019; Kolla and Houle, 2019) and various experimental animals (Kästner et al., 2019; Weinberg-Wolf and Chang, 2019). Like in mammals, aggressive birds usually exhibit lower 5-HT concentrations in specific brain regions such as the hypothalamus (Dennis et al., 2008). Additionally, the gut microbiota contributes to the synthesis of peripheral 5-HT. Due to the impermeable properties of the blood-brain barrier (BBB), peripheral 5-HT cannot pass this barrier to directly regulate brain function. As such, the pathophysiological roles of peripheral 5-HT in behavioral and motivational regulation are still unclear. It should be noted that the body's 5-HT can be derived from an essential amino acid, *L*-tryptophan, the precursor of 5-HT, which can pass through the BBB and directly and indirectly influences the mood, cognition, and behavioral exhibition of the host (Sandyk, 1992). Correspondingly, several gut bacterial taxa such as *Lactobacillus* influence the tryptophan synthesis of the host (Gao et al., 2018). Thus, it would be of interest to further investigate the effects of gut microbial modification on host aggressiveness.

Few studies have been conducted to evaluate the effects of gut microbial modulations on aggression. In poultry, the cecum contains the greatest microbial population and largest concentrations of microbial cells as compared with other segments of the gastrointestinal (GI) tract, which constantly attracts interest in investigating the function of gut microbiota on host biological homeostasis (Yan et al., 2017; Glendinning et al., 2020). Therefore, the objective of this study was to determine if early-life cecal microbiota transplantation (CMT) would potentially reduce aggressive behaviors through modulating the function of the central serotonergic and dopaminergic systems via the gut-brain axis.

3.3 Materials and methods

3.3.1 Birds, diets, and management

All experimental procedures were approved by the Purdue University Animal Care and Use Committee (PACUC# 1712001657); and the study was conducted in accordance with the guidelines set by the Federation of Animal Science Societies (2010). Chicken lines 6_3 and 7_2 developed by Avian Disease and Oncology Laboratory (East Lansing, MI) selected for Marek's disease resistance or susceptibility were used as donor chickens (Bacon et al., 2001).

Eighty-four 1-day-old male chicks of Dekalb XL, a commercial strain, were used as recipients. Water and feed were provided *ad libitum*. The general management, including vaccination, dietary formulation and nutrient contents, ambient temperature, and lighting program followed the Hy-line guidelines (2019).

3.3.2 Study design, treatment administration, and sample collection

At 60 week of age, cecal contents were randomly collected from 10 hens per line (6_3 and 7_2) and evenly pooled within the line. 5 grams of pooled cecal contents were suspended with gut microbiome media (Goodman et al., 2011) at a ratio of 1:10 and then kept in the -20^{o}C freezer for oral gavage.

Recipients were randomly assigned into 1 of 3 treatments with 7 cages per treatment and 4 birds per cage (n=7): CTRL (0.1 ml saline, control), 6_3-CMT (0.1 ml of line 6_3 cecal solution), and 7_2-CMT (0.1 ml of line 7_2 cecal solution) for a 16-week trial. Oral gavage was performed from d 1 to d 10, and the effectiveness of transplantation was boosted weekly from wk 3 to 5.

The hypothalamus and both sides of cecal contents were collected from 1 bird per cage at week 5 and 16, respectively (n=7). Cecal contents and hypothalamic tissues were snap-frozen and stored at -80°C until analysis.

3.3.3 Behavioral analysis

Paired behavioral test

Paired behavioral test was conducted among the treatments at wk 5, 11, and 16 following the procedure published previously (Dennis and Cheng, 2011). Briefly, birds with similar BW from the different treatments were randomly assigned to pairs for aggression tests (n=7). The test was conducted in novel cages allowing 750 cm^2/bird. 7 pairs were tested at the same time in the morning between 8:00 am -12:00 pm in the testing room and each pair was tested for 30 min. Afterward, the frequency of aggressive behavior was analyzed and counted as the average number of pecks per bird per cage in a 30-min period. The definition of aggression was "*Occurs when one bird raises head and forcefully stabs beak either once or multiple times at another bird. Aggressive pecks will usually be directed at the head but may also be directed at the body. The recipient will usually show avoidance behavior by ducking or moving away from aggressive birds"* (Daigle et al., 2014).

Home-cage aggressive behavior

The behavior of birds in the home-cage was recorded using digital video recording (DVR) systems (Geovision GV-1480) linked to overhead ceiling-mounted cameras (Panasonic WV-CP254H). Home-cage behavior was recorded for a 24-h period at wk 5 and 16 (n=5). Each week

of the recorded video was viewed using EZViewLog500 in real-time and collected from 1h after lights on and 1h before lights off (5:58 am - 6:58 am, 7:58 pm - 8:58 pm). The frequency of aggressive pecking was counted during the 2-h video segments and calculated as the average number of pecks per bird per hour (the number of pecks per cage per hour divided by the number of birds in each cage).

3.3.4 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA of the hypothalamic samples was extracted using RNeasy Mini Lipid Kit (Catalog #: 74804, Qiagen, Valencia, CA) following instructions provided by the company. The purity and concentration of total RNA were checked using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). The reverse transcription was conducted by using the Reverse Transcription Reagent Pack (Catalog #: N8080234, Applied Biosystems, Foster City, CA). A mixture of reverse transcription reagents consists of 2 μl RNase inhibitor, 2.5 μl multi-scribe reverse transcriptase, 5 μl random hexamers, 10 μl of TaqMan reverse transcription buffer, 20 μl deoxy nucleotides, and 22 μl of magnesium chloride. A total master mixture of each sample consists of 61.5 μl with the quantified RNA sample and RNase-free water for a final 100 μl. The RNA samples were reverse transcribed to cDNA by Techne TC-3000G PCR Thermal Cycler (Bibby Scientific Limited, Stone UK). To explore the microbiota modulation on the relative mRNA expression of the serotonin pathway, the mRNA expressions of related genes (Table 3.1) in the right side of hypothalamic tissues were detected by RT-qPCR using the methods described previously (Dennis and Cheng, 2011). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Results were quantitated by the standard curve method. Standards were measured in triplicates with a standard deviation of less than 2.0 and a coefficient of variation less than 2.0%.

3.3.5 High-performance liquid chromatography (HPLC)

To determine the serotonergic activities of brain samples, duplicate samples were run by HPLC (UltiMate^{TM} 3000 RSLCnano System, Thermo Fisher Scientific Inc., Waltham, MA), as previously described (Yan et al., 2020). Briefly, the left side of the hypothalamus was weighed and homogenized in 0.2 *M* perchloric acid at a ratio of 1:10 and then vortexed for 1 minute. Afterward, the supernatant was centrifuged at 15,000 × *g* for 10 min at 4°C for HPLC analysis; and then filtered through a 0.2 µm polyvinylidene fluoride filter into a sample vial. The flow rate of the mobile phase (Sigma, USA) was 0.7 mL/min, and the cell potential was set at 0 mV, 225 mV, and 550 mV. Each sample was tested twice at 10 µl per injection. The concentrations of 5-HIAA, 5-HT, tryptophan, NE, EP, DOPAC, and DA were calculated as nanograms per milliliter (ng/g) through a reference curve generated from corresponding standards.

3.3.6 Cecal microbial profiles

DNA extraction and 16S rRNA amplicon sequence

Total genomic DNA was extracted from cecal samples using the QIAamp Fast DNA Stool Mini kit (Catalog #: 51504 Qiagen, Valencia, CA) following the manufacturer's instructions. 16S rRNA gene sequence was conducted to characterize the effects of CMT on gut microbial diversity and community structures of recipient chickens (Caporaso et al., 2011). Briefly, the V4 region of the 16S rRNA gene was amplified from genomic DNA using 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT). Each 25 µL PCR reaction contains 9.5 µL of PCR-grade water (Certified DNA-Free), 12.5 µL of QuantaBio's AccuStart II PCR ToughMix (2x concentration, 1x final), 1 µL Golay barcode tagged Forward Primer (5 µM concentration, 200 pM final), 1 µL Reverse Primer (5 µM concentration, 200 pM final), and 1 µL of template DNA. The conditions for PCR were as follows: 94 °C for 3 minutes to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons are then quantified

using PicoGreen (Invitrogen). After quantification, volumes of each of the products were pooled into a single tube and cleaned up using AMPure XP Beads (Beckman Coulter), and then quantified using a fluorometer (Qubit, Invitrogen). After quantification, the molarity of the pool was determined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.75 pM with a 10% PhiX spike for sequencing on the Illumina MiSeq (Caporaso et al., 2012). The amplicons were sequenced using the Illumine Miseq platform (2 x 150 bp paired-end run) at the Argonne National Laboratory (Chicago, USA).

Quality control and sequence analyses

Amplicons were trimmed at the 13th base of each sequence for both the forward and reverse sequence and denoised with Divisive Amplicon Denoising Algorithm (DADA 2) pipelines via Qiime 2 (v2020.2). Amplicon sequence variants (ASVs) were generated using DADA 2; and the ASV sequences were classified using the 99% Silva v132 taxonomic database as a reference (https://data.qiime2.org/2020.2/common/silva-132-99-515-806-nb-classifier). Cecal samples were rarefied to 13,580 and 24,907 sequencing reads at wk 5 and 16, respectively.

α-diversity: The community richness and evenness of gut microbiota were reflected by Chao1 and evenness metrics respectively. The community diversity of gut microbiota was evaluated by Shannon and Faith's PD metrics. All the metrics of alpha diversity were analyzed using R studio (version 3.6.2). The Shapiro-Wilk test was used to analyze the normality of the data and Box-Cox was used for transformation. For the normalized data, statistical analysis was carried out one-way ANOVA and the Tukey-Kramer test was used to partition any significant differences among means due to treatment main effects. For the unnormalized data, Kruskal-Wallis test was used and followed by Dunn's test for multiple comparisons.

β-diversity: Unweighted UniFrac and weighted UniFrac distance metric (phylogenetic distance), Jaccard and Bray Curtis were conducted to compare the microbial community structure. To visualize the dissimilarity of microbiota communities, the plots of PCoA based on Bray-Curtis,

weighted UniFrac, unweighted UniFrac, and Jaccard metrics were performed using the Phyloseq, DESeq2, and ggplot2 packages in addition to custom scripts in the R studio (version 3.6.2). The treatment effects were evaluated using Adonis and the pairwise comparison was assessed using permutational multivariate analysis of variance (PERMANOVA) test and false discovery rate (FDR) adjusted *P*-value (q-value) was used for multiple comparisons in Qiime 2 (v.2020.2).

