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Effects of Anti-Coccidial Vaccination and Exogenous Enzymes on Chicken Growth Performance and Gut Health

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Declaration

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Date: 9th April 2020

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Publications

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Abstract

Gut health is the relationship between the host, gut microbes and diet. Therefore, comprehensive analyses of the host immune system, microbiota and diets in the same setting are required in order to study gut health. Correlations between the host immune system and the gut microbiota could provide a better understanding of the relationship between host and microbes. Knowledge of the relationships between the intestinal immune system and microbiota could help producers improve poultry health and nutrient utilisation. Application of this knowledge could result in better poultry growth performance, animal welfare and reduced environmental impact.

Two by two factorial arrangement study was conducted using Ross 308 broiler chickens. Anti-coccidial vaccination against *Eimeria* protozoa was used as a factor to create gut-damaged chickens. Combination of protease, xylanase and beta-glucanase enzymes was used as a dietary intervention factor. Chicken growth performance was significantly affected by the vaccination while enzymes supplement significantly improved the feed conversion ratio. Although coccidiosis lesions were observed in non-vaccinated chickens at 24-day-old, the feed efficiency of non-vaccinated chickens still outperformed the vaccinated chickens at this period. While at 35 days of age, vaccinated chickens showed compensatory growth with significantly better growth performance than non-vaccinated chickens. Therefore, samples for correlation analysis were selected from 24-day-old rather than 35-day-old chickens.

Using 16S rRNA metabarcoding analysis, the abundances of eleven bacterial genera at the ileum of 24-day-old chicken were significantly affected by the vaccination where two bacterial genera were significantly affected in the caecum. Although the enzymes supplement did not significantly affect the microbiota population at both ileum and caecum from 16S rRNA metabarcoding analysis, functional analysis using shotgun metagenomics and Carbohydrate Active enzymes database (CAzy) showed significant alteration by the enzymes supplement. From transcriptomic analysis, vaccination significantly affected the expression of 96 and 46 genes at the ileum and caecum respectively. In contrast, 4 and 3 genes were differentially expressed between the enzymes and non-enzymes supplement chicken at the ileum and caecum respectively. At both ileum and caecum, several immune-related genes were up-regulated while multiple absorption-related genes were down-regulated in vaccinated chicken. Interestingly, with more differentially expressed genes at the ileum than the caecum, no Gene Ontology (GO) term was significantly enriched at the ileum when compared between vaccinated and non-vaccinated chicken. Correlation between gut microbiota and gene expression using Weighted Gene Co-expression Network Analysis (WGCNA) showed multiple significant correlations between gene clusters and bacterial taxa. Using differentially expressed gene results from the RNA-seq analysis, 84 and 191 significant correlations were identified at ileum and caecum respectively.

In conclusion, anti-coccidial vaccination had a significant impact on chicken growth performance, gut microbiota and intestinal immune response. Alteration of the nutrition using exogenous enzymes improved feed efficiency with minor impact on the gut microbiota and host response. Correlation between gut microbiota and intestinal gene expression were identified. These bacteria and genes could be used as potential markers for gut health however, further investigation using these findings is required.

Lay summary

To increase the production of poultry and meet the huge demand from rising global population, health and nutrition play an important role. Disease resistance should improve animal welfare, decrease risk of potential disease transmitted from animal to human and decrease impact from drugs and chemicals used in disease prevention or treatment. Improvement of feed utilisation could lessen the environmental impact from nutrient waste including land and chemical use from crop production. Better understanding of gut health or the relationship between intestinal immune system and bacteria could help poultry producer improve the poultry industry.

A two by two factorial study was conducted using commercial broiler chickens. First factor, anti-coccidial vaccination usually used in the prevention of coccidia protozoan disease was used to alter the immune system of the chicken. Growth parameters of vaccinated or gut damged chickens were compared with non-vaccinated or control chickens. Second factor, exogenous enzymes commonly used to extract more nutrient from undigested sources such as fibre was given to create the variation in nutrient of the chicken diet. Non-enzymes supplementated chickens and enzymes supplemented chickens were compared in order to study the effects of nutrients on growth performance. Intestinal bacteria and response at the gene level were studied from chickens given these factors.

Body weight and feed consumption were calculated and compared between chickens given these factors. Gut-damaged chicken had lower body weight

when compared to normal chicken. While exogenous enzymes significantly reduced dietary consumption per body weight gain of the supplemented chicken.

Function and population of chicken gut bacteria were investigated. The abundance and function of intestinal bacteria were dramatically affected by the gut health intervention. Exogenous enzymes did not affect the intestinal bacterial population but the function related to the digestion of carbohydrate was affected by the exogenous enzymes supplement.

Gene expression analysis was used to identify the response of the animal to the given factors at the gene level. Gut-damaged chickens responded by increasing the expression of many immune-related genes and decreasing the expression of several digestion-absorption-related genes when compared to the normal chicken. Exogenous enzymes had minor effects on the intestinal gene expression.

Relationship between intestinal bacterial population and gene expression was studied. Several positive and negative relationships between bacteria and gene expression were identified.

In conclusion, damaged gut by the protozoa dramatically affected the growth, immune and digestive system including bacterial population and function. Improvement of nutrient utilisation with the exogenous enzymes increased the feed efficiency but the host response and gut bacteria were almost unchanged. These results showed that gut health is the complex relationship between host

and gut bacteria. The relationship between bacteria and host gene expression in this gut health model study could be used in further study in an attempt to manipulate the relationship or investigate the cause-effect relationship between them.

List of abbreviations

16S rRNA	16S ribosomal ribonucleic acid
AA	Auxiliary Activity
ANOVA	Analysis of variance
BIOM	Biological observation matrix
BLAST	Basic local alignment search tool
bp	Base pair
BP	Biological process
CAZy	Carbohydrate-active enzyme
CBM	Carbohydrase-Binding Module
CC	Cellular component
CE	Carbohydrase Esterase
CMI	Cell-mediated immunity
DGGE	Denaturant gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DSS	Dextran sulphate
ELISA	Enzyme-linked immunosorbent assay
EPEF	European production efficacy factor
FCR	Feed conversion ratio
FIT-C	Fluorescein Isothiocyanate
FMT	Faecal material transplant
GH	Glycoside Hydrolase

GI	Gastro-intestinal tract
GO	Gene ontology
GSEA	Gene set enrichment analysis
GT	Glycosyl Transferase
GTDB	Genome taxonomy database
IFN-gamma	Interferon-gamma
MAG(s)	Metagenome-assembled genome(s)
MF	Molecular function
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NCBI	National Center of Biotechnology Information
NF-kB	Nuclear factor kappa B
NGS	Next generation sequencing
NMDS	Non-Metric Multidimensional Scaling
NSP(s)	Non-starch polysaccharides
OTU(s)	Operational taxonomic unit(s)
PCA	Principal component analysis
PL	Polysaccharide Lyase
qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal database project
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
SCFA	Short chain fatty acid

SD	Standard deviation
SLH	S-layer Homology domain
SOPs	Standard operating procedures
TLR	Toll-like receptor
TPM	Transcripts per million
WGCNA	Weighted-gene co-expression network analysis

List of tables

List of figures

Table of contents

Chapter 1

Introduction

1.1 General introduction

The complex relationship between diet, host health and intestinal bacteria is generally known as gut health. Poor or imbalanced gut health could interfere with immune system and nutrient absorption systems which results in poor growth or feed efficiency of the chicken. With better understanding in health and feed efficiency, poultry production might be improved through comprehensive study of gut health.

Experiment with intervention on chicken nutrition and health is required to comprehensively study the relationship between nutrition, immune system and intestinal bacteria. The effects of the interventions on gut microbiota, host intestinal gene expression and their relationship could be studied with the use of Next Generation Sequencing (NGS) technology. Several experimental models for animal gut health study were used by many researchers, such as pathogen infection or irritation of the intestinal epithelium with chemicals. In this study, the use of the experimental model that resembles industrial practice is preferred to the laboratory experimental conditions. Therefore, the knowledge gain could be quickly adopted into the industrial practice.

In this Chapter, challenges for poultry production and how to overcome these challenges will be introduced. Definition of gut health and why it is of interested to poultry producers will be discussed. Previous gut health studies using NGS technology and their methodologies will be reviewed. Chicken experimental models used in gut health study and selected model in this study will be

discussed. Finally, the outline of this thesis will be introduced at the final section of this Chapter.

1.2 Poultry meat production

With the expected increase in global population from 7.7 to 9.7 billion people during 2019 to 2050, global demand of food is also expected to be increased in order to feed the rising population and per capita consumption (Farrell *et al.*, 2013; United Nations, 2019). Food producers are trying to produce food which contains sufficient nutrients, is pathogen-free, is sustainable and is cost-effective, in order to meet increasing demands from the world population. When compare between animal meats, poultry has more advantages than other types of domestic meat animals such as cattle or pig. Poultry spends less time to reach commercial weight, is more feed efficient, has less land requirements and less of an environmental impact when compared to other domestic livestocks (Gerber, Opio and Steinfeld, 2007; De Vries and De Boer, 2010; Shepon *et al.*, 2016). Poultry meat is also accepted by many religious beliefs where the consumption of pork or beef are restricted (Farrell *et al.*, 2013). Therefore, poultry meat is the major source of animal protein of the global population (OECD/FAO, 2019).

To increase poultry production in both quality and quantity, the significant challenges to poultry producers are cost-effective production, animal welfare and health, and taking into account direct and indirect environmental impacts from the whole production system. Less variable poultry meat production costs include animals, land and management, whereas highly variable costs include

diet and disease prevention strategies. These variable costs play a significant role in the total cost of production (van Horne and Bondt, 2013). Health risk management is one of the significant costs of production especially during disease outbreaks in order to prevent animals from infectious diseases and to reduce the threat of zoonotic or food-borne diseases to human (Gerber, Opio and Steinfeld, 2007). Vaccination and anti-microbials are used with the risk of increased pathogen resistance and consumers being exposed to vaccine or drug residues (Meeusen *et al.*, 2007; Bamidele Falowo and Festus Akimoladun, 2020). Chemical disinfectants are used extensively in the environment from the start of chick production to the end of meat processing. The cost of these disease prevention strategies has led poultry producers to search for more cost-effective solutions with less impact on humans and the environment. Using advances in animal genetics and breeding technologies, breeders aim to breed animals with increased disease resistance and better feed efficiency (Phocas *et al.*, 2016). Better disease resistance also leads to a reduction in the use of vaccine and anti-microbials used for disease prevention and treatment (Gunia *et al.*, 2018). Reduction of chemical usage also lessens the amount of chemical residue released into the environment and its impact (Bamidele Falowo and Festus Akimoladun, 2020).

Alongside breeding strategies, poultry nutritionists have focused on the improvement of animal feed efficiency by using alternative feed ingredients or feed additives. Poultry nutritionist can improve nutrient utilisation and reduce the pollutant from manure by refine the feed ingredients use, adjust feed

formulation or using feed additives (FAO, 2005; Carter and Kim, 2013). Improving nutrient utilisation in an animal can reduce its amount of feed consumption, which leads to more cost-effective production, less environmental impact on crop or grain production, and a reduction in undigested-unabsorbed nutrient waste, leading to less of an environmental impact (Chadd, 2007).

Overall, poultry industry is focusing on the improvement of nutrient utilisation and disease resistance of the animal to reduce indirect costs, improve animal welfare and lessen the environmental impact from poultry production. To improve poultry production through health and nutrient utilisation, a better understanding of the role of the chicken digestive and immune system is needed. However, the study of dynamic and complex relationship between health and the digestive system poses a major challenge.

1.3 Gut health

The relationship between intestinal health, nutrient digestion and absorption, including the gut microflora, is generally known as “gut health”. Gut health is a term that has been used repeatedly in human medicine and animal health although the definition is still unclear (Choct, 2009; Kogut and Klasing, 2009; Bischoff, 2011; Kogut *et al.*, 2017). Kogut et al. (2017), defined gut health as ‘the ability of the gut to perform normal physiological functions and to maintain homeostasis, thereby supporting its ability to withstand infections and non-infectious stressors’ (Kogut *et al.*, 2017). In human medicine, gut health has been defined using criteria such as, effective digestion and absorption of food,

absence of gastrointestinal (GI) tract illness, normal and stable intestinal microbiota (all micro-organisms found in the intestine) and effective immune status (Bischoff, 2011). From these definitions, it is clear that maintenance of optimal gut health is related with interdependent factors such as host genotype, immune system, microbiota and nutrition (Choct, 2009; Kogut and Klasing, 2009; Kogut *et al.*, 2017).

There has been an increased focus on animal gut health by animal producers was observed over the last 10 years (Choct, 2009; Kogut *et al.*, 2017; Oviedo-Rondón, 2019). As the meat production relies on the physiological roles of the animal intestinal tract, disruptions of the gut health could lead to the negative results of growth, feed efficiency and immune status (Oviedo-Rondón, 2019). Maintenance of gut health is essential to the welfare and productivity of the animal, especially after the removal of antibiotic growth promoters becomes a common practice worldwide (Choct, 2009; Cervantes, 2015; Oviedo-Rondón, 2019). To maintain homeostasis of the gut health, any feed additive, substance or environmental factor that affects the gut or animal as a whole can influence gut health, including the gut microbiota, nutrient digestion and immune function (Choct, 2009; Kogut *et al.*, 2017). Therefore, comprehensive studies on these factors are required in order to study gut health.

Studies of animal nutrition, intestinal immune systems and the gut microbiota of the chickens were conducted using several laboratory techniques such as quantitative polymerase chain reaction (qPCR) or denaturant gradient gel electrophoresis (DGGE) which separates DNA fragments according to their

mobilities under increaseingly denaturing conditions (Johansen, Bjerrum and Pedersen, 2007; Burkholder *et al.*, 2008; Gilbert *et al.*, 2010; Paris and Wong, 2013). These studies showed that our understanding of gut health could be improved with these molecular techniques. However, the disadvantage of these techniques were time-consuming, labour-intensive and low amount of generated information. The expression of a few specific genes or changes in the abundance of bacteria at a community level might be inadequate to observe many of the effects of factors relating to gut health. In the 2000s, the development of NGS technology has significantly reduced time and cost requirements for sequencing of large genomes. This technology would allow researchers of gut health to gain more detailed information which could not have been gained before the age of NGS technology (Kogut *et al.*, 2017).