Bacterial taxa: To identify the most differential bacterial taxa among treatment, analysis was performed by the R studio (version 3.6.2) to identify differentially enriched bacterial taxa on the summarized phylum and genus levels at the ASV level using the DESeq2 package (version 1.30.1). Significant differences in Log2 fold changes were determined by the Wald test with P-value correction by Benjamin and Hochberg. Levels of significance were determined when $P < 0.05$ and LDA score > 2.

3.3.7 Statistical analysis

The fixed effects were treatment and age. Physiological and behavioral data was considered as response variables. Data was analyzed using R studio (version 3.6.2) except for behavioral data of home-cage. The behavioral data of the home cages was analyzed using PROC MIXED repeated measures procedures in SAS 9.4 (SAS Institute Inc., Cary, NC). Days of the video recording within each week were considered as repeated measures. The frequency of aggression in home cages was normalized using Box-Cox transformation before analysis. The behavioral data of the paired test were analyzed using the Kruskal-Wallis test and followed by Dunn's test for multiple comparisons. Other normalized physiological data was carried out by one-way ANOVA and the Tukey-Kramer test was used to partition any significant differences among least square means due to treatment effect (Steel et al., 1997). For each treatment, Spearman's rank test was used to find the correlation between the significantly differential taxa and neurotransmitter concentrations. Significance was set at $P \leq 0.05$ and a trend was defined as $0.05 < P \leq 0.1$.

3.4 Results

3.4.1 Analysis of 16S rRNA gene sequence

Changes in the microbial community diversity and structures

α-diversity: 16S rRNA gene sequence of cecal contents were examined using to evaluate the efficiency of CMT on gut microbiota modification at wk 5 and 16. In donor birds, the selection based on resistance to Marek's disease caused variations in the structure of the gut microbial community. Birds of line 7_2 had a greater phylogenetic diversity than birds of line 6_3 (Figure 3.1A). Correspondingly, the CMT from the donor lines differently influenced the cecal microbial diversity of recipient birds. Regarding to alpha diversity, 7_2-CMT birds had the greater phylogenetic diversity (Faith's Pd index) compared with 6_3-CMT but not CTRL birds at wk 5 (Figure 3.1B). These differences were continuously detected at wk 16 (P = 0.090). However, there were no treatment differences on Chao1 richness (Figure 3.1D), evenness (Figure 3.1F), and Shannon diversity indices (Figure 3.1H) at both wk 5 and 16.

β-diversity: A summary of beta diversity is presented in Table 3.3. There were significant differences between donor lines in the beta diversity indicated by Bray Curtis (P = 0.001), Jaccard (P = 0.001), Unweighted (P = 0.011), and weighted UniFrac (P = 0.014) calculations. Similarly, treatment effects in recipients were found on Jaccard (P = 0.020) and Unweighted UniFrac calculations (P = 0.005) at wk 5 but not at wk 16 (P > 0.05, Table 3.3). The post hoc pairwise testing indicated there were significant differences in the microbial community structures between 6_3-CMT birds and 7_2-CMT birds (Unweighted UniFrac, P = 0.006, Table 3.4). The outcomes of the PCoA plots of these metrics were illustrated in Figure 3.2.

Differential bacterial taxa by treatment

The differential bacterial taxa were further examined. At wk 5, the number of differentially abundant bacterial taxa between 6_3-CMT birds vs. 7_2-CMT birds, 7_2-CMT birds vs. CTRL birds, and 6_3-CMT birds vs. CTRL birds were 38, 68, and 52 amplicon sequence variants (ASVs)

(Figures 3.3), respectively. Compared with CTRL birds, 10 genera (*GCA-900066225, Butyricicoccus, Lachnoclostridium, Christensenellaceae R-7 group, Flavonifractor, Ruminococcaceae UCG-014, Faecalibacterium, Alistipes, Fournierella, Ruminococcaceae UCG-005*) were abundant in 6_3-CMT birds; while 7_2-CMT birds had 13 enriched genera compared to CTRL birds, including *Alistipes, Candidatus Arthromitus, Dielma, Merdibacter, Faecalibacterium, Flavonifractor, GCA-900066225, Anaerofilum, Ruminococcus 2, Ruminococcaceae UCG-005, Ruminococcaceae NK4A214 group, Ruminococcaceae UCG-014, Lachnoclostridium*. Compared with 6_3-CMT birds, 10 genera (*Akkermansia*, *Dielma*, *Merdibacter*, *Anaeroplasma*, *Ruminococcaceae UCG-008*, *Faecalibacterium*, *GCA-900066225*, *Ruminococcaceae UCG-014*, *CAG-56*, *Blautia*) were more abundant in 7_2-CMT birds. Those ASVs belong to the phyla *Bacteroidota, Cyanobacteria, Firmicutes, Tenericutes, Verrucomicrobiota*.

At wk 16, there were 30, 14, and 25 amplicon sequence variant (ASV) differences between 6_3-CMT birds vs. 7_2-CMT birds, 7_2-CMT birds vs. CTRL birds, and. 6_3-CMT birds vs, CTRL birds, respectively (Figures 3.3). Compared with CTRL birds, 5 genera were more abundant (*Alistipes, Clostridia_UCG-014, Incertae_Sedis, Lachnoclostridium, Lachnospiraceae*) in 6_3-CMT birds, while 7_2-CMT birds had enriched *Bacteroides* and *Clostridia_UCG-014* enriched compared with CTRL. Compared with 6_3-CMT, 3 genera (*Bacillus*, *Escherichia-Shigella*, *Bacteroides*) were more abundant in 7_2-CMT birds. Those ASVs belong to the phyla *Bacteroidota, Firmicutes, Proteobacteria*.

3.4.2 Alterations in the neuroendocrine systems

The effects of early-life CMT on the central serotonergic and catecholaminergic systems of recipient birds are presented in Figure 3.4. At wk 5, 6_3-CMT birds had higher concentrations of hypothalamic 5-HT (P = 0.037) and 5-HIAA (P = 0.007, Figure 3.4A). At wk 16, the treatment-induced serotonergic difference was further increased, 6_3-CMT birds had the highest 5-HT

turnover among treatments at wk 16 (P = 0.011, Figure 3.4D). In addition, 6_3-CMT birds tended to have higher concentrations of hypothalamic tryptophan at both wk 5 (P = 0.089) and wk 16 (P = 0.063, Figure 3.4C). Microbiota modulation also impacted the activation of the catecholaminergic system, 7_2-CMT birds had the lowest NE and DA concentrations among treatments (P = 0.072 and 0.031, Figure 3.4E) at wk 5. There were no treatment effects on dopamine turnover (P = 0.447 and 0.957, Figure 3.4F, H) and the TPH2, Htr1a, Htr1b, and 5htt mRNA expressions (P > 0.05, Table 3.2) except that abundance of MAOA mRNA was increased in 6_3-CMT group at wk 16 (P = 0.024).

3.4.3 Correlations of neurotransmitter concentration with bacterial taxa

At wk 5, genus *Ruminococcaceae UCG-005* was positively correlated with brain serotonin levels in 6_3-CMT birds (P = 0.036, Figure 3.5), while genus *GCA-900066225* was negatively correlated with 5-HIAA in 7_2-CMT birds (P = 0.039, Figure 3.5).

3.4.4 Differences in behavioral exhibitions

Compared to 7_2-CMT and CTRL birds, 6_3-CMT birds had less frequent aggressive pecking both in the home cages (P = 0.039, Figure 3.6) and the paired test at wk 5 (P = 0.040, Figure 3.7). At wk 16, 7_2-CMT birds tended to exhibit more aggressive pecking during the paired test (P = 0.093), but no treatment effects were found on the frequency of aggressive pecking in home cages at wk 16 (P = 0.455, Figure 3.6).

3.5 Discussion

Growing evidence has highlighted the involvement of gut microbiota in the regulation of host physiological and behavioral homeostasis via the gut-brain axis and the potential implications of modification of gut microbiota in treating patients with psychiatric and mental disorders (Settanni et al., 2021). In the current study, to assess if the gut microbiota plays a similar role in

chickens, we transplanted cecal content from the divergent chicken lines with gentle or aggressive behavior into the GI tract of Dekalb XL roosters, a commercial strain. The recipient birds receiving the different cecal content were characterized by the changes in their diversity of the cecal microbial community and the activities of the central serotonergic and dopaminergic systems, which may be associated with the donor lines' unique behavioral exhibitions.

In chickens, the development of gut microbiota is usually in a succession manner where the microbial community diversity is age-dependent (Rychlik, 2020). During the early immature stage, the intestinal microbiota is highly variable in close with endocrine and immune development causing long-lasting effects. Much research, therefore, has been devoted to an early-life intervention of the gut microbiota composition in the host's health. In humans, it has been evidenced that selectively promoting the beneficial bacteria in infants, such as supplementation of prebiotics, is critical for the overall health status of the host in later life (Wall et al., 2009). Similarly, in chicks, in ovo delivery of probiotics promotes humoral and cellular immune responses (Slawinska et al., 2016). During a chick growth cycle, ongoing environmental exposures constantly change its gut microbial community diversity and ultimately establish a relatively stabilized microbiota until reaching adulthood (Videvall et al., 2019). In our study, the CMT-induced effects on the beta diversity were age-dependent; that is, the differences in beta diversity were no longer present when birds mature (around 16 wk of age). Additionally, birds were exposed to the same environment and diet throughout the trial. Taken together, this may indicate that CMT has greater influences on the establishment of gut microbiota at an early age, whereas at adulthoods, host genotype, environmental factors, and genetic-environmental interactions may have a more significant impact than CMT on the microbial community structures in recipient birds. Previous studies have shown that patients with neuropsychiatric disorders including Parkinson's disease (PD) (Zhang et al., 2020) and autism spectrum disorder (Wan et al., 2021), have a higher alpha diversity as compared with healthy individuals, which may indicate the association between the diversity and disease exist. Similarly, in broilers, *Eimeria* inoculation

(a model for coccidiosis) induces an increased cecal microbial richness and overall diversity than healthy control birds (Zhou et al., 2020). In line with this finding, we found that 7_2-CMT birds (received cecal content from 7_2 donors with susceptibility to Marek's disease) had a higher phylogenetic diversity as compared with 6_3-CMT, which may be associated with the susceptible properties to Marek's disease of donor 7_2. However, the underlying mechanisms regarding the diversity-disease relationship remain unclear.

It has been suggested that genus *Akkermansia* facilitated the degradation of the mucin layer and increased intestinal permeability, which may be associated with the development of neurodegenerative diseases such as Parkinson's disease (Nishiwaki et al., 2020). McGaughey et al. (2019) reported that relative abundances of *Akkermansia* spp. were positively correlated with behavioral metrics of both anxiety and depression. Additionally, previous studies found a positive correlation between the abundances of an *Akkermansia* spp. and tyrosine levels in cecal contents of layer-type chickens (Redweik et al., 2020). Increased tyrosine may influence levels of NE and affects brain physiology and behavior. Likely, the enriched genus *Akkermansia* in 7_2-CMT birds may play similar roles in regulating aggression at an early age. Furthermore, the enriched genera *Ruminococcaceae UCG-005* and *Butyricicoccus* in 6_3-CMT birds were reported to function as short chain fatty acids (SCFAs) producers, by which it may indirectly regulate the synthesis of neurotransmitters in the host (Li et al., 2021). For instance, Reigstad et al. (2015) reported that SCFAs could influence the levels of 5-HT and tryptophan in the colon. Taken together, it may suggest that gut microbiota may functionally regulate mental health by influencing the function of the 5-HTergic system via the gut-microbiota-brain axis. At wk 16, 7_2-CMT birds had enriched genera, *Bacillus*, *Escherichia/Shigella*, and *Bacteroides* as compared with 6_3-CMT birds. These genera are recognized as opportunistic pathogens producing exotoxins and accelerating inflammation in the host (Jiang et al., 2018). In chickens, enriched *Escherichia/Shigella* may disrupt the colonization of resident bacteria and further increase the susceptibility to other pathogens (Zhou et al., 2020). Exposure to pathogenic bacteria in the gut induces behavioral

problems in humans including depression-like behavior (Sun et al., 2019b). Goehler et al. (2008) also found that patients with cognitive issues exhibited overgrowth of *Escherichia/Shigella* in the gut. Overall, these findings imply that the potential link between the gut microbiota and abnormal behaviors such as aggression. It would be of interest for future studies to explore the association between these bacterial taxa and gut metabolomic profiling to identify the mechanism behind the behavioral changes.

Neurotransmitters are active messengers that promote communication between neurons and various organs and tissues, directly and indirectly regulating host behavior. In recent years, accumulating evidence supports the theory that gut microbiota plays an essential role in modulating brain function via the gut-brain axis by affecting the synthesis and release of various neurotransmitters (Strandwitz, 2018). Compared with control mice, decreased level of 5-HT has been found in the blood and colon in GF mice (Vincent et al., 2018). Interestingly, such changes can be recovered by administering a consortium of spore-forming species (Wikoff et al., 2009). Similarly, gut bacteria present a similar function in modulating neurotransmission in the current study. 6_3-CMT birds had significantly higher concentrations of hypothalamic 5-HT among treatments at wk 5. Previous studies have reported that the substantial role of central 5-HT in regulating aggression in animal and human trials (Rillich and Stevenson, 2018; Kästner, 2019). In rodents, killing behavior could be blocked by experimental elevating 5-HT in the brain, including the lateral hypothalamus (Tani et al., 1987; Nikulina, 1991). In addition, blocking 5-HT transporters has been used as an efficient treatment for patients with social impulsivity or aggression (Pierz and Thase, 2014). Dennis et al. (2018) also reported that excessive embryonic serotonin modulates aggressive and fearful behaviors during sexual maturity in chickens. Hence, the elevated 5-HT levels were in line with the behavioral results received from the home cages and paired test: 6_3-CMT birds had higher hypothalamic 5-HT concentrations and exhibited less aggression as compared with 7_2-CMT birds. Moreover, 5-HIAA, as the metabolite of 5-HT, is thought to be negatively correlated with aggression (Mehlman et al., 1994). Interestingly, 6_3-CMT

birds exhibited higher levels of 5-HIAA among treatment groups, which may indicate higher serotonergic activities. As discussed before, the SCFA-producer genus *Ruminococcaceae UCG-005* may indirectly enhance the 5-HT levels by influencing the tryptophan levels in the gut (Russo et al., 2019). Interestingly, we found a positive correlation between genus *Ruminococcaceae UCG-005* and 5-HT levels in 6_3-CMT birds. Furthermore, genus *GCA-900066225* was found to be negatively correlated with the concentrations of 5-HIAA in 7_2-CMT birds. Collectively, this may suggest that gut bacteria regulate aggressive behavior in recipient birds by enhancing hypothalamic serotonergic neurotransmission.

Given the chief role of serotonergic activities in aggression, CMT-induced expressions of several key genes involved in the synthesis and metabolism of 5-HT were further examined in the study. Generally, the activity of the 5-HTergic system is regulated by the enzyme tryptophan hydroxylase (TPH), a rate-limiting enzyme of 5-HT synthesis, and monoamine oxidase (MAO), an enzyme that catalyzes monoamines including 5-HT (Kriegebaum et al., 2010). Though the enzyme TPH presents in 2 isoforms including TPH1 and TPH2, the biosynthesis of brain 5-HT is primarily controlled by TPH2 (Israelyan and Margolis, 2019). It has been previously reported that TPH2 deficient mice have exaggerated aggression (Mosienko et al., 2012). However, we did not observe the CMT-induced changes in the mRNA abundance of TPH2 in our study. This may suggest the identified differential bacterial taxa may not affect the TPH2 levels in recipient birds. Subsequent studies have suggested that the catalytic activity of monoamine oxidase A (MAOA) in the brain may serve as a biomarker for assessing aggression (Hemmings et al., 2018). In a clinical study, individuals with antisocial personality disorders have lower levels of brain MAOA compared with controls (Kolla et al., 2015). Similarly, elevated levels of intermale aggression were presented in MAOA knockout mice, which further suggest the inversive correlation between the MAOA levels and aggression (Tricklebank and Petrinovic, 2019). In supporting the hypothesis, 6_3-CMT birds had a significantly higher MAOA gene expression at wk 16, which may correlate with a tendency of less aggression. In general, the up-regulation of MAOA is associated with

enhanced 5-HT turnover, pointing to the lower aggressive behavior (Ahmed et al., 2014; Roy and Linnoila, 1988). Interestingly, we found that 6_3-CMT tended to have higher concentrations of tryptophan and serotonin turnover at the same time point. Collectively, this may indicate gut microbiota may regulate aggressive behavior via alterations in the 5-HTergic system metabolism.

The substantial role of catecholamines in responding to various environmental stimuli and influencing behavioral patterns has long been appreciated (Antelman and Caggiula, 1977; Saboory et al., 2020). Catecholamines are a combination of stress-related hormones and neurotransmitters including NE, EP, and DA, which have multiple functions including fear response in animals (Dennis, 2016). Like 5-HT, catecholamines cannot penetrate the BBB under normal circumstances (Schwabe et al., 2012). However, as essential amino acid and a precursor of NE, tyrosine could be transported to brain neurons and synthesize catecholamines to influence brain function. In the current study, 7_2-CMT birds with more aggressive behavior had the lowest hypothalamic NE and DA at wk 5 but not at wk 16. The lower NE may indicate reduced activation of the noradrenergic system (Mizrahi et al., 2012). Therefore, in 7_2-CMT birds, the decreased levels of NE at wk 5 may be attributed to the negative feedback of the noradrenergic system when birds exhibit aggression. Similar to what was seen in 7_2-CMT birds, an early study in rodents also showed that stress-induced increases in NE could be attenuated by the exhibition of aggression (Tsuda, 1988). Furthermore, the altered dopaminergic transmission was observed among recipient birds, evidenced by the lowest concentrations of hypothalamic DA in 7_2-CMT birds at wk 5. Dopamine, as a precursor of NE, was negatively correlated with aggression in the midbrain (Leonard, 2001). As such, the reduced concentrations of DA in 7_2-CMT birds further supported our hypothesis that gut microbiota may work together with the catecholaminergic system to modulate aggression in recipient birds.

3.6 Conclusion

Our results indicate that CMT at an early age could induce donor-dependent alterations of the gut microbial composition and diversity and related changes in the neuroendocrine system, including the activities of serotonergic and catecholaminergic systems in recipient birds. The results provide a possible mechanism underlying aggressive behavior in poultry through the interactions between the gut microbiota and brain neurotransmitters. The outcomes indicate that early postnatal CMT has the potential to be a management strategy for reducing aggressive behavior in egg-laying strain chickens.

3.7 References

Ahmed, A. A., Ma, W., Ni, Y., Zhou, Q., and Zhao, R. (2014). Embryonic exposure to corticosterone modifies aggressive behavior through alterations of the hypothalamic pituitary adrenal axis and the serotonergic system in the chicken. *Hormones and Behavior*, *65*(2), 97-105.

Antelman, S. M., and Caggiula, A. R. (1977). Norepinephrine-dopamine interactions and behavior. *Science*, *195*(4279), 646-653.

Bacon, L. D., Hunt, H. D., and Cheng, H. H. (2001). Genetic resistance to Marek's disease. *Marek's Disease*, 121-141.

Berman, M. E., Tracy, J. I., and Coccaro, E. F. (1997). The serotonin hypothesis of aggression revisited. *Clinical Psychology Review*, *17*(6), 651-665.

Buss, D. M., and Duntley, J. D. (2006). The evolution of aggression. *Evolution and social psychology*, 263-286. Psychology Press, New York.

Cannon, W. B. (1909). The influence of emotional states on the functions of the alimentary canal. *The American Journal of the Medical Sciences*, *137*, 480-487.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M. and Gormley, N. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*, *6*(8), 1621-1624.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N. and Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, *108*(S1), 4516-4522.