1.4 The impact of Next Generation Sequencing technology on gut health study

With the development of NGS technology in the 2000s, vast amounts of DNA could be sequenced more rapidly than using the previous Sanger sequencing method (Kulski, 2016). Massive DNA sequencing is performed in parallel with extremely high throughput from multiple samples at considerably reduced cost (Mardis, 2011; Kulski, 2016). Apart from whole genome sequencing, NGS technology can also be used for other analyses, such as whole transcriptome shotgun sequencing (also known as RNA sequencing or RNA-seq or transcriptomics), whole-exome sequencing, targeted sequencing and methylation sequencing (Kulski, 2016). RNA-seq can be used to identify

transcriptional activities within a cell or tissue sample with more precise and sensitive measurement of gene expression than previous technologies such as microarrays (Ozsolak and Milos, 2011; Wang *et al.*, 2011). Microbiome studies (the study of the whole genome or genetic materials of the microbes in a specific community) also gained enormous advantages from NGS technology. Bacterial populations and their functions could be identified using whole-genome sequencing, shotgun metagnomic and targeted sequencing such as 16S ribosomal RNA (rRNA) sequencing (Caporaso *et al.*, 2011; Jovel *et al.*, 2016). Bacteria which could not be cultured or identified via conventional bacterial identification and classification methodologies could be identified using NGS technology. Without labour-intensive and time-consuming steps, such as growing the bacteria on selective growth media, knowledge of the microbiome rapidly advanced, and significant amounts of data were generated. Therefore, microbiome studies with the use of NGS technology have significantly improved our knowledge in many research areas, such as health, disease and many environmental issues (Jovel *et al.*, 2016). Overall, within the gut health research area, RNA-seq, shotgun metagenomics and 16S metabarcoding analysis have the potential to provide a more in-depth understanding in the relationship between the host intestinal immune system and gut bacteria.

1.4.1 RNA-seq analysis

A gene expression study is a study that compares the amount of messenger RNAs (mRNA) in the tissues within an individual or between groups (Wang,

Doyle and Mark, 1989; Gibson, Heid and Williams, 1996; Heid *et al.*, 1996; VanGuilder, Vrana and Freeman, 2008). The central dogma of molecular biology outlines that genes are stored as DNA, transcribed into mRNA and translated into proteins (Crick, 1970). Therefore, according to the central dogma, the quantity of mRNA is expected to reflect the amount of protein synthesis by the cells (De Sousa Abreu *et al.*, 2009). The expression of this genetic information characterises the phenotype of the organism (Kukurba and Montgomery, 2015). Therefore, changes in transcription should have a phenotypic consequence (Liao and Meng, 2015). Comparison of the quantity of mRNA between samples is generally known as differential gene expression analysis. A researcher can study the response of the cell or tissue to an intervention by identifying the differentially expressed genes. By generating information on the response of the animal at the cellular level, gene expression analysis can improve our understanding of chicken gut health.

Using NGS technology, RNA-seq analysis can measure the abundance of thousands of mRNAs present in tissue or cells, therefore; not only single or selected mRNAs are studied, but in theory, every mRNA in the sample (Schena *et al.*, 1998; VanGuilder, Vrana and Freeman, 2008; Lovén *et al.*, 2012). With bioinformatic analysis, researchers can study the metabolic pathways of tissues or cells using this global gene expression analysis (B.-W. Kong *et al.*, 2011; Bottje *et al.*, 2012). RNA-seq analysis could lead to more detailed, molecular-level information about feed efficiency, disease resistance mechanisms or host immune responses. In chickens, many RNA-seq studies

related to feed efficiency or immunity have been performed. A gene expression analysis of the muscular tissues were compared between high and low feed efficiency chickens (Zhou *et al.*, 2015). The researchers found that anabolic pathways such as the growth hormone signalling pathway were enriched in high-feed efficiency chickens which may explain the increased breast muscle yield and feed efficiency (Zhou *et al.*, 2015). Another group reported the effects of chicken breeding on the expression of genes in immunity pathways (Truong, Hong and Lillehoj, 2015; Truong *et al.*, 2017). Experimental infection of *Eimeria* protozoa in chickens induced the changes of gene expression at the caecal mucosa (Li *et al.*, 2019). These results showed that RNA-seq could be used in the study of gut health and identified the genes or pathways related with growth or immune system of the chicken.

The goal of studying gene expression in the digestive and immune systems of chickens is to identify gene markers or gene sets and to try manipulate their expression (Richards, 2003; Burt, 2005). As of today, in many countries, consumer perception was still negative toward the application of genetic engineering or modification in food animals (Marques, Critchley and Walshe, 2015; Schuppli, Molento and Weary, 2015; Ishii, 2017; Cui and Shoemaker, 2018; Critchley *et al.*, 2019). Without genetic engineering, poultry breeders can select the chicken line that has the highest level of expression of these genes to improve chicken disease resistance or feed efficiency. While poultry feed nutritionists can try to manipulate gene expression by using diets or feed additives. Knowledge of the host response to diets and pathogens gained from

gene expression studies may help improve poultry health and nutrition (Frésard *et al.*, 2013; Triantaphyllopoulos, Ikonomopoulos and Bannister, 2016).

1.4.2 Microbiome analysis

1.4.2.1 Targeted sequencing using 16S rRNA

Gut microbiota related with gut health have been studied using bacterial indentification and classification methods. These bacterial culture-dependent techniques have been used by researchers in the animal gut health studies in the identification of interesting bacteria (Vahjen *et al.*, 1998; Annett *et al.*, 2002; Jia *et al.*, 2009). Although bacterial culture using selective media could identify bacteria in the sample qualitatively and quantitatively, these methods are time-consuming and labour-intensive which lead to limited numbers of bacterial genera or species in the study. Moreover, another disadvantage of the culture-dependent method is ommission of non-culturable bacteria, e.g. those that cannot be grown in the laboratory conditions. Therefore, DNA sequence-based techniques or culture-independent techniques such as qPCR and DGGE have also been applied to gut microbiota studies by using the microbial 16S ribosomal RNA (rRNA) gene for genetic characterisation, as pioneered by Carl Woese and George Fox in 1977 (Woese and Fox, 1977). This 16S rRNA is present in all prokaryotes (i.e. bacteria and archaea) and is highly conserved as the ribosomal subunit expressed by the 16S rRNA gene has essential functions in prokaryotes (Woese *et al.*, 1975). Along the 16S rRNA gene,

hypervariable regions are interspersed between conserved regions (**Figure 1.1**). These hypervariable regions are highly variable between bacterial taxa and therefore, can be used as a means for bacterial classification (Chakravorty *et al.*, 2007). Universal primers can be designed to amplify the hypervariable regions of interest for DNA sequencing and further analysis. In total, there are 9 hypervariable regions (V1-V9) of different sizes and locations along the 16S rRNA gene which can be used for bacterial taxonomic classification (Chakravorty *et al.*, 2007).

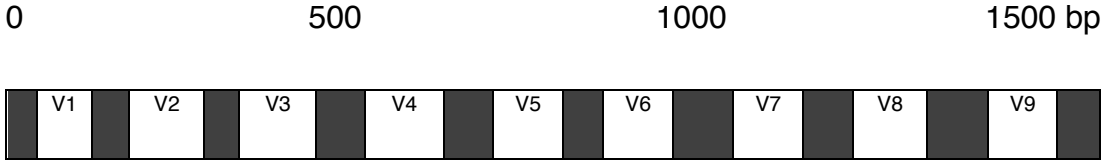


Figure 1.1 Schematic diagram of the 16S rRNA gene, showing conserved regions (black) and hypervariable regions (white) with the approximate location in the gene in base pairs (bp).

These hypervariable regions have been used as targets for microbial population analysis in many gut health-related studies (Johansen, Bjerrum and Pedersen, 2007; Burkholder *et al.*, 2008). The effects of feed withdrawal and rearing temperature on chicken gut microbiota and the susceptibility to the *Salmonella enteritidis* colonisation have been studied using the V3 region of 16S rRNA genes and DGGE (Burkholder *et al.*, 2008). Using DGGE,

researchers can compare microbial communities and visualise compositional diversity (Ferris, Muyzer and Ward, 1996). Using a similar technique with universal 16S rRNA primers, researchers investigated the effects of the anti-coccidial drug salinomycin on the gut microbiota of broiler chickens (Johansen, Bjerrum and Pedersen, 2007). With the help of sequence-based methods, researchers could observe the overall community structure of the gut microbiota rather than a few specific bacteria from bacterial culture methods.

With the advancement of sequencing technology, today researchers can sequence the 16S rRNA gene, or selected hypervariable regions of multiple bacterial taxa present in a sample in a single run (Woese and Fox, 1977; Arnold, Roach and Azcarate-Peril, 2016). With NGS technology, qualitative and quantitative analysis of the selected hypervariable region of 16S rRNA of all bacteria in the samples could be performed. It is also possilbe to sequence multiple samples within a single run on an NGS machine. With the use of a unique nucleotide sequence tag or barcode for each sample, this allows multiple samples to be combined and sequenced before the results are later de-convolved by the bioinformatics analysis. Sequencing results are analysed with bioinformatics software to produce taxonomic and abundance data about the bacterial populations in a sample (Arnold, Roach and Azcarate-Peril, 2016). Since the birth of NGS technology, studies have described the populations of micro-organisms in locations such as the ocean (Sunagawa *et al.*, 2015), soil (Gilbert, Jansson and Knight, 2014), human body (Turnbaugh

et al., 2007), human gut (Spor, Koren and Ley, 2011) or space (Checinska *et al.*, 2015).

The chicken intestinal microbiome has been studied by many researchers using 16S metabarcoding analysis (Danzeisen *et al.*, 2011; Torok *et al.*, 2011; Yeoman *et al.*, 2012; Stanley *et al.*, 2013; Oakley *et al.*, 2014). Host factors such as breed, sex, age, intestinal region and external factors such as diet, disease, medication, environment can affect the intestinal microbiome of the chicken (Danzeisen *et al.*, 2011; Luo *et al.*, 2013; Stanley *et al.*, 2013; Oakley *et al.*, 2014; Oakley and Kogut, 2016; Siegerstetter *et al.*, 2017; Kers *et al.*, 2018; Ocejo, Oporto and Hurtado, 2019; Shi *et al.*, 2019; Chintoan-Uta *et al.*, 2020). Therefore, it is important to understand the factors that could affect the 16S metabarcoding analysis results in order to control or minimise confounding factors in the proposed gut health studies.

The effects of chicken genotype or breed on gut microbiota have been reported in several studies. Gut microbiota showed significant differences between chicken breeds (Pandit *et al.*, 2018; Richards *et al.*, 2019). Chicken age is another factor that affects the gut microbiota as shown in several studies. In commercial broiler chickens and layer hens, at a young age (less than one week old), the gut microbiota was dominated by phylum Proteobacteria while Firmicutes or Bacteroidetes dominated in gut microbiota of the older chickens (over one week old) (Ballou *et al.*, 2016; Oakley and Kogut, 2016; Ocejo, Oporto and Hurtado, 2019). In broiler chickens, after 14 days of chicken age, the bacterial diversity is increased and stabilised through until the chicken

reaches its commercial weight (approximate 35 to 42 days old) (Oakley and Kogut, 2016; Ocejo, Oporto and Hurtado, 2019), although these birds will live for 3-4 years if not slaughtered.

Different intestinal regions along the chicken gastro-intestinal (GI) tract also harbour different groups of bacteria,as shown in **Figure 1.2**. The bacterial genus *Lactobacillus spp* is often the dominant population in the crop, gizzard, and small intestine (duodenum, ileum and jejunum) while within the caecum, where bacterial fermentation occurs, high bacterial diversity has been observed, with the phyla Bacteroidetes or Firmicutes often observed as the dominant population (Yeoman *et al.*, 2012; Stanley *et al.*, 2013; Oakley and Kogut, 2016; Xiao *et al.*, 2017). From these results, it is shown that host factors have an effect on the gut microbiota of the chicken; however, the microbial populations greatly varied across studies due to differing diets and environmental conditions

In chickens, the ileum and caecum are the most interesting intestinal region for microbiome study. Caecal content is one of the most studied areas as it has been suggested that the bacterial populations in the caecum might be an indicator for feed efficiency of the chicken (Apajalahti and Vienola, 2016). Residues from nutrient digestion and absorption in the proximal part of the small intestine could become substrates for specific bacteria in the caecum. Therefore, study of the caecal microbiome could demonstrate gut health in the proximal intestinal regions and the caecum itself (Apajalahti and Vienola, 2016). The ileum is the major site of nutrient absorption and regulation of the

gut immune system as it is the location of Peyer's patches, lymphoid structures scattered along the intra-epithelium which play an important role in host defense against microbial infection (Lillehoj and Trout, 1996; Svihus, 2014). Therefore, focusing on the ileal and caecal microbiota may help to provide an explanation of the relationship between gut microbiota and host in relation to the immune and digestive system.

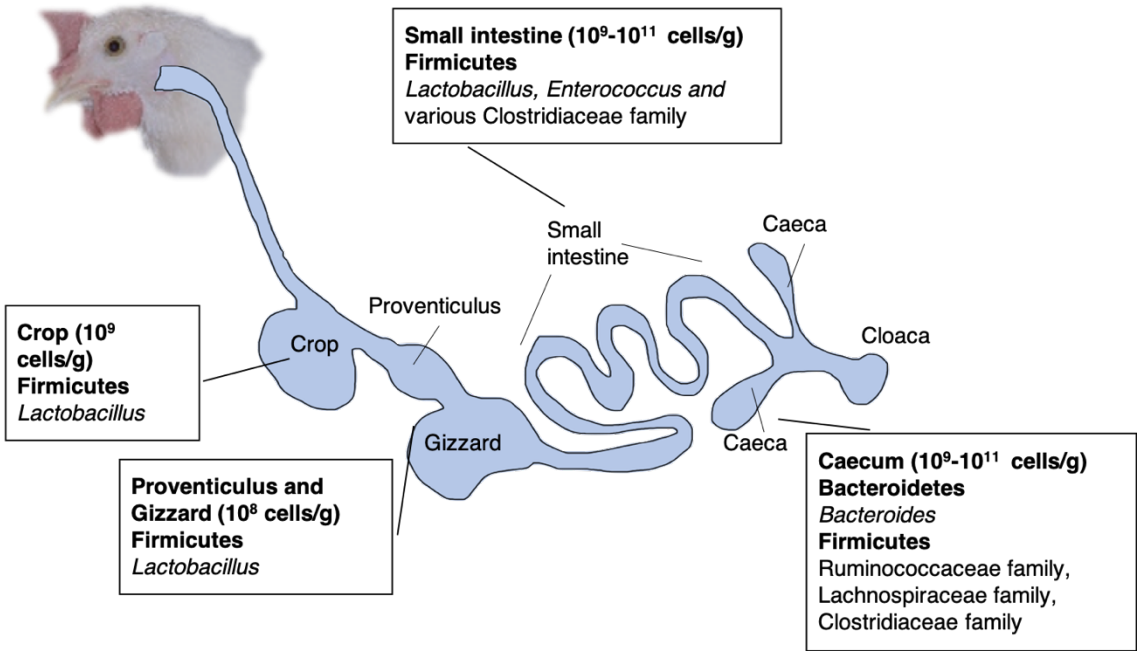


Figure 1.2 Major bacterial taxa of the chicken gastrointestinal tract (phylum and genus level are shown in bold and italic font respectively). Data on taxa distribution are taken from Oakley et al., 2014.