Cheng, H. W., and Muir, W. M. (2007). Mechanisms of aggression and production in chickens: genetic variations in the functions of serotonin, catecholamine, and corticosterone. *World's Poultry Science Journal*, *63*(2), 233-254.

Cryan, J. F., and Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience*, *13*(10), 701-712.

Daigle, C. L., Rodenburg, T. B., Bolhuis, J. E., Swanson, J. C., and Siegford, J. M. (2014). Use of dynamic and rewarding environmental enrichment to alleviate feather pecking in non-cage laying hens. *Applied Animal Behaviour Science*, *161*, 75-85.

Dennis, R. L. (2016). Adrenergic and noradrenergic regulation of poultry behavior and production. *Domestic Animal Endocrinology*, *56*, S94-S100.

Dennis, R. L., and Cheng, H. W. (2011). The dopaminergic system and aggression in laying hens. *Poultry Science*, *90*(11), 2440-2448.

Dennis, R. L., Chen, Z. Q., and Cheng, H. W. (2008). Serotonergic mediation of aggression in high and low aggressive chicken strains. *Poultry Science*, *87*(4), 612-620.

Dennis, R. L., Fahey, A. G., and Cheng, H. W. (2013). Alterations to embryonic serotonin change aggression and fearfulness. *Aggressive behavior*, *39*(2), 91-98.

Drake, K. A., Donnelly, C. A., and Dawkins, M. S. (2010). Influence of rearing and lay risk factors on propensity for feather damage in laying hens. *British Poultry Science*, *51*(6), 725-733.

Gao, J., Xu, K., Liu, H., Liu, G., Bai, M., Peng, C., Li, T. and Yin, Y. (2018). Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. *Frontiers in Cellular and Infection Microbiology*, *8*, 13.

Gershon, M. D. (2003). Serotonin and its implication for the management of irritable bowel syndrome. *Reviews in Gastroenterological Disorders*, *3*, S25-S34.

Glendinning, L., Stewart, R. D., Pallen, M. J., Watson, K. A., and Watson, M. (2020). Assembly of hundreds of novel bacterial genomes from the chicken caecum. *Genome Biology*, *21*(1), 1-16.

Goehler, L. E., Park, S. M., Opitz, N., Lyte, M., and Gaykema, R. P. (2008). Campylobacter jejuni infection increases anxiety-like behavior in the holeboard: possible anatomical substrates for viscerosensory modulation of exploratory behavior. *Brain, Behavior, and Immunity*, *22*(3), 354-366.

Goodman, A. L., Kallstrom, G., Faith, J. J., Reyes, A., Moore, A., Dantas, G., and Gordon, J. I. (2011). Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proceedings of the National Academy of Sciences*, *108*(15), 6252-6257.

Hand, T. W., Vujkovic-Cvijin, I., Ridaura, V. K., and Belkaid, Y. (2016). Linking the microbiota, chronic disease, and the immune system. *Trends in Endocrinology and Metabolism*, *27*(12), 831-843.

Hartcher, K. M., Tran, K. T. N., Wilkinson, S. J., Hemsworth, P. H., Thomson, P. C., and Cronin, G. M. (2015). The effects of environmental enrichment and beak-trimming during the rearing period on subsequent feather damage due to feather-pecking in laying hens. *Poultry Science*, *94*(5), 852-859.

Hemmings, S.M.J., Xulu, K., Sommer, J., Hinsberger, M., Malan-Muller, S., Tromp, G., Elbert, T., Weierstall, R. and Seedat, S. (2018). Appetitive and reactive aggression are differentially associated with the STin2 genetic variant in the serotonin transporter gene. *Scientific Reports*, *8*(1), 1-9.

Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., Codelli, J.A., Chow, J., Reisman, S.E., Petrosino, J.F. and Patterson, P.H., (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell*, *155*(7), 1451-1463.

Hubbard, A. J., Foster, M. J., and Daigle, C. L. (2021). Impact of social mixing on beef and dairy cattle—a scoping review. *Applied Animal Behavior Science*, *241*, 105389.

Israelyan, N., and Margolis, K. G. (2019). Reprint of: Serotonin as a link between the gut-brain-microbiome axis in autism spectrum disorders. *Pharmacological Research*, *140*, 115-120.

Jiang, H. Y., Zhang, X., Yu, Z. H., Zhang, Z., Deng, M., Zhao, J. H., and Ruan, B. (2018). Altered gut microbiota profile in patients with generalized anxiety disorder. *Journal of Psychiatric Research*, *104*, 130-136.

Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z., Yin, Y., Wang, W., Tang, W., Tan, Z., Shi, J. and Li, L. (2015). Altered fecal microbiota composition in patients with major depressive disorder. *Brain, Behavior, and Immunity*, *48*, 186-194.

Kang, D.W., Adams, J.B., Coleman, D.M., Pollard, E.L., Maldonado, J., McDonough-Means, S., Caporaso, J.G. and Krajmalnik-Brown, R., (2019). Long-term benefit of Microbiota Transfer Therapy on autism symptoms and gut microbiota. *Scientific Reports*, *9*(1), 1-9.

Kästner, N., Richter, S.H., Urbanik, S., Kunert, J., Waider, J., Lesch, K.P., Kaiser, S. and Sachser, N. (2019). Brain serotonin deficiency affects female aggression. *Scientific Reports*, *9*(1), 1-9.

Klasen, M., Wolf, D., Eisner, P.D., Eggermann, T., Zerres, K., Zepf, F.D., Weber, R. and Mathiak, K., (2019). Serotonergic contributions to human brain aggression networks. *Frontiers in Neuroscience*, *13*, 42.

Kolla, N. J., and Houle, S. (2019). Single-photon emission computed tomography and positron emission tomography studies of antisocial personality disorder and aggression: a targeted review. *Current Psychiatry Reports*, *21*(4), 1-11.

Kolla, N.J., Matthews, B., Wilson, A.A., Houle, S., Bagby, R.M., Links, P., Simpson, A.I., Hussain, A. and Meyer, J.H. (2015). Lower monoamine oxidase-a total distribution volume in impulsive and violent male offenders with antisocial personality disorder and high psychopathic traits: An [11 C] Harmine positron emission tomography study. *Neuropsychopharmacology*, *40*(11), 2596-2603.

Kriegebaum, C., Song, N.N., Gutknecht, L., Huang, Y., Schmitt, A., Reif, A., Ding, Y.Q. and Lesch, K.P. (2010). Brain-specific conditional and time-specific inducible Tph2 knockout mice possess normal serotonergic gene expression in the absence of serotonin during adult life. *Neurochemistry International*, *57*(5), 512-517.

Lagoda, M. E., O'Driscoll, K., Marchewka, J., Foister, S., Turner, S. P., and Boyle, L. A. (2021). Associations between skin lesion counts, hair cortisol concentrations and reproductive performance in group housed sows. *Livestock Science*, *246*, 104463.

Leonard, B. E. (2001). Stress, norepinephrine and depression. *Journal of Psychiatry and Neuroscience*, *26*, S11.

Li, Q., Hu, J., Nie, Q., Chang, X., Fang, Q., Xie, J., Li, H. and Nie, S. (2021). Hypoglycemic mechanism of polysaccharide from Cyclocarya paliurus leaves in type 2 diabetic rats by gut microbiota and host metabolism alteration. *Science China Life Sciences*, *64*(1), 117-132.

McGaughey, K.D., Yilmaz-Swenson, T., Elsayed, N.M., Cruz, D.A., Rodriguiz, R.M., Kritzer, M.D., Peterchev, A.V., Roach, J., Wetsel, W.C. and Williamson, D.E. (2019). Relative abundance of Akkermansia spp. and other bacterial phylotypes correlates with anxiety-and depressive-like behavior following social defeat in mice. *Scientific Reports*, *9*(1), 1-11.

Mehlman, P.T., Higley, J.D., Faucher, I., Lilly, A.A., Taub, D.M., Vickers, J., Suomi, S.J. and Linnoila, M. (1994). Low CSF 5-HIAA concentrations and severe aggression and impaired impulse control in nonhuman primates. *The American Journal of Psychiatry*, *151*, 1485-1491.

Mizrahi, R., Addington, J., Rusjan, P.M., Suridjan, I., Ng, A., Boileau, I., Pruessner, J.C., Remington, G., Houle, S. and Wilson, A.A. (2012). Increased stress-induced dopamine release in psychosis. *Biological Psychiatry*, *71*(6), 561-567.

Mosienko, V., Bert, B., Beis, D., Matthes, S., Fink, H., Bader, M., and Alenina, N. (2012). Exaggerated aggression and decreased anxiety in mice deficient in brain serotonin. *Translational psychiatry*, *2*(5), e122-e122.

Nikulina, E. M. (1991). Neural control of predatory aggression in wild and domesticated animals. *Neuroscience and Biobehavioral Reviews*, *15*(4), 545-547.

Nishiwaki, H., Hamaguchi, T., Ito, M., Ishida, T., Maeda, T., Kashihara, K., Tsuboi, Y., Ueyama, J., Shimamura, T., Mori, H. and Kurokawa, K. (2020). Short-Chain Fatty Acid-Producing Gut Microbiota Is Decreased in Parkinson's Disease but Not in Rapid-Eye-Movement Sleep Behavior Disorder. *Msystems*, *5*(6), e00797-20.

Pierz, K. A., and Thase, M. E. (2014). A review of vilazodone, serotonin, and major depressive disorder. *The Primary Care Companion for CNS Disorders*, *16*(1), PCC.13r01554.

Pourhamzeh, M., Moravej, F.G., Arabi, M., Shahriari, E., Mehrabi, S., Ward, R., Ahadi, R. and Joghataei, M.T., (2021). The Roles of Serotonin in Neuropsychiatric Disorders. *Cellular and Molecular Neurobiology*, 1-22.

Raine, A. (2002). Biosocial studies of antisocial and violent behavior in children and adults: A review. *Journal of Abnormal Child Psychology*, *30*(4), 311-326.

Redweik, G. A., Daniels, K., Severin, A. J., Lyte, M., and Mellata, M. (2020). Oral treatments with probiotics and live Salmonella vaccine induce unique changes in gut neurochemicals and microbiome in chickens. *Frontiers in microbiology*, *10*, 3064.