Diet or dietary supplementation could shift or alter the gut microbiota of the chicken (Choct, 2009; Pan and Yu, 2013; Oviedo-Rondón, 2019). Abundance of pathogenic bacteria affected by feed ingredients and its nutritional

composition have been demonstrated (Annett *et al.*, 2002; Lourenco *et al.*, 2019). Several bacterial species were also favored by some feed ingredients such as the increased abundance of *Lactobacillus crispatus* in sorghum fed chickens and *Clostridium leptum* in wheat fed chickens (Crisol-Martínez *et al.*, 2017). Moreover, the nutritional value differences between broiler-type and layer-type diets also shifts the gut microbiota of the chickens and significantly changes the expression of immune-related genes after infection with *Campylobacter jejuni* (Han *et al.*, 2016). This finding suggested that not only the feed ingredients but also the nutritional value of the diet affects the gut microbiota.

Dietary supplementation or feed additives such as probiotics (micro-organisms used as a supplement with proven beneficial effects on health), prebiotics (poorly digested feed supplements that promote growth and activity of beneficial bacteria), phytochemicals and exogenous enzymes also have effects on the gut microbial populations, such as promoting the growth of specific bacteria, maintaining normal flora or controlling pathogens (Stanley, Hughes and Moore, 2014; Pourabedin and Zhao, 2015; Sugiharto, 2016; Y. Wang *et al.*, 2017; Borda-Molina, Seifert and Camarinha-Silva, 2018). For example, in one study L-theanine (an amino acid found in green tea extract) increased the population of *Lactobacillus spp* and affected the mRNA expression of several cytokine genes such as tumour necrosis factor-alpha and interferon-gamma in the chicken intestine (Saeed *et al.*, 2019). The enrichment of the genus *Lactobacillus spp* has also been observed in the

caecum of chickens supplemented with xylo-oligosaccharide prebiotics (Pourabedin, Guan and Zhao, 2015). These findings show that diet or dietary supplementation has direct or indirect effects on intestinal bacterial populations of the chicken. A better understanding of the relationship between nutrition and the gut microbiota may help poultry producers to modulate chicken growth performance and health.

Several infectious diseases of the GI tract significantly affect the gut microbiome (Martynova-Van Kley *et al.*, 2012; Videnska *et al.*, 2013; Awad *et al.*, 2016; Macdonald *et al.*, 2017). Chickens infected with the protozoa *Eimeria*, which damages the gut epithelium, had increased abundance of bacteria in the genus *Escherichia/Shigella* (Martynova-Van Kley *et al.*, 2012) and other taxa belonging to the Enterobacteriaceae family (Macdonald *et al.*, 2017). Another study found that *Campylobacter jejuni* infected chickens had a higher abundance of *Clostridium spp* when compared to negative control chickens (Awad *et al.*, 2016). Chicken infected with *Salmonella enteritidis* had an increased percentage abundance of *Lactobacillus ultunensis* in the caecum when compared to the non-infected chickens (Videnska *et al.*, 2013). Results from these studies show that shifting of the gut microbial population could be the direct result of pathogen colonisation of the chicken intestine or the alteration of the host defensive mechanisms. More studies on the relationship between pathogens, healthy gut microbiota and the intestinal immune system will allow us to design better disease controls and preventions in the future.

Environmental factors such as biosecurity level of the animal house, animal housing system, litter management, feed access and climate including geographical location can affect the chicken gut microbiota (Kers *et al.*, 2018). Rearing room was found to affect the non-dominant bacteria of the caecal microbiota (Ludvigsen, Svihus and Rudi, 2016). Geographical location affecting the chicken gut microbiota has been observed in several studies (Zhou *et al.*, 2016; Siegerstetter *et al.*, 2017). Litter or bedding of chicken pens shares several bacterial taxa with chicken faecal samples, therefore, re-using litter could transfer the bacteria from a previous flock and influence the gut microbiota of the future flock (Oakley *et al.*, 2013; Pan and Yu, 2013). Although environmental effects on the chicken gut microbiota have been studied widely, they are still poorly understood as there are many factors within and between studies that may affect the results. Therefore, it is difficult to evaluate the specific environmental aspects that might influence the results among these studies (Kers *et al.*, 2018).

These studies show that 16S rRNA metabarcoding analysis could be used in the gut health related study such as the effects of nutrition and pathogens on gut microbiota. However, a well-controlled experiment is required, as several confounding factors could affect the gut microbiome. Moreover, downstream laboratory procedures such as freezing the sample, DNA extraction methodology, bioinformatics pipeline selection also affect 16S metabarcoding results, which might affect the interpretation of the results (Pollock *et al.*, 2018).

1.4.2.2 Shotgun metagenomic analysis

Shotgun metagenomic analysis is performed similarly to whole genome analysis in human genome studies where DNA from bacteria, virus, archaea and host cells in the intestinal content are expected to be sequenced. After downstream bioinformatics analysis, functional properties of the micro-organisms can be identified by the presence of genes in their genomes.

Shotgun metagenomic analysis gained notable attention by researchers after its potential in the study of the relationship between gut microbiota and human health was demonstrated (Turnbaugh *et al.*, 2006; Turnbaugh, Hamady, *et al.*, 2009; Turnbaugh, Ridaura, *et al.*, 2009). These results led to many shotgun metagenomic studies in human gut health related diseases, such as Crohn's disease, irritable bowel syndrome and intestinal cancer, which provided detailed and insightful information that significantly improved human medical knowledge (Erickson *et al.*, 2012; Gevers *et al.*, 2014; Huttenhower, Kostic and Xavier, 2014; Zeller *et al.*, 2014).

The selection of microbiome analyses depends on the research question and the type of information expected from the results. 16S metabarcoding is suited for analysis of large number of samples and longitudinal studies (Jovel *et al.*, 2016), Shotgun metagenomics is usually more expensive but offers the advantage of species or strain level classification of bacteria while 16S rRNA metabarcoding is typically limited to genus level classification depending on the database and classifiers used (Rausch *et al.*, 2019). Shotgun metagenomics also allows researchers to discover new bacterial genes and

genomes, examine the metabolic function of bacteria and also simultaneously study archaea, viruses, phages and eukaryotes (Jovel *et al.*, 2016).

In chickens, microbiome studies using shotgun metagenomic analysis have been performed less than 16S metabarcoding analyses. From the shotgun metagenomics outcomes, genes encoded on the bacterial genome such as for enzymes acting on specific carbohydrates can reflect the metabolic function of the bacteria. Quantitative analysis of the abundance of these genes could indicate the functional changes or effects of the intervention on chickens.

Danzeizen et al. (2011) studied the function of chicken caecal bacteria and compared populations between anti-microbial growth promoter treatments. Although no significant difference was observed regarding the resistance gene count, it demonstrated the capability of shotgun metagenomic analysis to be able to study functional aspects of the chicken gut microbiota. De Cesare et al. (2019), used shotgun metagenomics and identified cysteine desulfurase as the most abundant functional genes of the chicken gut bacteria, followed by alpha-galactosidase and serine hydromethyltransferase (De Cesare *et al.*, 2019). Different metabolic functions of the bacteria between intestinal regions can also be studied using shotgun metagenomic analysis. Metabolic capacity of the chicken intestinal bacteria was studied and differences were found between different age groups in the foregut (duodenum, jejunum and ileum) and hindgut (caecum and colorectum) of the chicken (Huang *et al.*, 2018). Breed and diet have also been shown to have an effect on the carbohydrate active enzymes involved in metabolising cellulose and hemicellulose using

shotgun metagnomic analysis (Glendinning *et al.*, 2019). Not only the effects of the host and external factors on the gut microbiome can be studied, but also the discovery of novel bacterial strain or species can be performed using shotgun metagenomics, as shown in a recent chicken microbiome study (Glendinning *et al.*, 2019). These studies show that shotgun metagenomics has the capability to identify useful information about the functionality of the chicken gut bacteria. This knowledge could be applied by nutritionists, as the metabolic function of the bacteria could reflect the availability of nutrients in the gut and may help nutritionists formulate a better diet for the balance between the host and the gut bacteria.

1.5 Experimental model of gut health study

As the homeostasis of the gut or “good” gut health cannot be identified, researchers generally generated a gut-damaged to induce “bad” gut health and compare several parameters such as intestinal morphology, intestinal gene expression or gut microbiota, against the non-gut-damaged or “control” animals. With several attempts and multiple comparisons between gut-damaged and control animals, it could be possible to identify the homeostatic markers of the host and microbiota such as specific immune-related genes or bacteria. In chickens, several methods were used such as inoculation of pathogens or chemical agents to damage the gut, and compared the outcomes to the control chickens (Silva *et al.*, 2009; Chen *et al.*, 2015; Kuttappan *et al.*, 2015; Gilani *et al.*, 2017). Using pathogen infection model to study gut health, gut microbial populations have been compared between infected and control

animals (Borewicz *et al.*, 2015; Thibodeau *et al.*, 2015; Awad *et al.*, 2016). These results show that not only the relationship between bacteria and host but also the relationship between different bacteria can be studied using these experimental models.

Another model to induce gut-damaged in animals is the use of a chemical substance that irritates or damages the intestinal epithelium of the host. For example, Dextran Sulphate Sodium (DSS), lactulose and mannitol have been used in many gut health studies as they can irritate the intestinal epithelium and result in the malfunction of the gut barrier system (Balda *et al.*, 1996; Kuttappan *et al.*, 2015; Lindblom *et al.*, 2017). A fluorescence substance such as Fluorescein Isothiocyanate (FIT-C) can then be inoculated to determine the level of intestinal damage from the amount of the substance measured in the blood stream (Kuttappan *et al.*, 2015; Vicuna, 2015). This model has proved useful to investigate the intestinal integrity during studies of gut damage; however, these chemical substances are toxic to both human and animals. These experiments should only be performed by a specialist or under veterinary suggestion.

The disadvantage of some infectious models is a potential biohazard risk to humans and other animals, while chemical irritation models could pose harm to both researcher and animal. However, these potentially harmful models can be performed in a small scale, well-controlled laboratory setting. On the contrary, in a large scale or commercial scale settings, the risk from the infectious agent or chemical reagent is higher from larger number of animals

in these less-controlled settings. Therefore, a lower risk model to create gut-damaged animals is required in large scale research such as could be achieved with nutritional studies. A recent study showed the advantage of using a vaccine against coccidiosis or anti-coccidial vaccination as a model for chicken gut health study (Chen *et al.*, 2015). Researchers can study the effects of damage caused to the gut by vaccination on intestinal immune responses related to the bacterial populations. The knowledge of chicken intestinal immune response and gut microbiome affected by the vaccination could provide us with a better understanding of coccidiosis disease and may help us develop the solution to prevent or treat the affected animals.

1.5.1 Coccidiosis and anti-coccidial vaccination

Coccidiosis, caused by protozoa in *Eimeria spp*., is one of the major threats to the poultry industry worldwide (Williams, 1999; Bera *et al.*, 2010; Peek and Landman, 2011). The estimated loss from coccidiosis in the British poultry industry in 1995 was £38 million (Williams, 1999). While the annual cost of management related to coccidiosis worldwide has been estimated to exceed 3 billion US dollars (Noack, Chapman and Selzer, 2019). *Eimeria* protozoa carry out their life cycles in the intestine of the host and damage the intestinal epithelium during several developmental stages as shown in **Figure 1.3** which results in intestinal inflammation (Blake and Tomley, 2014; Chapman, 2014). *Eimeria* protozoa have several species which develop their life cycles at different regions of the GI tract and have different pathogenicity as shown in **Table 1.1**. Mild infection of coccidiosis may lead to secondary infection of

opportunistic bacteria and result in subclinical infection which affects growth and feed efficiency of the poultry (Williams, 1999; Gussem, 2006; Bera *et al.*, 2010). Severe infection of *Eimeria* protozoa can cause bloody diarrhoea and result in moderate to high mortality (Gussem, 2006).

In order to prevent, control, treat or eradicate the coccidiosis, many strategies were developed such as the use of a vaccine, anti-microbials, chemical agents, probiotics and prebiotics (Williams, 2002; Luquetti, Furlan and Alarcon, 2012; Chapman, 2014; Quiroz-Castañeda and Dantán-González, 2015). However, the most effective prevention strategy is the use of anti-coccidial drugs (Peek and Landman, 2011). The consequence of extensive use of anti-coccidial drugs is increased resistance of the protozoa to the treatment (Chapman, 1997); therefore, possible upcoming bans on the prophylactic use of anti-coccidial drugs are being discussed (Williams, 2002; Peek and Landman, 2011). Although many alternative solutions to anti-coccidial drugs have been developed, results from these alternatives are inconsistent (Williams, 2002; Allen, 2003; Lee *et al.*, 2007; S. H. Lee *et al.*, 2011; Sugiharto, 2016). Without demonstrating effective prevention of coccidiosis, these alternatives are not fully adopted by poultry producers, and the prophylactic use of the anti-coccidial drugs cannot be eliminated.

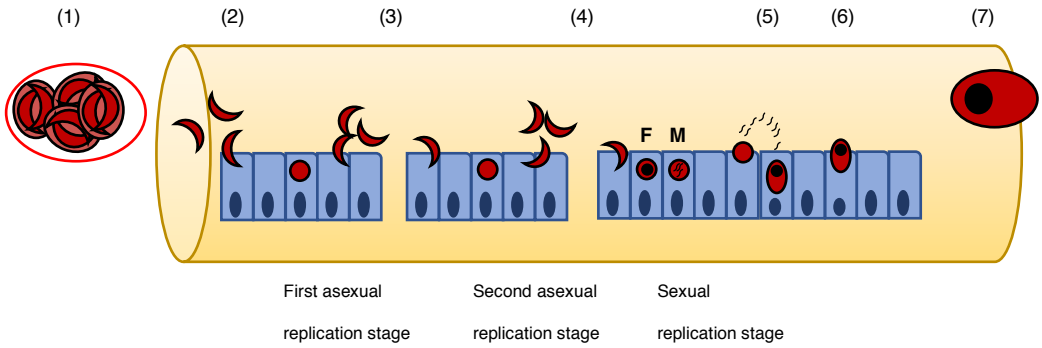


Figure 1.3 The life cycle of protozoa *Eimeria* in poultry. In brief, (1) ingestion of sporulated oocyst by poultry, (2) sporocysts released from each oocyst in GI tract of the host and release sporozoites which enter the intestinal epithelial cells and develop asexually, (3) first-generation merozoites escape from the cell and re-enter to another cell which then develop into secondary merozoites, (4) secondary merozoites leave the cell and re-enter to another cell to develop macrogametocyte (female or F) or microgametocyte (male or M) in sexual replication stage, (5) mature microgametes leave the cell and enter the mature macrogamete to form the zygote, (6) mature zygote leaves the cell and enters the GI tract, (7) zygotes develop into unsporulated oocysts and leave the host to the environment. The unsporulated oocyst requires moisture, warmth and oxygen to become a sporulated oocyst which is ready to infect the new host (Blake and Tomley, 2014).