Reigstad, C.S., Salmonson, C.E., III, J.F.R., Szurszewski, J.H., Linden, D.R., Sonnenburg, J.L., Farrugia, G. and Kashyap, P.C. (2015). Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells. *The FASEB Journal*, *29*(4), 1395-1403.

Rillich, J., and Stevenson, P. A. (2018). Serotonin mediates depression of aggression after acute and chronic social defeat stress in a model insect. *Frontiers in Behavioral Neuroscience*, *12*, 233.

Roy, A., and Linnoila, M. (1988). Suicidal behavior, impulsiveness and serotonin. *Acta Psychiatrica Scandinavica*, *78*(5), 529-535.

Russo, E., Giudici, F., Fiorindi, C., Ficari, F., Scaringi, S., and Amedei, A. (2019). Immunomodulating activity and therapeutic effects of short chain fatty acids and tryptophan post-biotics in inflammatory bowel disease. *Frontiers in Immunology*, *10*, 2754.

Rychlik, I. (2020). Composition and function of chicken gut microbiota. *Animals*, *10*(1), 103.

Saboory, E., Ghasemi, M., and Mehranfard, N. (2020). Norepinephrine, neurodevelopment and behavior. *Neurochemistry International*, *135*, 104706.

Sandyk, R. (1992). L-tryptophan in neuropsychiatry disorders: a review. *International Journal of Neuroscience*, *67*(1-4), 127-144.

Schwabe, L., Joëls, M., Roozendaal, B., Wolf, O. T., and Oitzl, M. S. (2012). Stress effects on memory: an update and integration. *Neuroscience and Biobehavioral Reviews*, *36*(7), 1740-1749.

Settanni, C. R., Ianiro, G., Bibbò, S., Cammarota, G., and Gasbarrini, A. (2021). Gut microbiota alteration and modulation in psychiatric disorders: Current evidence on fecal microbiota transplantation. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *109*, 110258.

Slawinska, A., Plowiec, A., Siwek, M., Jaroszewski, M., and Bednarczyk, M. (2016). Long-term transcriptomic effects of prebiotics and synbiotics delivered in ovo in broiler chickens. *PloS One*, *11*(12), e0168899.

Steel, R. G. D., J. H. Torrie, and D. J. Dickey. (1997). Principles and procedures of statistics: A biometrical approach. 3rd ed. McGraw Hill Book Co., New York, NY.

Strandwitz, P. (2018). Neurotransmitter modulation by the gut microbiota. *Brain Research*, *1693*, 128-133.

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.N., Kubo, C. and Koga, Y. (2004). Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *The Journal of Physiology*, *558*(1), 263-275.

Sun, J., Xu, J., Ling, Y., Wang, F., Gong, T., Yang, C., Ye, S., Ye, K., Wei, D., Song, Z. and Chen, D., (2019a). Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. *Translational Psychiatry*, *9*(1), 1-13.

Sun, L., Zhang, H., Cao, Y., Wang, C., Zhao, C., Wang, H., Cui, G., Wang, M., Pan, Y., Shi, Y. and Nie, Y. (2019b). Fluoxetine ameliorates dysbiosis in a depression model induced by chronic unpredicted mild stress in mice. *International Journal of Medical Sciences*, *16*(9), 1260.

Tani, Y., Kataoka, Y., Sakurai, Y., Yamashita, K., Ushio, M., and Ueki, S. (1987). Changes of brain monoamine contents in three models of experimentally induced muricide in rats. *Pharmacology Biochemistry and Behavior*, *26*(4), 725-729.

Tricklebank, M. D., and Petrinovic, M. M. (2019). Serotonin and aggression. In *The Serotonin System*, 155-180. Academic Press, London.

Tsuda, A., Tanaka, M., Ida, Y., Shirao, I., Gondoh, Y., Oguchi, M., and Yoshida, M. (1988). Expression of aggression attenuates stress-induced increases in rat brain noradrenaline turnover. *Brain Research*, *474*(1), 174-180.

Turner, S.P., Farnworth, M.J., White, I.M., Brotherstone, S., Mendl, M., Knap, P., Penny, P. and Lawrence, A.B., (2006). The accumulation of skin lesions and their use as a predictor of individual aggressiveness in pigs. *Applied Animal Behaviour Science*, *96*(3-4), 245-259.

Videvall, E., Song, S.J., Bensch, H.M., Strandh, M., Engelbrecht, A., Serfontein, N., Hellgren, O., Olivier, A., Cloete, S., Knight, R. and Cornwallis, C.K., (2019). Major shifts in gut microbiota during development and its relationship to growth in ostriches. *Molecular Ecology*, *28*(10), 2653-2667.

Vincent, A. D., Wang, X. Y., Parsons, S. P., Khan, W. I., and Huizinga, J. D. (2018). Abnormal absorptive colonic motor activity in germ-free mice is rectified by butyrate, an effect possibly mediated by mucosal serotonin. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *315*(5), G896-G907.

Wall, R., Ross, R. P., Ryan, C. A., Hussey, S., Murphy, B., Fitzgerald, G. F., and Stanton, C. (2009). Role of gut microbiota in early infant development. *Clinical medicine: Pediatrics*, *3*, 45-54.

Wan, Y., Zuo, T., Xu, Z., Zhang, F., Zhan, H., Dorothy, C.H.A.N., Leung, T.F., Yeoh, Y.K., Chan, F.K., Chan, R. and Ng, S.C., (2021). Underdevelopment of the gut microbiota and bacteria species as non-invasive markers of prediction in children with autism spectrum disorder. *Gut*.

Weinberg-Wolf, H., and Chang, S. W. (2019). Differences in how macaques monitor others: Does serotonin play a central role?. *Wiley Interdisciplinary Reviews: Cognitive Science*, *10*(4), e1494.

Wikoff, W. R., Anfora, A. T., Liu, J., Schultz, P. G., Lesley, S. A., Peters, E. C., and Siuzdak, G. (2009). Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proceedings of the National Academy of Sciences*, *106*(10), 3698-3703.

Wolf, S., and Wolff, H. G. (1943). Human gastric function: An experimental study of a man and his stomach. Oxford University Press.

Yan, F. F., Wang, W. C., and Cheng, H. W. (2020). Bacillus subtilis–based probiotic promotes bone growth by inhibition of inflammation in broilers subjected to cyclic heating episodes. *Poultry Science*, *99*(11), 5252-5260.

Yan, W., Sun, C., Yuan, J., and Yang, N. (2017). Gut metagenomic analysis reveals prominent roles of Lactobacillus and cecal microbiota in chicken feed efficiency. *Scientific Reports*, *7*(1), 1-11.

Yu, M., Jia, H., Zhou, C., Yang, Y., Zhao, Y., Yang, M., and Zou, Z. (2017). Variations in gut microbiota and fecal metabolic phenotype associated with depression by 16S rRNA gene sequencing and LC/MS-based metabolomics. *Journal of Pharmaceutical and Biomedical Analysis*, *138*, 231-239.

Zhang, F., Yue, L., Fang, X., Wang, G., Li, C., Sun, X., Jia, X., Yang, J., Song, J., Zhang, Y. and Guo, C., (2020). Altered gut microbiota in Parkinson's disease patients/healthy spouses and its association with clinical features. *Parkinsonism and Related Disorders*, *81*, 84-88.

Zheng, P., Wu, J., Zhang, H., Perry, S.W., Yin, B., Tan, X., Chai, T., Liang, W., Huang, Y., Li, Y. and Duan, J. (2021). The gut microbiome modulates gut–brain axis glycerophospholipid metabolism in a region-specific manner in a nonhuman primate model of depression. *Molecular Psychiatry*, *26*(6), 2380-2392.

Zheng, P., Zeng, B., Zhou, C., Liu, M., Fang, Z., Xu, X., Zeng, L., Chen, J., Fan, S., Du, X. and Zhang, X. (2016). Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. *Molecular Psychiatry*, *21*(6), 786-796.

Zhou, B. H., Jia, L. S., Wei, S. S., Ding, H. Y., Yang, J. Y., and Wang, H. W. (2020). Effects of Eimeria tenella infection on the barrier damage and microbiota diversity of chicken cecum. *Poultry science*, *99*(3), 1297-1305.

Zhu, F., Guo, R., Wang, W., Ju, Y., Wang, Q., Ma, Q., Sun, Q., Fan, Y., Xie, Y., Yang, Z. and Jie, Z., (2020). Transplantation of microbiota from drug-free patients with schizophrenia causes schizophrenia-like abnormal behaviors and dysregulated kynurenine metabolism in mice. *Molecular Psychiatry*, *25*(11), 2905-2918.

Table 3.1 Relevant genes involved in the serotonin pathway.

Function	Genes	Assay ID
Synthesis	TPH2	Gg03345550_m1
Reuptake	5-HTT	Gg03349687_m1
Auto-receptor signaling	Htra1a, Htr1b	Gg07157052_s1, Gg07157117_s1
Metabolism	MAOA	Gg03350714_m1

Table 3.2 Effects of cecal microbiota transplantation on relative mRNA expression of genes involved in serotoninergic pathway in the hypothalamus of roosters at week 5 and 16.

Treatment	MAOA	TPH2	5-HTT	Htr1a	Htr1b
Week 5					
6_3-CMT	1.507	0.333	1.121	1.465	1.193
7_2-CMT	1.561	0.454	0.724	1.440	1.455
Control	1.449	0.395	0.917	1.500	1.358
SEM	0.116	0.054	0.165	0.113	0.129
P- value	0.796	0.307	0.259	0.932	0.373
Week 16					
6_3-CMT	2.517^{a}	0.829	1.625	0.862	2.352
7_2-CMT	1.797^{b}	0.844	1.535	0.774	2.316
Control	1.899^{ab}	1.000	2.041	0.808	2.119
SEM	0.045	0.124	0.213	0.070	0.092
P- value	**0.024**	0.449	0.227	0.671	0.183

Values are least square means ± SEM, n=7. ^{a,b} indicates significant difference ($P \leq 0.05$).

Table 3.3 PERMANOVA testing of the effects of cecal microbiota transplantation on beta diversity of donor and recipient birds.

Overall effect: Donor birds	*P* - value
Bray Curtis	**0.001**
Jaccard	**0.001**
Unweighted UniFrac	**0.011**
Weighted UniFrac	**0.014**
Recipient birds	
Overall effect of CMT at week 5	*P* - value
Bray Curtis	0.346
Jaccard	**0.020**
Unweighted UniFrac	**0.005**
Weighted UniFrac	0.530
Overall effect of CMT at week 16	*P* - value
Bray Curtis	0.533
Jaccard	0.724
Unweighted UniFrac	0.475
Weighted UniFrac	0.672

Donors: line 6_3 and 7_2, n=10; Recipients: 6_3-CMT, 7_2-CMT, CTRL, n=7.

Table 3.4 Pairwise PERMANOVA results of Unweighted_UniFrac and Jaccard metrics at week 5.

Pairwise PERMANOVA - Unweighted_UniFrac: effects of CMT at wk 5					
Group 1	Group 2	Permutations	pseudo-F	*P*-value	*q*-value
CTRL	7_2-CMT	999	1.605	0.036	**0.036**
CTRL	6_3-CMT	999	2.751	0.036	**0.036**
7_2-CMT	6_3-CMT	999	4.587	0.002	**0.006**
Pairwise PERMANOVA - Jaccard: effects of CMT at wk 5					
Group 1	Group 2	Permutations	pseudo-F	*P*-value	*q*-value
CTRL	7_2-CMT	999	1.639	0.010	**0.030**
CTRL	6_3-CMT	999	1.384	0.048	**0.072**
7_2-CMT	6_3-CMT	999	1.183	0.134	0.134

Donors: line 6_3 and 7_2, n=10; Recipients: 6_3-CMT, 7_2-CMT, CTRL, n=7.

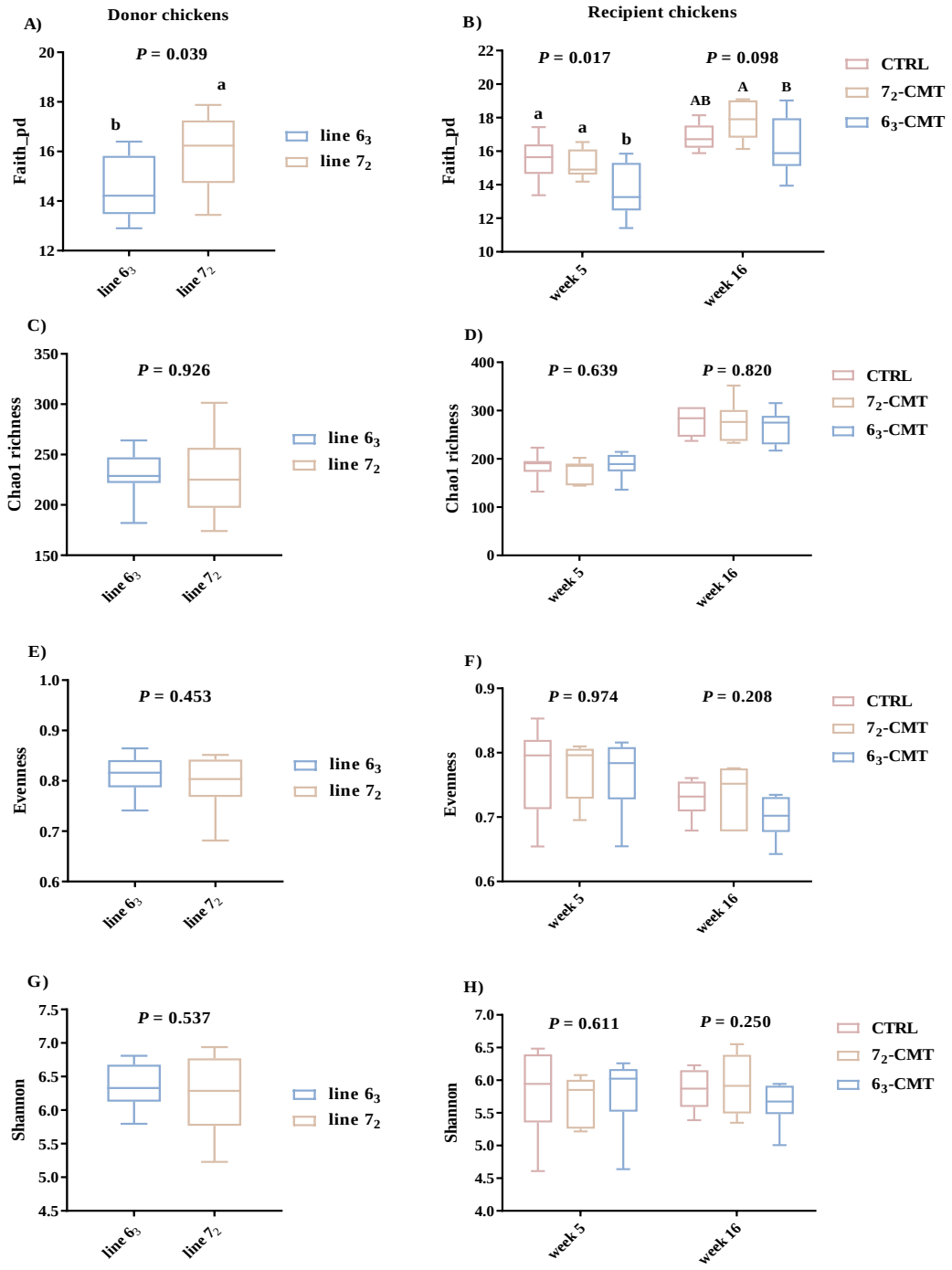

Figure 3.1 Effects of cecal microbiota transplantation on alpha-diversity (evenness, chao1 richness, faith_pd, and shannon) of the roosters at week 5 and 16.

Values are median ± SEM, n=7. [a,b] indicates significant differences ($P \leq 0.05$), [A,B] shows trend differences ($0.05 < P \leq 0.1$). Donor birds: Figures A, C, E, and G; Recipient birds: Figures B, D, F, and H.

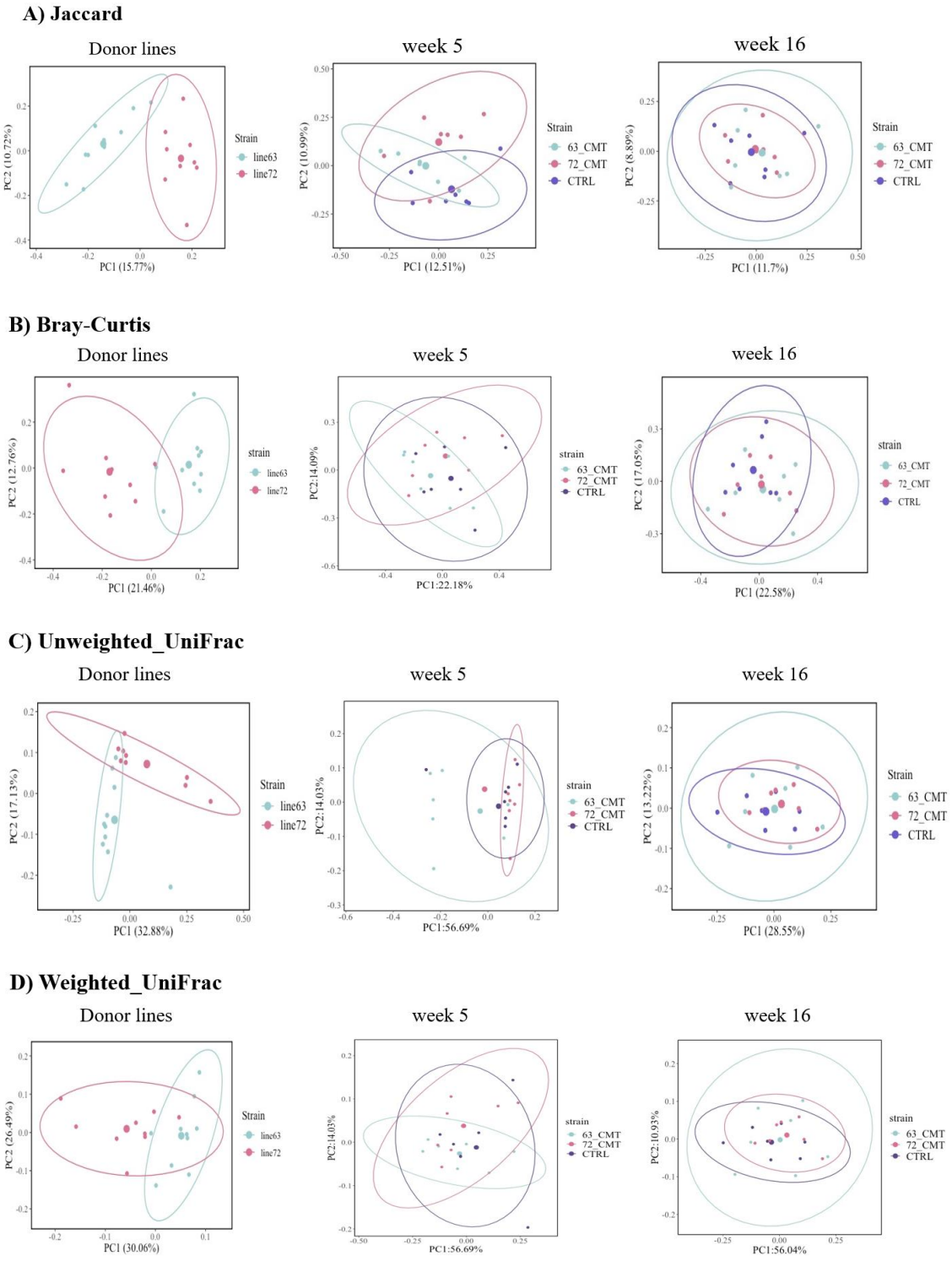

Figure 3.2 Principal coordinate analysis of beta diversity metrics (A, Jaccard; B, Bray_curtis; C, unweighted_uniFrac; D, weighted_uniFrac) of roosters at week 5 and 16 (n=7). Each dot represents one bird, PCo1 and PCo2 represent the percentage of variance explained by each coordinate.