Table 1.1: Site of life cycle development and pathogenicity of *Eimeria* species*

Species	Affected intestinal region	Pathogenicity
Eimeria hagani	Duodenum, jejunum and ileum	Least pathogenic
Eimeria praecox	Duodenum, jejunum	Least pathogenic
Eimeria acervulina	Duodenum, ileum	Less pathogenic
Eimeria mitis	Ileum	Less pathogenic
Eimeria mivati	Duodenum, rectum	Less pathogenic
Eimeria maxima	Jejunum, ileum	Moderatley-high pathogenic
Eimeria brunetti	Caeca and rectum	Highly pathogenic
Eimeria tenella	Caeca	Highly pathogenic
Eimeria necatrix	Jejunum, ileum and caeca	Highly pathogenic

* Data taken from Quiroz-Castañeda and Dantán-González, 2015.

Anti-coccidial vaccination is another preventive measure that had been created and commercialised since the 1950s (Williams, 2002). Anti-coccidial vaccines are usually a mixture of multiple species of *Eimeria* oocysts given to the chickens, as the immune responses to the *Eimeria* are not fully cross-protective between species (Lillehoj and Trout, 1993; Williams, 2002). Different types of vaccines (attenuated, non-attenuated or recombinant) and various mixture of *Eimeria* species or strains are commercially available for poultry producers worldwide (Williams, 2002; Tewari and Maharana, 2011). However, these differences resulted in different immune response and adverse effects on growth performance to the chickens (Williams, 2002; Tewari and Maharana, 2011).

As the oocysts from the vaccine enter the chicken GI tract, they develop their life cycle and cause a mild infection which results in the development of the immune response to the protozoa (Williams, 2002; Hong *et al.*, 2006). However, the development of immunity from vaccination is a time-consuming process. Therefore, it was commonly used in longer life-span chickens such as egg-laying hens and broiler breeders (Chapman, 2000). Using anti-coccidial vaccination in long life-span chickens, the resistance of the protozoa to the coccidiostat by long-term use can be reduced (Chapman, 1997). The protective efficacy of the vaccine is quite reliable but the major disadvantage of the vaccine is growth retardation as the vaccine usually contains a low number of live-oocysts (Williams, 1998). As the oocysts in the vaccine enter and start their life cycles in the gut epithelial lining cells, the protozoa damage

cells without life-threatening effects on the chicken but significantly decrease their growth and feed efficiency (Williams, 1998). Therefore, the use of the anti-coccidial vaccine is not fully adopted in the fast-growing commercial broiler industry worldwide.

Another factor that might play an essential role in the disadvantage of anti-coccidial vaccine is diet. Cereal types included in diet are known to have different effects on the severity of coccidiosis (Williams, 1992). When compared to the maize-based diet, the wheat-based diet tended to promote the enteric disease in poultry as wheat contains more non-starch polysaccharides (NSPs) than maize (Choct *et al.*, 1995; Williams, 2005). This polysaccharide is favoured by some bacteria such as *Clostridium spp* which increase pathogenicity of coccidiosis (Annett *et al.*, 2002). However, the role of diet on disease might not be an outcome from the diet only but also the relationship between gut microbiota, diet and host.

Using anti-coccidial vaccination as a gut-damage model could create a situation where the gut epithelium is damaged which should change the homeostatic relationship between host and gut microbiota. It is also an excellent model to study adverse effects from the vaccination on growth performance. A solution to the reduction of chicken growth performance needs to be found in order to reduce the use of anti-coccidial medication and introduce the use of anti-coccidial vaccination to commercial broiler chicken production. One possible solution is the use of exogenous enzymes to provide

the gut-damaged chicken with more nutrients, including the reduction of NSPs that favourable the growth of the opportunistic bacteria.

1.5.2 Exogenous enzymes supplementation

Exogenous enzymes have been used in commercial poultry production for more than 3 decades and have shown positive effects on animal growth performance (Cowieson and Adeola, 2005; Cowieson, Hruby and Pierson, 2006; Ravindran, 2013). Targets of enzyme inclusion in the diet are; to provide animals with more suitable enzymes to digest nutritional substrates (e.g. starch, protein, lipid); to digest substrates with enzymes that the animal does not produce (e.g. cellulose) and to digest substrates that have anti-nutritive effects (e.g. beta-glucans, phytate) (Ravindran, 2013). Several enzymes are produced and currently being used in the commercial poultry production such as protease, amylase, lipase, phytase, xylanase, beta-glucanase and many more. The benefits from the addition of these enzymes to the diets are; to improve animal growth performance, reduce poultry excreta moisture which improves welfare related with poor litter quality such as foot lesions and hock burns, to improve intestinal morphology and integrity, to lower manure output and unabsorbed nutrients which result in better nutrient utilisation and reduces environmental impact (Cowieson and Adeola, 2005; Adeola and Cowieson, 2011; Bedford and Cowieson, 2012; Ravindran, 2013). These benefits showed that the addition of the enzymes could help the poultry industry in many aspects; however, it is crucial to select the enzymes that match with the substrates found in feed ingredients (Ravindran, 2013). With the recent

advancements in biotechnology, targeted substrates in feed ingredients have been studied with fine-tuned production of enzymes that are specific to these substrates (Ravindran, 2013). This advancement in enzyme production results in cost-effective diets that help improve nutrient digestibility and increase feed efficiency and growth.

Phytase was one of the first enzymes used in the animal feed industry (Ravindran, 2013). The substrate of the phytase enzymes is phytate or phytic acid which is a polyionic molecule that chelates important positively charged nutrients such as phosphorus, calcium, zinc and trace minerals (Selle and Ravindran, 2007). Around 59.9% – 79.5% of total phosphorus in feed ingredients are in phytate form, which is poorly digested and absorbed by the animal (Selle and Ravindran, 2007). Phytase supplementation reduces the amount of phosphorus in chicken excreta for up to 50% (Simons *et al.*, 1990). Phosphorus is one of the most common causes of eutrophication in the rivers, lakes and reservoirs (Correll, 1999). Therefore, the inclusion of phytase in the animal diet improves phosphorus availability and reduces excreta waste which is toxic to the environment. Inclusion of phytase was shown to have significant benefits on growth performance, energy utilisation, nutrient digestibility and environmental impact (Selle and Ravindran, 2007; Adeola and Cowieson, 2011).

Xylanase and beta-glucanase are the enzymes in the carbohydrase group predominantly used in the poultry industry. Xylanase and beta-glucanase help digest NSPs such as arabinoxylans or beta-glucans in the grain-type

ingredients in the diet (Cowieson and Adeola, 2005; Adeola and Cowieson, 2011; Ravindran, 2013). The presence of soluble NSPs in the diet can lead to high viscosity of the digesta that decreases the contact between endogenous enzymes and substrates, therefore, decreases digestion and animal growth performance (Choct *et al.*, 1995; Liu and Kim, 2017). Inclusion of these carbohydrase enzymes has been shown to have significant benefits on growth performance, energy utilisation and nutrient digestibility of chickens (Józefiak *et al.*, 2006; Kalmendal and Tauson, 2012; Munyaka *et al.*, 2015; Stefanello *et al.*, 2015).

It is generally known that a valuable amount of non-digested protein passes through the GI tract as the inclusion of exogenous protease on the chicken diet increased the crude protein digestibility by 4.7% to 5.1% (Wang and Parsons, 1998; Angel *et al.*, 2011). Therefore, protease is one of the enzymes used regularly in the animal feed industry, with positive effects on protein digestibility and feed efficiency (Angel *et al.*, 2011; Pasquali *et al.*, 2016; Erdaw, Wu and Iji, 2017; Mohammadigheisar and Kim, 2018; Cowieson *et al.*, 2019). Improvement of protein and amino acid digestibility results in improved growth performance, less feed consumption, cost-effective production and less nutrient waste in the excreta (Adeola and Cowieson, 2011; Angel *et al.*, 2011; Cowieson *et al.*, 2019). Protease also has indirect effects on the proteinaceous anti-nutrients (e.g. trypsin inhibitors in a soya-bean meal), antigenic proteins and damaged proteins from processing (Ghazi *et al.*, 2002; Cowieson, Hruby and Pierson, 2006). Protease also affects the mucous layer of the intestine by

increased the turnover rate of the mucous and thereby helps to alleviate the detrimental effects of coccidial infection (Peek *et al.*, 2009).

The most beneficial effects from exogenous enzyme inclusion can be found in the combined use of several enzymes. Xylanase has been shown to increase the permeability of the aleurone layer of wheat where phytate is storage (Parkkonen *et al.*, 1997). Therefore, the animal could gain more benefits from the combination of phytase and xylanase enzymes on a wheat-based diet. Inconsistent beneficial effect interactions among enzymes have been reported in multiple studies (Selle *et al.*, 2003; Wu *et al.*, 2004; Cowieson and Adeola, 2005; Juanpere *et al.*, 2005; Cowieson and Bedford, 2009; Selle, Ravindran and Partridge, 2009; Cowieson, Bedford and Ravindran, 2010). Adeola and Cowieson (2011), suggested that the benefits from the combination of enzymes could be feedstuff specific or dietary composition dependent and more studies are required to understand the optimum combination of enzymes.

As exogenous enzymes have a direct effect on digesting and extracting nutrients from ingredients, changes in the quality and quantity of nutrients are expected to have consequent effects on gut microbiota (Bedford and Cowieson, 2012). Several studies have been conducted on the effects of enzyme supplementation on gut bacterial populations using bacterial culture-dependent methods. Reduction of total anaerobic bacteria throughout the intestine was observed in chickens fed with a xylanase supplemented diet (Hübener, Vahjen and Simon, 2002). In addition, supplementation of multiple carbohydrase enzymes on wheat-based diets improved feed efficiency,

lowered the digesta viscosity and altered the lactic acid bacteria count (Engberg *et al.*, 2004; Jia *et al.*, 2009). Multiple exogenous enzymes (xylanase and beta-glucanase) supplements significantly reduced *Escherichia coli* count in the ileum of chickens fed a wheat-based diet, whereas the *Lactobacilli* count was not significantly affected (Roofchaei *et al.*, 2019). With the use of NGS technology, more data could provide more detailed information on the shift in gut microbiota the caused by enzyme supplementation. Munyaka et al. (2015), reported changes in the ileal microbiota of chickens fed a carbohydrase supplemented diet using 16S rRNA metabarcoding analysis. Various bacterial taxa such as genus *Ruminococcus, Blautia* and family Lachnospiraceae, Lactobacillaceae showed an association with the enzyme supplement on a maize-based diet (Munyaka *et al.*, 2015). However, due to the lack of knowledge related to the effect of exogenous enzymes on the gut microbiota, more comprehensive studies using NGS technology are still required, as many confounding factors could affect the results between studies.

Studying the effect of exogenous enzymes on chicken growth performance, gut health and gut microbiota should provide a greater understanding of chicken gut health and maybe a solution to the detrimental effects of coccidiosis vaccination on chicken growth performance. Parker et al. (2007), reported improved feed intake of anti-coccidial vaccinated chickens fed with a mixture of xylanase, amylase and protease supplemented diet. The group suggested that the improvement may be due to the change of gut environment which reduced the coccidiosis lesion score and led to a higher production of

volatile fatty acids (Parker *et al.*, 2007). Bacterial cells counts in the ileum and immunoglobulin A concentration from Enzyme-linked Immunosorbent Assays (ELISA) were significantly affected by the anti-coccidial vaccination and enzyme supplementation but the responses varied depending on the crude protein level in the diet (Parker *et al.*, 2007). With the use of NGS technology on a similar topic, more comprehensive results on the effects of the exogenous enzymes and anti-coccidial vaccination and their relationship could be investigated.

1.5.3 Relationship between the gut microbiota and health

As the gut health is the relationship between health, diet and microbiota, alteration in one factor is expected to change the outcome of the others or the phenotype of the host (Choct, 2009). With this concept, manipulation of the gut microbiota may be able to improve growth performance or disease resistance (Oakley and Kogut, 2016; Shah *et al.*, 2019). A clear example of the health improvement through manipulation of the gut microbiota is the use of faecal material transplantation (FMT) in the treatment of several human diseases (Colman and Rubin, 2014; Chu *et al.*, 2017). In chickens, many studies have used FMT in an attempt to improve growth performance, feed efficiency or increase disease resistance (Gilroy *et al.*, 2018; Siegerstetter *et al.*, 2018; Metzler-Zebeli *et al.*, 2019; Chintoan-Uta *et al.*, 2020). However, the results from these studies in chickens were inconsistent.

Although the FMT proved that the gut microbiota relates with the host health or phenotype, the mechanisms behind the relationship are still unclear in every

condition (Kelly *et al.*, 2015). It is not known whether the whole bacterial community or single bacterium or other components presented in the transplant materials is responsible for the outcomes. Therefore, another attempt that can be used in gut health study in order to observe the relationship between health, diet and microbiota is the correlation study.

Correlation study between gut microbiota and host health could be performed using intestinal gene expression data as part of a microbiome study. The finding from correlation study could provide a better understanding of how the host relates to the gut microbiota. Positive or negative correlation between abundance of gut microbiota and gene expression could be identified. In poultry production, using the correlation finding to manipulate specific bacteria might alter outcome of the bird such as improve disease resistance or feed efficiency (Oakley and Kogut, 2016; Shah *et al.*, 2019). The production of desirable chicken phenotype from gut microbiota manipulation could be a solution for the poultry producer without using genetic enginerering approach on the chicken genome.

Correlation studies using gene expression analysis and intestinal bacteria as part of gut health studies have been studied in human and animals under various circumstances (Larsson *et al.*, 2012; Morgan *et al.*, 2015; Sun *et al.*, 2015; Han *et al.*, 2016; Slawinska *et al.*, 2019). In chickens, correlation between gene expression and intestinal bacteria under the effects of several factors such as age, nutrition and feed efficiency on gut health have been studied (Oakley and Kogut, 2016; Saeed *et al.*, 2019; Shah *et al.*, 2019).

Although few correlation studies was conducted in the chickens, these results show the potential of incorporating gene expression data into microbiota studies, to provide more information on the reationship between gut health and host-gut bacteria.

In this study, dietary and host health interventions affect on gut microbiota and intestinal gene expression were studied. Correlation analysis between gut microbiota and intestinal gene expression were performed which could provide in-depth knowledge on chicken gut health and improve knowledge of the use of anti-coccidial vaccination and exogenous enzymes to the poultry industry.

1.6 Thesis outline and main objectives

As discussed in **Chapter 1**, the poultry producers want to understand gut health in order to maximise the production with sustainability. However, due to the complex and dynamic environment of the gut, the relationship between host immunity, gut microbiota and nutrition is still unclear. Using new 'omics' technology could help researchers and poultry producers understand gut health.

Before starting my gut microbiome analysis on experimental samples, a comparison between several 16S metabarcoding pipelines to find the most accurate pipelines was performed. In **Chapter 2**, three bioinformatic pipelines were compared using public open-source data (Bokulich *et al.*, 2016). These pipelines were mothur (Schloss *et al.*, 2009), QIIME (Caporaso *et al.*, 2010) and Microbiome Helper (Comeau, Douglas and Langille, 2017). The most

accurate pipeline is then used in the 16S metabarcoding analysis in the following chapter.

In **Chapter 3**, chickens were reared and allocated into a 2 by 2 factorial arrangement study. Anti-coccidial vaccination was used as a host health intervention factor while exogenous enzymes were used as a dietary intervention factor. Chicken growth performance was calculated and statistically compared between factors. Finally, to identify the benefits of exogenous enzymes supplementation on improving growth performance of gut damaged chicken, the effects of enzymes supplements on growth performance between vaccinated and non-vaccinated chickens was compared.

In **Chapter 4**, gut microbiome analysese were performed in order to study the effects of anti-coccidial vaccination and exogenous enzyme supplementation on the gut bacteria. Intestinal contents from ilea and caeca from the chickens in the experiment from **Chapter 3** were collected and DNA was extracted. Quantitative analysis of the gut microbiome was performed with 16S metabarcoding analysis using the most accurate pipeline from the comparison in **Chapter 2**. Statistical analysis was performed to find bacterial taxonomies that were significantly affected by the vaccination and enzyme supplementation. Qualitative and quantitave analysis of the chicken gut microbiota was analysed using shotgun metagnomic analysis. Carbohydrate active enzymes (CAZyme) of the chicken gut bacteria were classified and compared between groups.

In **Chapter 5**, transcriptomic analysis was performed to identify the significantly differentially expressed genes affected by anti-coccidial vaccination and exogenous enzyme supplementation. Intestinal tissues collected from the ilea and caeca during the experiment described in **Chapter 3** underwent RNA extraction and this RNA was used for the gene expression analysis. RNA-seq analysis was performed to identify differentially expressed genes that resulted from treatment given to the chickens. Pathways or functions of the expressed gene were analysed using Gene set enrichment analysis (GSEA) and compared between factors to identify differentially enriched gene sets.

In **Chapter 6**, the correlation between the gut microbiome and intestinal gene expression was studied to identify the relationship between them. Network analysis of gene expression results from **Chapter 5** was analysed, including the correlation between significantly differentially expressed genes and the gut microbiome at each intestinal region. Correlations between differentially expressed genes and bacterial Operational Taxonomic Units (OTUs), a definition used to classify groups of closely related bacteria, were identified. These findings demonstrate the relationship between specific bacteria and host gene expression.

Finally, in **Chapter 7**, a general discussion of the findings in this thesis will be presented, with suggestions and comments for potential future studies.

Chapter 2

Comparison between three 16S rRNA metabarcoding analysis methods

2.1 Introduction and Aims

To study bacterial community compositions with 16S rRNA metabarcoding analysis, several methods can be used to analyse and interpret the sequencing results, such as QIIME1 (Caporaso *et al.*, 2010), mothur (Schloss *et al.*, 2009), MG-RAST (Glass and Meyer, 2011), DADA2 (Callahan *et al.*, 2016), Kraken (Wood and Salzberg, 2014), CLARK (Ounit *et al.*, 2015), One Codex (Minot, Krumm and Greenfield, 2015) and many more. However, as these methods utilise different algorithms, operating systems, and have different computer resource requirements, a comparison between these pipelines is essential to identify the differences between them and to choose a pipeline for further analyses in this thesis.

Many studies have compared the advantages and disadvantages between these methods, such as analysis time, computer requirements and the most critical feature, accuracy (D'Argenio *et al.*, 2014; Nilakanta *et al.*, 2014; Plummer and Twin, 2015; Siegwald *et al.*, 2017; López-García *et al.*, 2018). D'Argenio et al. (2014), demonstrated that QIIME1 was more accurate and faster than the web-based pipeline, MG-RAST. Another study reported that the best 2 methods are QIIME1 and mothur as they have more comprehensive features, easy to follow tutorials and support documentation (Nilakanta *et al.*, 2014). In addition, the results from another study showed that QIIME1, mothur and MG-RAST were comparable, but MG-RAST was suggested for researchers with low command-line experience (Plummer and Twin, 2015). Siegwald et al. (2017), compared between several 16S rRNA metabarcoding

analyses and found that mothur and QIIME1, were faster and required less computing resources and also performed better than Kraken and CLARK, in identifying novel or poorly-annotated members of the microbiome (Siegwald *et al.*, 2017). Lopez-Garcia et al. (2018), compared QIIME1 and mothur using rumen microbiome data and found no significant differences between these pipelines. However, when changing the reference database, there were significant differences between the 2 pipelines in low abundance bacteria (López-García *et al.*, 2018). These studies showed that the pipelines, reference databases and datasets used in these comparisons affected the results of 16S metabarcoding analysis; therefore, it is worth conducting a comparison study to find the most accurate pipeline that matches with the researcher's specific needs.

In early 2017, another bioinformatics pipeline for 16S rRNA metabarcoding analysis called Microbiome helper was published (Comeau, Douglas and Langille, 2017). This method is based on the QIIME1 algorithm, but several steps are added and improved from the QIIME1 developer's standard operating procedures (SOPs). Microbiome helper is also more user-friendly for a researcher with limited bioinformatics experience than mothur. The final part of Microbiome helper transforms the text-based data into a format that can be opened in STAMP software for point-and-click data visualisation and statistical analysis (Parks *et al.*, 2014). However, as the pipeline has been developed recently, the accuracy of the Microbiome helper pipeline should be compared with other pipelines, before use. To my knowledge, there have been no

publication comparing the accuracy between the Microbiome helper, mothur and QIIME1 pipelines at the time of writing.

In order to determine the accuracy of the 16S rRNA metabarcoding analysis method, a known dataset that can be tested across these methods is essential, with earlier studies using their custom or clinical datasets for this purpose (D'Argenio *et al.*, 2014; Nilakanta *et al.*, 2014; Plummer and Twin, 2015; Siegwald *et al.*, 2017; López-García *et al.*, 2018). Datasets, called Mockrobiota, have been published publicly for researchers to use as standard datasets to learn to analyse 16S rRNA metabarcoding data (Bokulich *et al.*, 2016). With these datasets, it is possible to compare the accuracy between analysis pipelines to decide upon a reproducible pipeline which could be used in further analysis.

In this chapter, a comparison between Microbiome Helper (version 1), mothur (version 1.39.5) and QIIME (version 1.9.1) will be performed with 7 Mockrobiota datasets. The first comparison between pipelines was performed using custom reference databases, which were manually created from nucleotide sequences provided by the Mockrobiota's developer. The second comparison was performed using the reference databases, followed by the developer's standard operating procedures (SOPs). From these comparisons, the most accurate pipeline will be identified and used in the microbial population analyses included in the following chapters of this thesis.

2.2 Materials and Methods

2.2.1 Datasets

The 7 Mockrobiota datasets selected for this comparison were mock13, mock14, mock15, mock18, mock19, mock22 and mock23 datasets as shown in (Bokulich *et al.*, 2016). These datasets were selected in order to represent the range of factors which might differ between as yet unknown field samples, including factors such as community composition, read quality, read number and community evenness as shown in **Table S1**. Three datasets (mock13, mock14 and mock15) have similar bacterial compositions with different total numbers of reads and read qualities (and **Table S2**). In mock18, there are 15 bacterial strains with even distribution, while mock19 shares similar bacterial strains to mock18 with the addition of 12 synthetic spike-ins (**Table S3**). The other 2 datasets, mock22 and mock23, shares similar bacterial strains but with even and uneven distributions respectively (**Table S4**). All 7 datasets are Illumina Miseq paired-end read sequenced with 515f/806r primers specific to the variable 4 (V4) region of the bacterial 16S rRNA gene (Caporaso *et al.*, 2011). The V4 region was shown to have less error when compared to V3-V4 and V4-V5 region (Kozich *et al.*, 2013). Therefore, this V4 region will also be used in further analysis in this thesis. Primers were removed with Cutadapt (Martin, 2013). After the removal of primers and adapters, the approximate size of the sequencing reads was 250 base pair (bp).

2.2.2 Mockrobiota custom reference database

Mockrobiota's developer provides the nucleotide sequences of all bacteria present in each dataset. These sequences were combined and adjusted to match the format required by each pipeline. In total, there were 3 custom reference databases; the first database was for mock13, mock14 and mock15. Another database was for mock18 and mock19 datasets. The last database was for the mock22 and mock23 datasets.

Table 2.1: Details of the Mockrobiota* datasets selected for pipeline comparison

Datasets	Number of reads	Mean Phred quality score of forward sequence	Mean Phred quality score of reverse sequence
mock13	602,819	30.21	26.81
mock14	613,091	30.22	25.53
mock15	1,012,097	30.60	25.54
mock18	169,516	33.93	34.47
mock19	153,841	32.82	32.53
mock22	356,563	34.50	32.46
mock23	336,667	34.53	32.02

*Bokulich et al., 2016

2.2.3 OTU classification using a custom reference database

Three bioinformatics pipelines, Microbiome helper, mothur and QIIME 1.9.1 were used in this comparison with the custom reference databases from Mockrobiota's developer. Selected datasets (shown in **Table S1**) were obtained from the Mockrobiota developer's website (https://github.com/caporaso-lab/mockrobiota/tree/master/data). All bioinformatics pipelines used in this analysis were performed following the developer's SOPs published on their website: Microbiome helper version 1 (https://github.com/LangilleLab/microbiome_helper/wiki/16S-Bacteria-and-Archaea-Standard-Operating-Procedure), mothur (https://www. mothur.org/wiki/MiSeq_SOP) (Kozich *et al.*, 2013) and QIIME 1.9.1 (https://nbviewer.jupyter.org/github/biocore/qiime/blob/1.9.1/examples/ipynb/illumina_overview_tutorial.ipynb). One exception was the addition of a chimaera sequence removal step in QIIME 1.9.1. Each pipeline was performed based on the reference databases shown in Table S1 – S3. The pipeline SOPs were obtained from the developer's website during June-October 2017. A detailed comparison of each step in all 3 bioinformatics pipelines is shown in **Table 2.2**.

Table 2.2: Comparison of the methods and algorithms used in each step between 3 bioinformatic pipelines, based on the developer's standard operating procedures published on the developer's website.

Command	Microbiome helper	Mothur	QIIME 1.9.1
Join paired end reads	PEAR (Zhang *et al.*, 2014)	make.contigs	fastq-join (Aronesty, 2013)
Quality filtration (quality score and length)	read_filter.pl	screen.seqs	split_libraries
Chimaera removal	Vsearch (Rognes *et al.*, 2016)	UCHIME (Edgar *et al.*, 2011)	Usearch (Edgar and Bateman, 2010)
Reference for chimaera removal	Ribosomal Database Project (RDP) (Cole *et al.*, 2014)	SILVA (Quast *et al.*, 2013)	Greengenes (Desantis *et al.*, 2006)
Picking OTUs method	SortMeRNA (Kopylova, Noé and Touzet, 2012) and SUMACLUST (Bonin *et al.*, 2014)	classify.seqs	UCLUST (Edgar and Bateman, 2010)
Reference for OTU taxonomies	Greengenes (Desantis *et al.*, 2006)	RDP (Cole *et al.*, 2014)	Greengenes (Desantis *et al.*, 2006)
Data visualisation	Create BIOM file Open in R	Create BIOM file Open in R	Create BIOM file Open in R

2.2.3.1 Microbiome helper version 1

From the developer's SOPs, paired-end reads were merged using PEAR (Zhang *et al.*, 2014). PEAR is a fast and accurate paired-end reads merger that uses both quality scores and sequence matches to merge reads without specifying read length (Zhang *et al.*, 2014). Merged reads were filtered by score and length with the *read_filter.pl* command which filters out sequences with quality scores of less than 30 at 90% of sites and those with length shorter than 240 bp. Chimaeric sequences or artificial sequences created from 2 or more sequences during the PCR amplification process (Smyth *et al.*, 2010), were detected with VSEARCH (Rognes *et al.*, 2016). VSEARCH uses the UCHIME algorithm (Edgar *et al.*, 2011) which divides each query into 4 segments and looks for their potential parents based on the Ribosomal Database Project (RDP) reference trainset version 15 (Cole *et al.*, 2014). Detected chimaeric sequences were removed from the samples before OTU classification. QIIME wrapper scripts version 1.9.1 were used for 2-step OTU classification. First, reference-based OTU picking was performed, where reads were clustered against the Mockrobiota custom reference databases using the SortMeRNA method with minimum 97 per cent query coverage (Kopylova, Noé and Touzet, 2012). Second, *de novo* OTU picking was performed, where any reads which did not hit the reference were subsequently clustered without reference using the SUMACLUST method followed by taxonomic assignment (Bonin *et al.*, 2014). OTUs that were assigned less than 0.1% of the total number of sequences were then filtered out by using *remove_low_*

confidence_otus.py command. Finally, samples were rarefied by using the lowest number of reads from any sample in the analysis. OTU abundance and classification of each sample were summarised into Biological observation matrix (BIOM) format table for further analysis.

2.2.3.2 mothur

From the developer's SOPs, paired-end reads were joined and quality controlled with the *make.contigs* and *screen.seqs* commands respectively. Quality control was performed at the *make.contigs* step where the quality score was set to higher than 25 and the *deltaq* score (difference between quality scores of a mismatched base) of the overlapping region was set at lower than 6. At *screen.seqs*, the maximum number of ambiguous bases per sequence was set to 0 and minimum sequence length was set at 240 bp. Reads were removed if they did not meet the quality criteria defined for these commands. The dataset was simplified using the *unique.seqs* command to merge identical sequences before alignment to reduce the size of data and computer resources required. Alignment of sequences to improve quality and reliability of downstream comparisons was performed with the *align.seqs* command using SILVA database release 102 as a reference (Quast *et al.*, 2013). The alignment was then filtered using the *screen.seqs* command to select only the V4 region of the 16S rRNA gene and the *filter.seqs* command was used to remove the gap characters generated by the alignment, to accelerate downstream computational speed. To reduce errors from sequencing, the *pre.cluster* command was used to split the sequences, sort them by

abundance and merge sequences within 2 nucleotides difference of each other. Then chimaeric sequences were detected with the *chimera.uchime* command using the UCHIME algorithm (Edgar *et al.*, 2011), as described in section 2.2.3.1, and were removed by the *remove.seqs* command. Taxonomic classification of reads was performed with *classify.seqs* using the mothur-formatted Mockrobiota custom reference databases. Unclassified reads and reads from non-bacteria (such as archea, chloroplasts and mitochondria) were removed by using the *remove.lineages* command. Sequences were binned and clustered by using phylotype (database dependent approach) at the genus level. The sequence abundance of each OTU was calculated with the *make.shared* command. The *classify.otu* command was used to classify the taxonomy of each OTU. Singletons (read that present exactly once throughout the analysis) were removed with the *remove.rare* command. Samples were rarefied by using the lowest number of reads from any sample in the analysis. Finally, OTU abundance and classification of each sample were summarised into BIOM table format for further analysis.