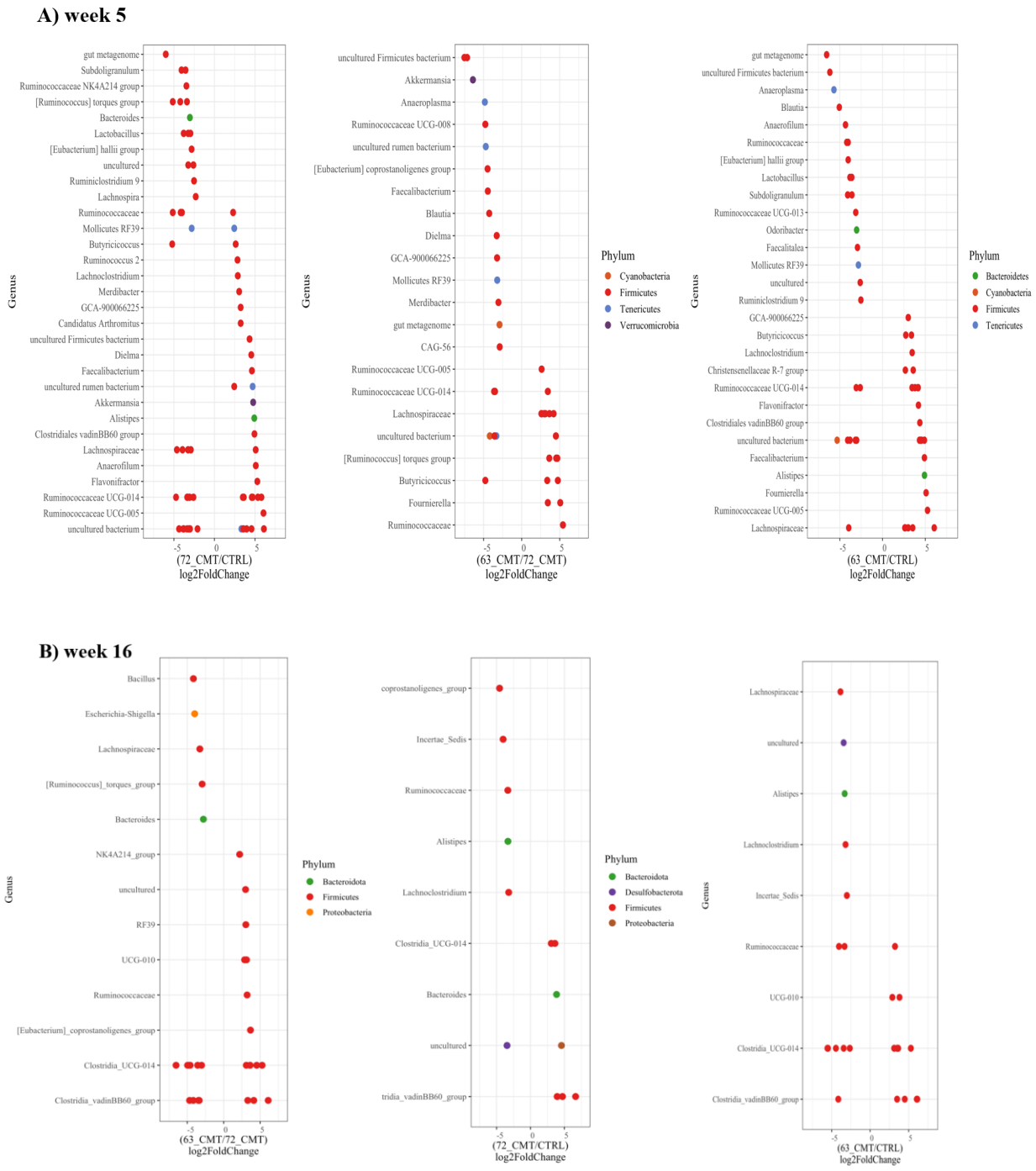

Figure 3.3 DESeq2 analysis of differentially abundant ASVs between A) 6_3-CMT group and CTRL group B) 6_3-CMT group and 7_2-CMT group c) 7_2-CMT group and CTRL group at week 5 (top) and 16 (bottom).

Estimations of log2 fold change values for each ASV were computed and each point represents an ASV that was significantly different ($P < 0.05$). Each color represents a phylum; Bacteroidetes (green), Firmicutes (red), Cyanobacteria (orange) and Verrucomicrobia (purple).

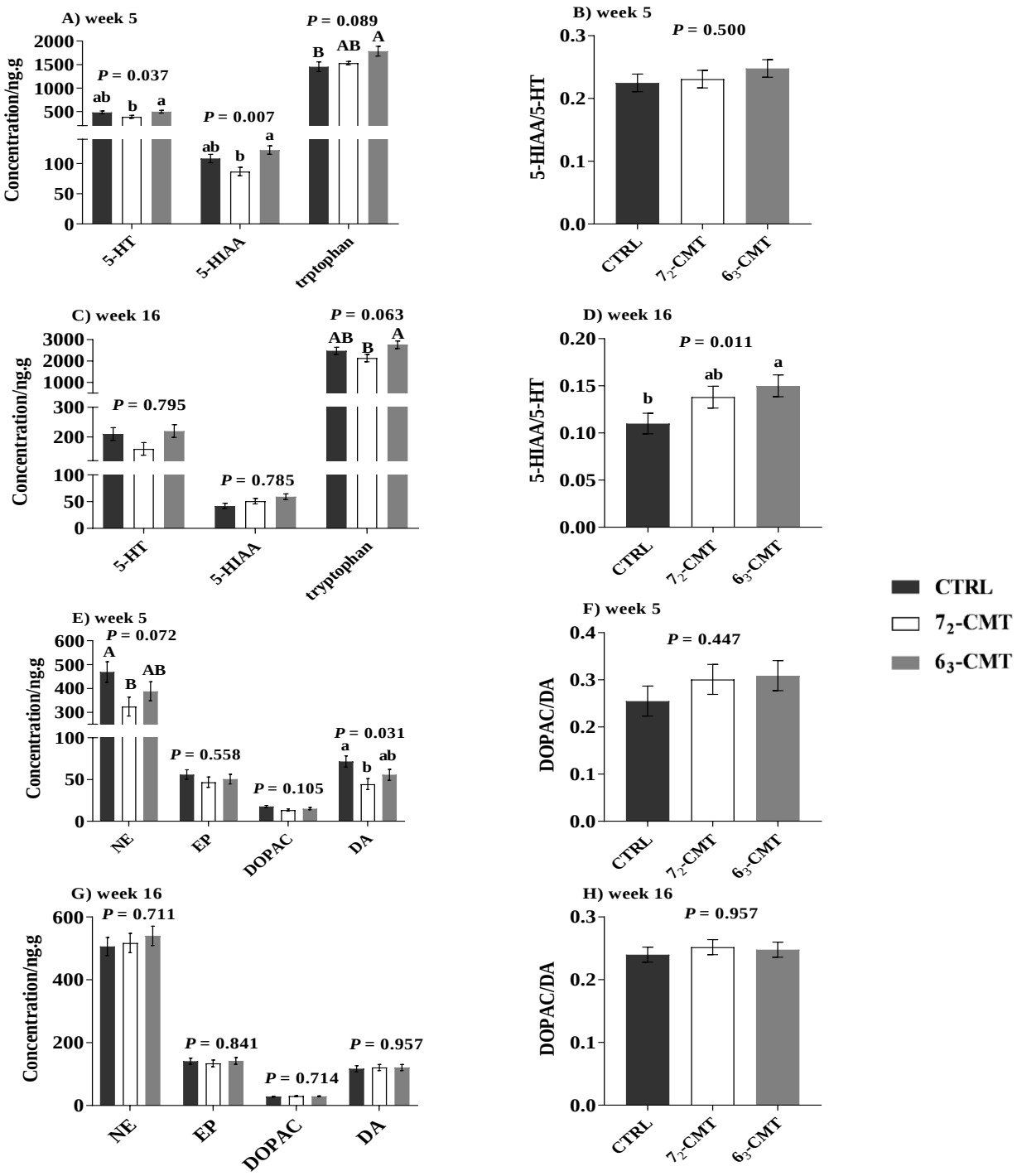

Figure 3.4 Effects of cecal microbiota transplantation on serotonergic activities in the hypothalamus of roosters at week 5 and 16.

Values are least square means ± SEM, n=7. ^{a,b} indicates significant differences ($P \leq 0.05$), and ^{A,B} shows trend differences ($0.05 < P \leq 0.1$).

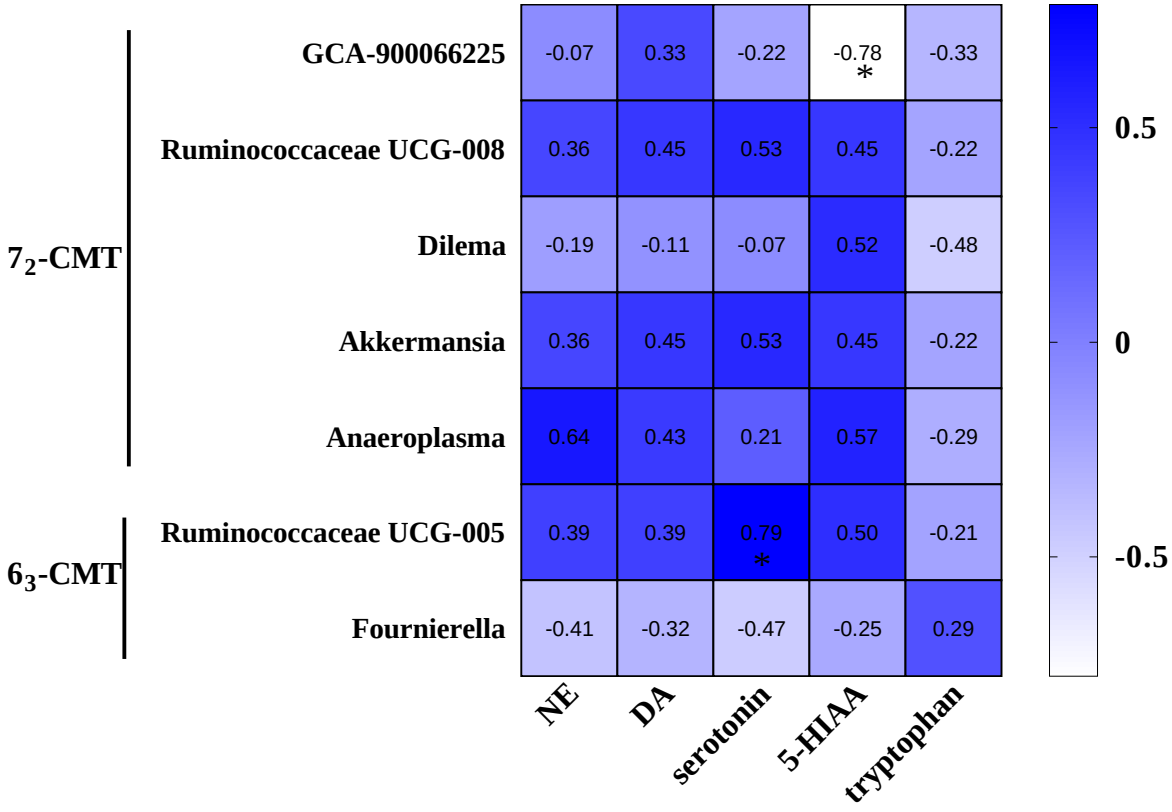

Figure 3.5 Spearman correlations of neurotransmitter (and its metabolites) concentration with bacterial taxa (ASVs) in 7_2-CMT and 6_3-CMT birds at week 5. * indicates significant differences ($P \leq 0.05$).