2.2.3.3 QIIME 1.9.1

From the developer's SOPs, fastq-join, which uses squared distance scoring and which allows it to be highly selective and result in high speed and sensitivity, was used to join paired-end reads (Aronesty, 2013). Quality filtration was performed with *split_libraries_fastq.py* command with the default minimum quality score = 3 (Bokulich *et al.*, 2013). Usearch (Edgar and Bateman, 2010) was used to identify chimaeric sequences with Greengenes

database version 13_8 as a reference (Desantis *et al.*, 2006). Chimaeric sequences were filtered out with the *filter_fasta.py* command. OTUs were picked with 2-step OTUs classification, reference-based and *de novo* OTU picking, with the default UCLUST algorithm (Edgar and Bateman, 2010). UCLUST selects a read as a centroid and clustered with other reads that have more than 97% identity to the centroid. Read with lower than 97% identity to the centroid will represent as a centroid of the new cluster. Finally, taxonomic classification was performed on each cluster. Mockrobiota custom reference databases were used for reference-based OTU picking. Singletons were removed with the *filter_otus_from_otu_table.py* command. Samples were rarefied by using the lowest number of reads from any sample in the analysis. OTUs abundance and classification of each sample were summarised into BIOM table format for further analysis.

2.2.3.4 Accuracy of OTUs classification

The percentage relative abundance of bacteria classified by each pipeline in each dataset was calculated and visualised with the *ggplot2* package (Wickham, 2016) using R software version 3.5.3 (R Core Team, 2018). Principal Component Analysis (PCA) graphs were plotted at the genus level. The Euclidean distances between the results from each pipeline and the actual percentage abundances were calculated for each dataset. The pipeline with the lowest distance to the actual percentage was considered as the most accurate pipeline.

2.2.4 OTUs classification using the developer's suggested reference database

Mockrobiota's custom reference databases were replaced with the developer's suggested reference database for OTUs classification before all the pipelines were re-analysed according to step 2.2.3. From the SOPs published by the developers of each pipeline, different reference databases were recommended. The Greengenes database version 13_8 was used at the reference-based OTUs picking step of Microbiome helper and QIIME 1.9.1 as recommended on the developer's website (Desantis *et al.*, 2006). The RDP version 14 was used as a reference at the OTUs classification step of mothur (Cole *et al.*, 2014). The number of observed OTUs was summarised and compared between pipelines. The abundance of bacteria and Euclidean distance to the actual percentage was calculated and summarised similarly to the method described in section 2.2.3.4.

2.3 Results

2.3.1 OTUs classification results using Mockrobiota custom reference database

From the 7 mockrobiota datasets used in this comparison, the number of reads rarefied in each pipeline based on the reference database was shown in **Table S5**. The numbers of OTUs classified by each pipeline were higher than the actual number of strains in each dataset, with an especially high number of observed OTUs analysed with QIIME 1.9.1 (**Table 2.3**). Microbiome helper

showed the closest number of observed OTUs in 5 datasets, while mothur and Microbiome helper shared the closest observed OTUs in mock18 and mock19 datasets.

Table 2.3: Number of observed OTUs classified by each pipeline using the custom reference databases.

Dataset	Number of strains	Microbiome helper	mothur	QIIME 1.9.1
mock13	21	25	30	1,097
mock14	21	25	31	1,262
mock15	21	24	31	1,135
mock18	15	26	26	342
mock19	27	27	27	386
mock22	20	23	24	1,174
mock23	20	23	25	907

When analysing the percentage relative abundance of bacteria in each dataset using the custom database provided by the Mockrobiota's developer, each pipeline performed poorly on several datasets. In mock13, mock14 and mock15, all 3 pipelines performed poorly in the classification of bacteria in genus *Propionibacterium* (**Figure 2.1, Figure 2.2** and **Figure 2.3**). The abundance of bacteria in the genus *Propionibacterium* classified by all pipelines was less than 0.2% while the actual percentage was 4.76%. QIIME

1.9.1 performed differently from the other 2 pipelines in the classification of the genera *Bacteroides, Helicobacter* and *Pseudomonas*. The percentage of genus *Bacteroides* classified by QIIME 1.9.1 was at 12.48%-15.55% where the actual percentage was at 4.76%. Microbiome Helper and mothur classified the genus Bacteroides at 5.53%-6.06% and 5.23%-6.19% respectively. Genera *Helicobacter* and *Pseudomonas* classified by Microbiome Helper and mothur were close to the actual percentage. In contrast, QIIME 1.9.1 classified genera *Helicobacter* and *Pseudomonas* at 1.24%-1.29% and 2.66%-2.85% respectively. Microbiome helper was not able to classified genus Lactobacillus in mock13,mock14 and mock15. In contrast, mothur and QIIME 1.9.1 classified genus *LactoBacillus* at 1.20%-1.31% and 0.04%-0.05% respectively.

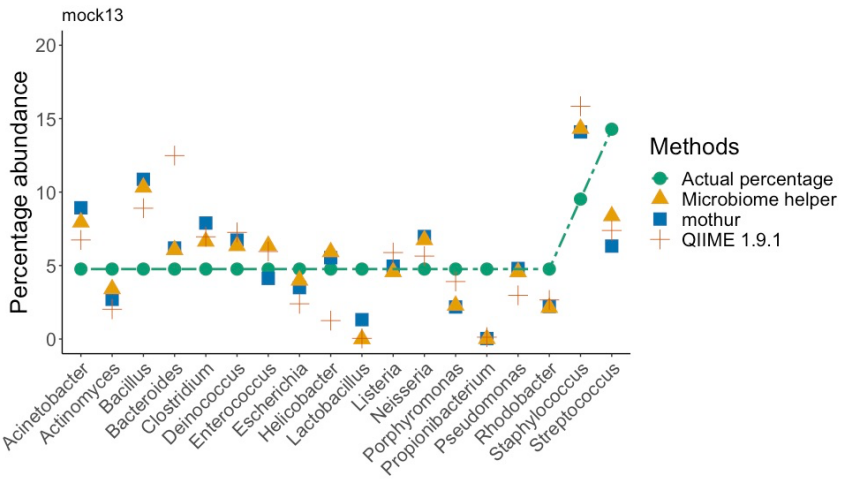


Figure 2.1: Percentage abundance of OTUs classified by 3 pipelines, compared to the actual percentage at genus level of the mock13 dataset using a custom database.

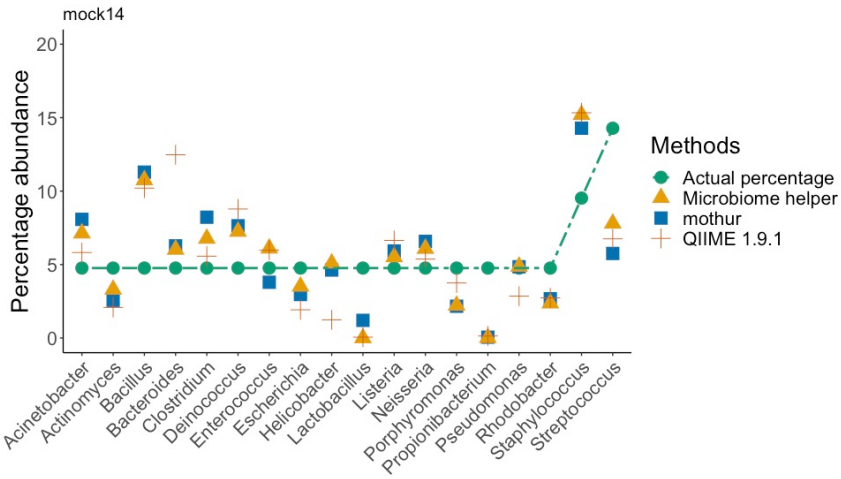


Figure 2.2: Percentage abundance of OTUs classified by 3 pipelines, compared to the actual percentage at genus level of the mock14 dataset using a custom database.

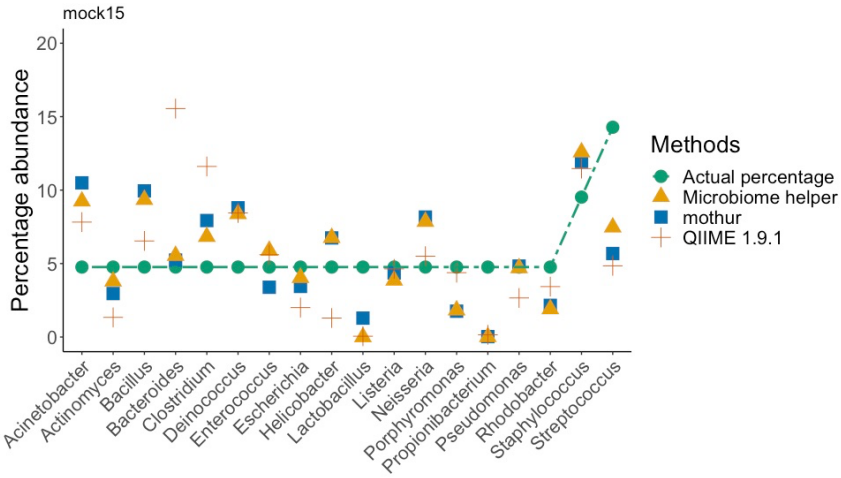


Figure 2.3: Percentage abundance of OTUs classified by 3 pipelines, compared to the actual percentage at genus level of the mock15 dataset using a custom database.

In mock18 and mock19 all pipelines performed quite similarly when using the custom reference databases (**Figure 2.4**, **Figure 2.5**, **Figure 2.6** and **Figure 2.7**). The small difference between pipelines was the underestimation of the abundance of bacteria in genus *Anaerolina* by Microbiome helper while the other pipelines overestimated it in mock18 and mock 19. Interestingly, all pipelines were not able to classify bacteria in the genus *Microlunatus* in the mock18 dataset (**Figure 2.4**). In mock22 and mock23, Microbiome helper could not identify the genus *Lactobacillus.* QIIME 1.9.1 could identify this genus at 0.02% from the actual percentage at 5% in mock22 and less than 0.01% from 0.22% in mock23. In mock22 and mock23, genus *Enterococcus* classified by Microbiome helper and QIIME 1.9.1 was higher than the actual values while mothur classified this genus close to the actual value.

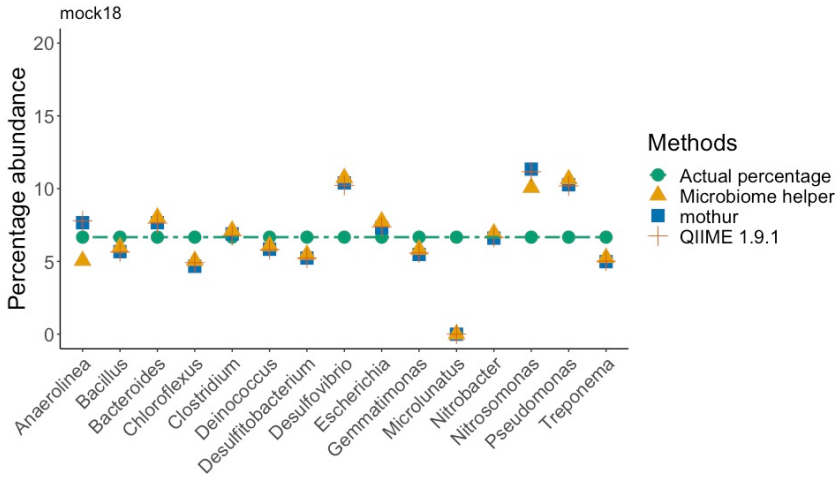


Figure 2.4: Percentage abundance of OTUs classified by 3 pipelines, compared to the actual percentage at genus level of the mock18 dataset using a custom database.

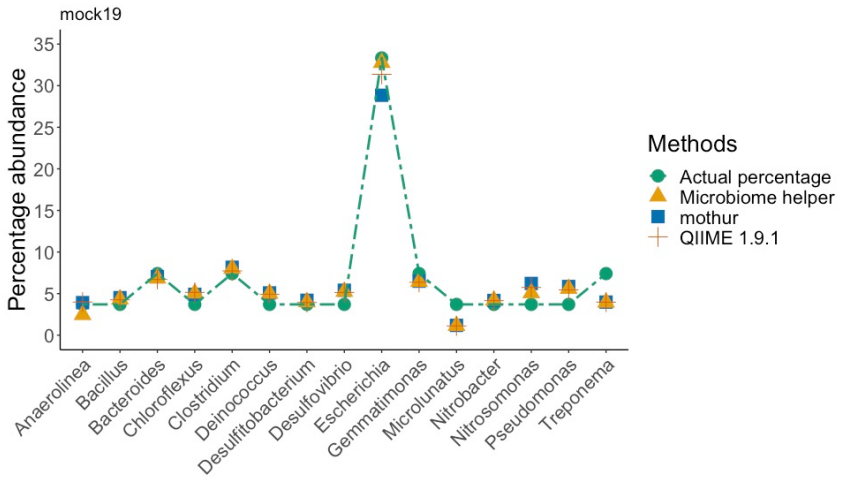


Figure 2.5: Percentage abundance of OTUs classified by 3 pipelines, compared to the actual percentage at genus level of the mock19 dataset using a custom database.

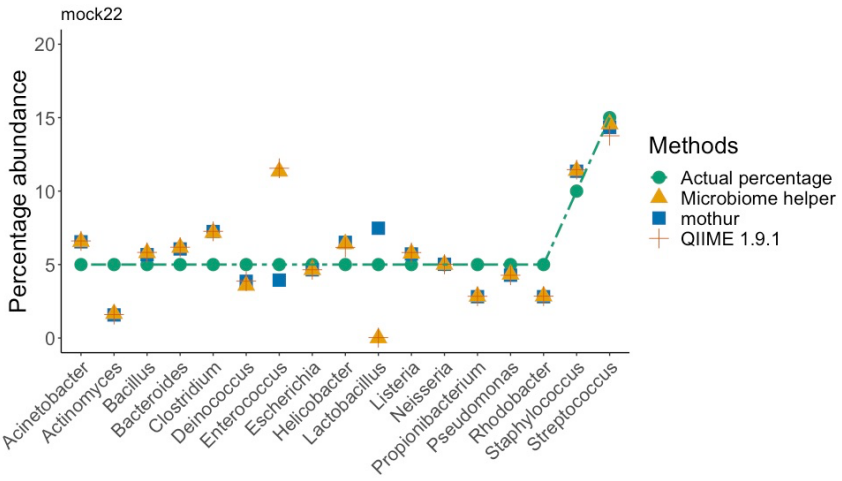


Figure 2.6: Percentage abundance of OTUs classified by 3 pipelines, compared to the actual percentage at genus level of the mock22 dataset using a custom database.