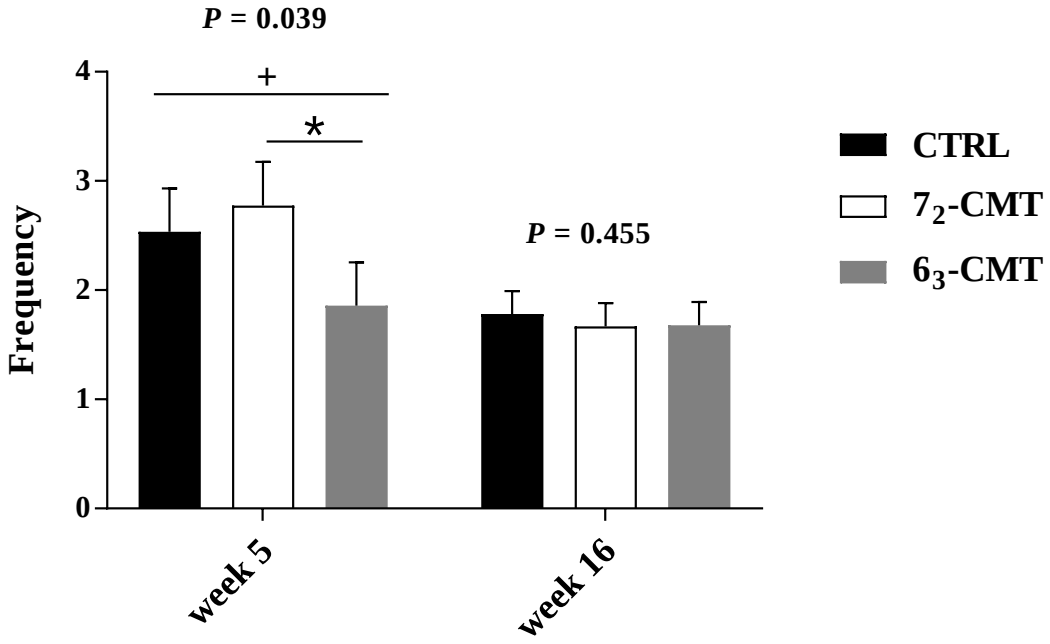

Figure 3.6 Frequency of aggressive pecking of roosters in the home cages (± SEM) at week 5 and 16.

Values are least square means ± SEM, n=5. * indicates significant differences ($P \leq 0.05$), and + shows trend differences ($0.05 < P \leq 0.1$).

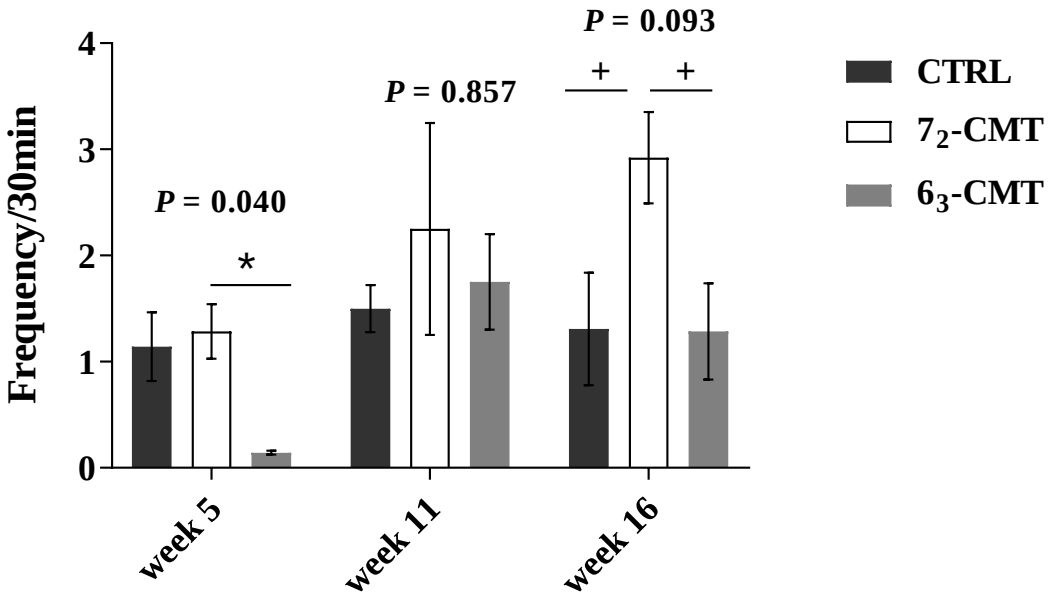

Figure 3.7 Frequency of aggressive pecking of roosters during paired tests (± SEM) at week 5, 11, and 16.

Values are means ± SEM, n=7. * indicates significant differences ($P \leq 0.05$), and + shows trend differences ($0.05 < P \leq 0.1$).

CHAPTER 4. SUMMARY

Aggression is a highly complex behavior. From an evolutionary point of view, aggression in animals is related to survival, growth, and reproduction (Buss et al., 1997). Some routine management practices used in the current poultry production systems, such as mixing and regrouping chickens, can commonly disrupt the established social structure and induce excessive social stress and aggression (Hubbard et al., 2021). On commercial poultry farms, aggression leads to feather and tissue damage, pain, and subsequently mortalities, which compromises poultry production, health, and welfare. Although several approaches such as beak trimming, genetic selection, and lighting control have been implemented, the use of these alternatives is controversial. Thus, developing an effective approach to prevent such behaviors is critical for substantial poultry production.

Emerging evidence has indicated that the maintenance of gastrointestinal homeostasis is essential for the overall health of the host. Gut microbiota modulates the nutrient resorption, immunity, neuroendocrine function, and stress capability of the host by interacting with various organs including the intestine, spleen, brain, and adrenal gland (Feng et al., 2018). Much recent research, therefore, has focused on the modulation of gut microbiota including fecal microbiota transplantation in treating patients with gastrointestinal disease or mental disorders. The aim of this project was to investigate if cecal microbiota transplantation (CMT) presents similar efficiency in improving the health and welfare status by reducing aggressive behavior in egg-laying chickens. Chicken lines 6_3 (gentle birds) and 7_2 (aggressive birds) were used as donors and a commercial strain Dekalb XL was used as recipients for CMT. Cecal microbiota transplantation was conducted through oral gavage once daily from day 1 to day 10 and then boosted once weekly from week 3 to week 5. Results indicated that CMT led to the line-correlated effects on the immune capability of the recipient birds, which was reflected by changes in the concentrations of antibodies such as IgG and production of inflammatory cytokines including IL-6, IL-10, and TNF-α at both mRNA

and protein levels. In addition, CMT led to divergent changes in the behavioral exhibition of recipients, which was accompanied by the changes in cecal microbial compositions and central serotonergic activities in recipient birds. The identified correlations between bacterial taxa and serotonin levels in recipients may provide a possible mechanism underlying aggressive behavior in poultry through communication between the gut microbiota and brain serotonergic activity. Previous studies have shown that gut microbiota can bidirectionally communicate with the central nervous system via the gut-brain axis and affect the metabolism or biosynthesis of neuroactive compounds and neurotransmitters, by which it regulates cognition, mood, and exhibition of behaviors (Lyte, 2013). Especially, serotonin, a key modulatory neurotransmitter in the central nervous system, regulates aggression in humans and various species of animals including rodents and chickens (Dennis et al., 2008; Manchia et al., 2017).

In conclusion, results from this study indicate that postnatal CMT has the potential to modify aggression in chickens through the regulation of gut microbiota composition, central serotonergic activity, peripheral immunity, and stress response via the gut-immune-brain axis. The results provide novel insights into targeting gut microbiota to prevent aggression in poultry and offer a theoretical basis for preventing abnormal behavior in other farm animals.

4.1 References

Buss, D. M., and Shackelford, T. K. (1997). Human aggression in evolutionary psychological perspective. *Clinical Psychology Review*, *17*(6), 605-619.

Dennis, R. L., Chen, Z. Q., and Cheng, H. W. (2008). Serotonergic mediation of aggression in high and low aggressive chicken strains. *Poultry Science*, *87*(4), 612-620.

Feng, Q., Chen, W. D., and Wang, Y. D. (2018). Gut microbiota: an integral moderator in health and disease. *Frontiers in Microbiology*, *9*, 151.

Hubbard, A. J., Foster, M. J., and Daigle, C. L. (2021). Impact of social mixing on beef and dairy cattle—a scoping review. *Applied Animal Behaviour Science*, 105389.

Lyte, M. (2013). Microbial endocrinology in the microbiome-gut-brain axis: how bacterial production and utilization of neurochemicals influence behavior. *PLoS Pathogens, 9*(11), e1003726.

Manchia, M., Carpiniello, B., Valtorta, F., and Comai, S. (2017). Serotonin dysfunction, aggressive behavior, and mental illness: exploring the link using a dimensional approach. *ACS Chemical Neuroscience, 8*(5), 961-972.