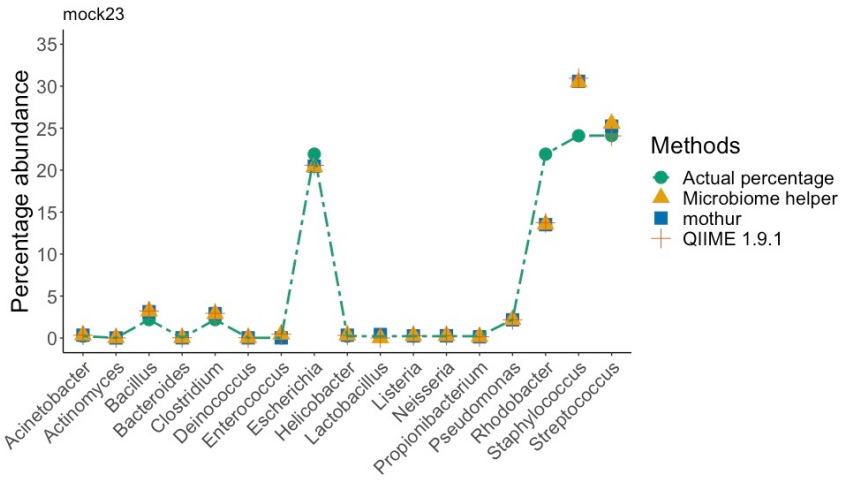


Figure 2.7: Percentage abundance of OTUs classified by 3 pipelines, compared to the actual percentage at genus level of the mock23 dataset using custom database.

The PCA plot showed that all pipelines produced similar results when using the custom reference databases obtained from the Mockrobiota developer's website. In each dataset, all 3 pipelines clustered together (**Figure S1**). Therefore, the Euclidean distance between each pipeline to the actual percentage at the genus level was close (**Table 2.4**). When comparing the Euclidean distance at each dataset, Microbiome helper had the lowest distance in 6 datasets while mothur had the lowest distance in only one dataset.

Table 2.4: Euclidean distance values between the expected percentage abundances for each pipeline and the actual percentage at genus level of each dataset using a custom database.

Dataset	Microbiome helper	mothur	QIIME 1.9.1
mock13	13.24	14.62	16.13
mock14	13.88	15.20	16.82
mock15	13.93	15.46	19.09
mock18	10.10	10.37	10.16
mock19	5.85	7.63	6.13
mock22	10.11	6.68	10.30
mock23	10.81	10.84	10.84

2.3.2 OTUs classification results using reference database suggested by pipeline's SOPs

From each of the 7 mockrobiota datasets used in this comparison, the number of reads rarefied in each pipeline was 80,160 reads, 116,052 reads and 52,930 reads from Microbiome helper, mothur and QIIME 1.9.1 respectively. The numbers of OTUs classified by each pipeline were higher than the actual number of strains in each dataset, with an especially high number of observed OTUs from the mock13, mock14 and mock15 datasets analysed with QIIME 1.9.1 (**Table 2.5**). Microbiome helper showed the closest number of observed OTUs between the mock13, mock14 and mock15 datasets, while mothur had the closest observed OTUs in the rest of the datasets.

Table 2.5: Number of observed OTUs classified by each pipeline using suggested database from pipeline's SOPs

Dataset	Number of strains	Microbiome helper	mothur	QIIME 1.9.1
mock13	21	58	106	1,358
mock14	21	59	112	1,472
mock15	21	58	106	1,398
mock18	15	28	22	279
mock19	27	30	25	351
mock22	20	53	35	557
mock23	20	46	38	349

OTUs classified by each pipeline were simplified into phylum and genus level for comparison. The expected percentage relative abundances of OTUs classified from each pipeline were compared to the actual percentage. As mock13, mock14 and mock15 shared the same abundance of bacteria, the results of these 3 datasets from each pipeline were closely similar between datasets. From mock13, mock14 and mock15, QIIME 1.9.1 showed different results from Microbiome helper and mothur at the phylum level (**Figure S2**, **Figure S3** and **Figure S4**). QIIME 1.9.1 overestimated the percentage abundance of phylum Bacteroidetes and underestimated the percentage abundance of phylum Proteobacteria while the other 2 pipelines estimated the abundance of OTUs at phylum level in the opposite direction to QIIME 1.9.1.

At the genus level, the abundance of bacteria in the genus *Propionibacterium* classified by all pipelines was less than 0.15% while the actual percentage was 4.76% (**Figure 2.8, Figure 2.9** and **Figure 2.10**). Similar to the pipeline comparison using the custom reference database, QIIME 1.9.1 performed differently from the other 2 pipelines in the classification of the genera *Bacteroides, Helicobacter* and *Pseudomonas*. Microbiome helper could not identify bacteria in the genus *Escherichia* and *Listeria*. In contrary, mothur identified the genus *Bacillus* at 0% - 0.01% from the actual percentage at 4.76%.

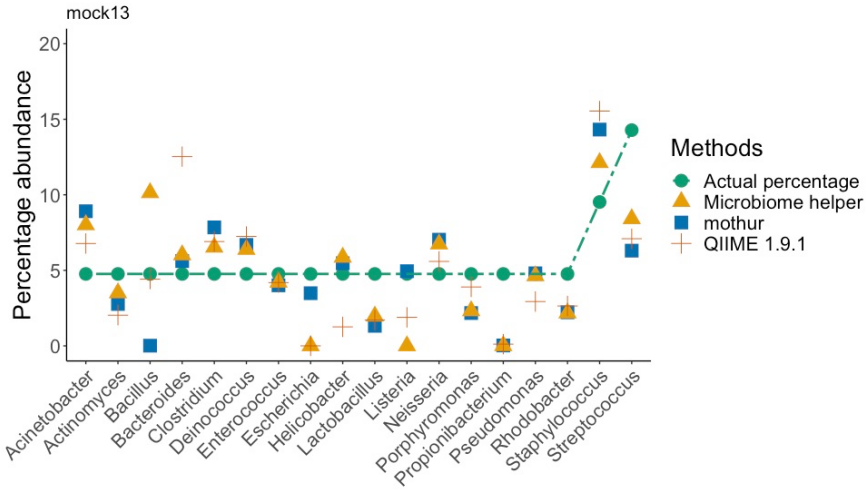


Figure 2.8: Percentage abundance of OTUs classified by 3 pipelines compared to the actual percentage at genus level of the mock13 dataset.

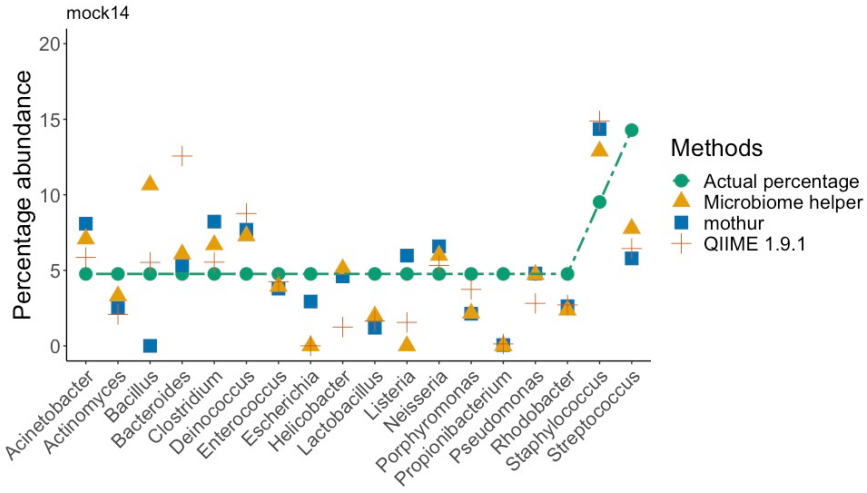


Figure 2.9: Percentage abundance of OTUs classified by 3 pipelines compared to the actual percentage at genus level of the mock14 dataset.

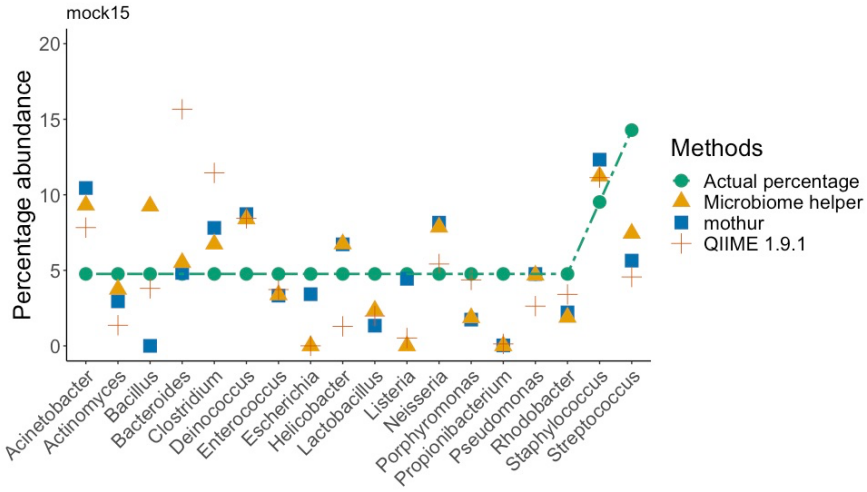


Figure 2.10: Percentage abundance of OTUs classified by 3 pipelines compared to the actual percentage at genus level of the mock15 dataset.

All 3 pipelines performed similarly in the classification of OTUs at phylum level in the mock18 dataset. All pipelines could not identify the bacteria from phylum Actinobacteria and overestimated the bacteria in phylum Proteobacteria (**Figure S5**). At the genus level, bacteria in genus *Microlunatus* was not classified by any pipelines (**Figure 2.11**). QIIME 1.9.1 and Microbiome helper were not able to identify bacteria in the genera *Escherichia*, *Desulfitobacterium*, *Nitrobacter* and *Nitrosomonas*. Moreover, only Microbiome helper pipeline that could not classified the genus *Desulfovibrio*.

In mock19, mothur identified Proteobacteria and being low abundance compared to the other pipelines while QIIME 1.9.1 and Microbiome helper identified Firmicutes as being higher abundance when compared to mothur (**Figure S6**). At the genus level, all pipelines performed similarly to the mock18 dataset except that bacteria in genus *Microlunatus* were only able to be classified by all pipelines at low abundances (**Figure 2.12**).

From mock22 and mock23, all pipelines performed similarly at the phylum level. At the genus level, bacteria in genus *Escherichia* were not classified by Microbiome helper and QIIME 1.9.1 (**Figure 2.13** and **Figure 2.14**). Microbiome helper could not classify the bacteria from the genus *Listeria* while mothur could not classify the bacteria from the genera *Bacillus*. Moreover, Microbiome helper underestimated the percentage abundance of the bacteria in the genus *Pseudomonas* and *Staphylococcus*.

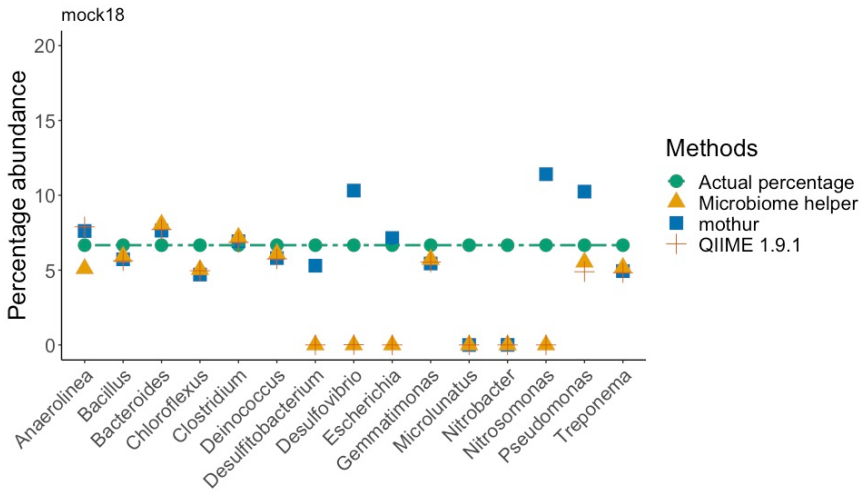


Figure 2.11: Percentage abundance of OTUs classified by 3 pipelines compared to the actual percentage at genus level of the mock18 dataset.

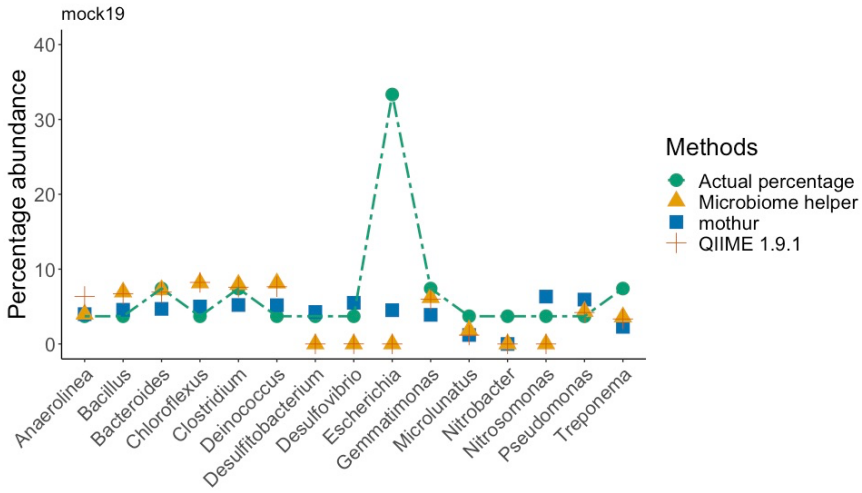


Figure 2.12: Percentage abundance of OTUs classified by 3 pipelines compared to the actual percentage at genus level of the mock19 dataset.

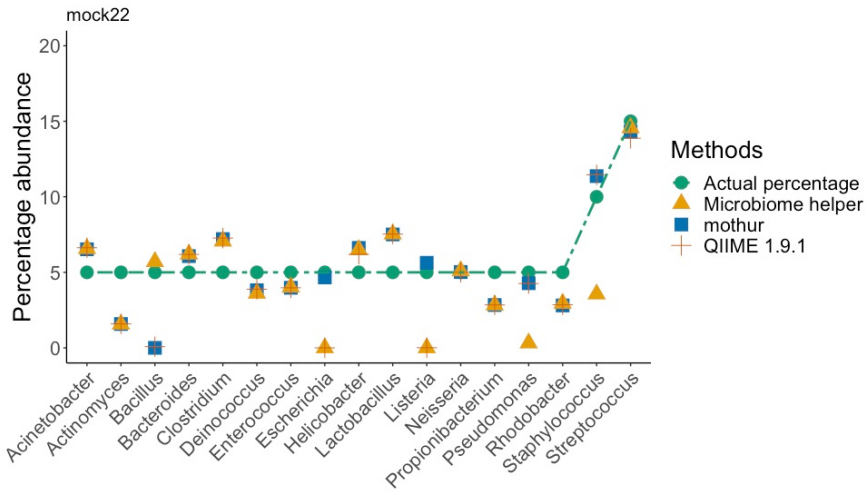


Figure 2.13: Percentage abundance of OTUs classified by 3 pipelines compare to the actual percentage at genus level of the mock22 dataset.

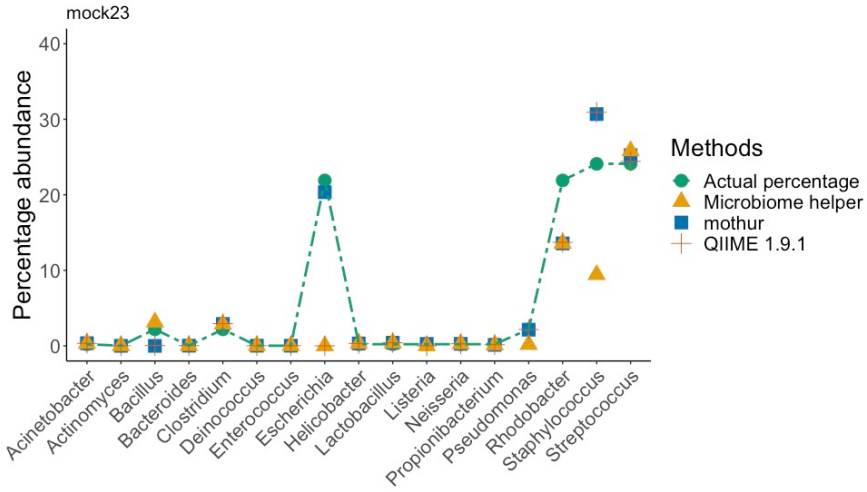


Figure 2.14: Percentage abundance of OTUs classified by 3 pipelines compare to the actual percentage at genus level of the mock23 dataset.

From the PCA plot at the phylum level (**Figure S7A**), mock 13, mock14 and mock15 samples classified by QIIME 1.9.1 clustered separately from the other pipelines. In the mock19 dataset, Microbiome helper and QIIME 1.9.1 were clustered together and closer to the actual percentage than mothur. The other datasets classified by each pipeline were clustered close together. At genus level (**Figure S7B**), all pipelines were clustered tightly (mock13, mock14, mock15, mock18 and mock19 datasets) except the mock22 and mock23 datasets classified by Microbiome helper that were clustered separately from the other 2 pipelines.

The Euclidean distance of pipelines from the actual percentage at phylum and genus level are shown in **Table 2.6** and **Table 2.7** respectively. At the phylum level, the pipeline with the lowest Euclidean distance from actual percentage was Microbiome Helper (3 out of 7 datasets each) while mothur and QIIME 1.9.1 had the lowest distance in 2 datasets each. At the genus level, mothur better than other pipelines by classifying the bacteria closest to the actual percentage in 4 out of the total 7 datasets. The other 3 datasets were classified closely to the actual percentage by Microbiome helper.

Table 2.6: Euclidean distance values between the expected percentage abundances for each pipeline and the actual percentage at phylum level of each dataset using the suggested reference database of each pipeline's SOPs.

Dataset	Microbiome helper	mothur	QIIME 1.9.1
mock13	7.24	8.24	13.07
mock14	7.85	8.49	14.36
mock15	9.41	11.07	15.65
mock18	23.07	21.80	21.68
mock19	10.36	19.47	10.33
mock22	8.33	8.14	8.26
mock23	13.82	13.67	13.09

Table 2.7: Euclidean distance values between the expected percentage abundances for each pipeline and the actual percentage at genus level of each dataset using suggested reference database of each pipeline's SOPs.

Dataset	Microbiome helper	mothur	QIIME 1.9.1
mock13	13.54	14.12	15.95
mock14	14.08	14.51	16.21
mock15	14.67	15.32	19.64
mock18	16.71	12.30	16.78
mock19	35.16	30.36	35.26
mock22	12.43	8.32	10.88
mock23	27.80	11.05	24.47

2.4 Discussion

From both comparisons using the custom reference database and developer's suggested reference database, the number of observed OTU results from Microbiome helper and mothur were close to the actual number of bacterial strains of the Mockrobiota datasets. The inflation of the number of observed OTUs from QIIME 1.9.1 was similar to previous research (Edgar, 2017). The spurious number of OTUs in the standard mock community which had low diversity raised the concerns over the unknown community which may have high diversity and may result in the more inflated number of OTUs (Edgar, 2017). One possible explanation for OTU inflation may be an error of filtering or denoising of the sequencing reads (Edgar, 2017). This explanation may be supported by the better accuracy of the Microbiome Helper pipeline compared to QIIME 1.9.1, as Microbiome helper is an improved pipeline from QIIME 1.9.1, notably that the cutoff value during the quality filtration steps is set at 25 instead of 3. The previous study also showed that the use of SortMeRNA and SUMACLUST methods instead of the default UCLUST method at the OTU-picking step reduced the spurious number of OTUs in the results from QIIME pipeline (Kopylova *et al.*, 2016). As the Microbiome Helper pipeline adopted this replacement, the number of observed OTUs analysed by Microbiome Helper was closer to the actual number of strains of the datasets than QIIME 1.9.1.

When the custom reference database provided by the Mockrobiota developer was applied to the analysis pipelines, the Euclidean distance results showed

that the classification of bacteria at genus level was quite similar between pipelines in each dataset. Microbiome helper had the lowest Euclidean distance to the actual percentage in 6 out of 7 datasets. In mock18, mock19, mock22 and mock23, the Euclidean distances of all pipelines in each dataset were close together. However, Microbiome helper still performed better than the other pipelines in mock13, mock14 and mock15 as the Euclidean distance were notably lower than the other pipelines. Although the datasets and reference databases were similar, these results show that different approaches in quality filtration and OTU classification steps between pipelines affect the accuracy of 16S metabarcoding analysis.

Mothur has a unique process which generates the alignment of the reads with reference 16S rRNA sequences. This step was expected to increase the robustness of OTU assignment (Schloss, 2010; Plummer and Twin, 2015); however, the Euclidean distance values of the Microbiome Helper pipeline were the lowest for most datasets. Another unique point was the default value used at the quality filtrating step of QIIME 1.9.1. The default Phred quality score cutoff of QIIME 1.9.1 was 3 while mothur and Microbiome helper used default quality score cutoff at 25 and 30. The allowance of these low-quality score reads through to the next steps of the pipeline may offer an explanation for the poor results in the analysis of mock13, mock14 and mock15 by QIIME 1.9.1.

Interestingly, several bacterial genera were not classified by pipelines when using developer's suggested reference database but were classified using

custom reference database. For example, using the developer's suggested reference database, the mothur pipeline was unable to classify bacteria in the genus *Bacillus* in mock22 and mock23 datasets. Microbiome helper pipelines could not classify the genus *Escherichia* and *Listeria*. However, these pipelines were able to classify these bacteria at the family level as unknown genera. These results showed that all pipelines with the developer's suggested reference databases have their own limitations in OTU-classification of bacteria at genus level. Therefore, for unknown samples, it might be better to classify bacteria at a higher taxonomic level than genus or switch to another reference database.

In the mock18 dataset, all of the pipelines were not able to classify the bacteria in genus *Microlunatus* using both the custom reference database and the developer's suggested database. The nucleotide basic local alignment search tool (BLAST) from BLAST+ software version 2.9.0 (Camacho *et al.*, 2009) was used to identify the *Microlunatus* sequencing reads in the raw dataset. From the BLAST results using The National Center of Biotechnology Information (NCBI) reference (Sayers *et al.*, 2019) showed that there was no sequencing read matched with the genus *Microlunatus*. It is likely that there is no read from the bacteria genus Microlunatus in the raw file of mock18 dataset. Therefore, none of the 3 pipelines could able to classify this OTU as a *Microlunatus*.

The Mockrobiota dataset is an excellent public source for practising and studying 16S metabarcoding analysis. A pipeline developer can also use these datasets to develop their new pipeline or adjust it for better accuracy. The

dataset has some low-quality sequences which can reflect the real situation when working with unknown field samples. Overall, Mockrobiota provides an idea of how the quality of the reads, pipeline algorithms and reference databases can affect the final results of 16S metabarcoding analyses.

In January 2018, the QIIME developer stopped supporting QIIME 1.9.1 and released a new version of QIIME (called “QIIME 2”) with improvement in the quality filtration and OTU classification. However, QIIME 1.9.1 is still supported by the developer and used by many researchers across the globe at the time of this analysis.

Although the results from these 3 pipelines showed low accuracy when using the custom reference database, analyses using the developer’s suggested reference database showed more inaccurate results. However, it is important to acknowledge that all the pipelines and databases had their own deficiencies. Researcher should use consistent methods with the positive mock-community control to ensure that the pipeline works similarly across datasets or studies (Pollock *et al.*, 2018). Moreover, in all pipelines, there are some commands that can adjust or filter the dataset, which may give better results from the analyses. The different reference databases used in each method may also affect the bacterial composition results produced from these pipelines. Therefore, adjustment of the pipeline with the right combination of database references may help to improve the accuracy of analysis. However, comparisons in this study were strictly based on the defaults settings and recommended databases taken from the developer’s SOPs on their websites.

By following the SOPs strictly, researchers all over the world can used the same pipeline found on the developer's website , and it would be expected to produce the same results between labs or researchers. Therefore, these results could be reproduced by any researchers and compared between studies.

In summary, Microbiome helper pipeline showed the best accuracy when using the custom reference database while mothur showed the best accuracy when using the developer's suggested database. Although mothur pipeline with the RDP reference database is more accurate than Microbiome helper with Greengenes database, mothur poorly handles the analysis with high number of samples and huge file size. Mothur requires a huge amount of computer resource in order to generate the distance matrix between samples which often results in error simulation as reported in previous study (Siegwald *et al.*, 2017). In **Chapter 4,** huge number of samples and sequencing reads from 16S metabarcoding analysis were performed with mothur and always resulted in a crash during the distance matrix generation. Therefore, Microbiome helper pipeline with Greengenes database was used in further 16S rRNA metabarcoding analysis because Microbiome helper was the most accurate pipeline when using the custom reference database and able to handle large number of samples. The analysis will be performed at the family level as it was shown in this chapter that all pipelines performed poorly at the genus level.

Chapter 3

Effects of exogenous enzyme supplementation and anti-coccidial vaccination on chicken growth performance

3.1 Introduction and Aims

Conventional feed research must be conducted to study the effects of exogenous enzymes and anti-coccidial vaccination in order to collect chicken growth performance data before attempting to study the relationship between host and microbiome. There are several factors which need to be taken into account before conducting a chicken microbiome experiment: Animal breed, feed ingredients, management practices and the environment can all influence growth performance, feed efficiency and gut microbiome of the chicken (Borda-Molina, Seifert and Camarinha-Silva, 2018; Clavijo and Flórez, 2018; Kers *et al.*, 2018). Therefore, this experiment was conducted at the project sponsor's facilities in Thailand with the chicken breed, rearing conditions, vaccination programme, raw diet ingredients and dietary formulating technology that the sponsor uses in their standard industrial practice. Moreover, the knowledge gained from this experiment could be adopted quickly by the sponsor in the future.

Anti-coccidial vaccination has previously been used as an experimental model to induce gut-damage in chickens (Chen *et al.*, 2015). Damage of the chicken intestinal epithelium due to the protozoal infection results in decreased growth performance from poor nutrient absorption (Chapman, 2014). By using an anti-coccidial vaccination, we are able to produce an impact on the immune system and gut health, as mild protozoal infection is expected to decrease growth performance and affect gut microbiota including intestinal gene expression. Therefore, it is possible to study growth performance, gut microbiota and

intestinal gene expression within one single experiment when using this model. Moreover, using anti-coccidial vaccination to study gut health is more realistic from the industry point of view than a pathogen or chemical model as it is commonly used in poultry production. In addition, large scale experimental settings for nutritional research are also not suitable for pathogen inoculation or chemical irritation models, as discussed in **Chapter 1**, section 1.5. Knowledge of the effect on growth performance and gut health produced by the anti-coccidial vaccination could be useful for the poultry producer, to allow them to handle or manage the disadvantages of subclinical infection from the vaccine.

Exogenous enzyme supplementation have also been shown to improve growth performance in many studies (Ravindran and Bryden, 1999; Selle *et al.*, 2003; Selle, Ravindran and Partridge, 2009; Cowieson *et al.*, 2019; Olukosi, Cowieson and Adeola, 2019). Improved nutrient digestibility by exogenous enzyme supplementation increases feed efficiency, growth performance and decreases environmentally harmful excreta waste (Adeola and Cowieson, 2011; Ravindran, 2013). Changes in nutritional quality and quantity, could also affect the gut microbiota and intestinal gene expression, especially expression of digestive and absorptive related genes (Bedford and Cowieson, 2012; Cowieson *et al.*, 2019).

Another objective of this animal experiment is to test whether there is a beneficial effect on growth performance when the exogenous enzyme is supplied to gut-damaged chickens due to anti-coccidial vaccination. The

damaged intestinal epithelium of the vaccinated chicken may be able to absorb more digested nutrients with the help of exogenous enzyme and thereby lead to improved growth. Therefore, the inclusion of exogenous enzyme to a diet may be the solution to the application of the anti-coccidial vaccination and may help reduce the use of anti-coccidial drugs in the future.

In summary, in this chapter, an experiment was conducted in order to study the interactive effects of exogenous enzymes and anti-coccidial vaccination on chicken performance. The experiment was conducted at the sponsor's facilities in Thailand. Growth performance parameters such as average body weight, feed consumption and feed conversion ratio were collected, calculated and analysed. Tissue and digesta samples were collected for further analysis. The effects of dietary and host health interventions on intestinal gene expression and the gut microbiome will be analysed and described in the following chapters.

3.2 Ethical statement

The experiment was conducted at the Feed Research and Innovation Center, Charoen Pokphand Group's facilities located at Chonburi province, Thailand. Experimental protocols were approved by the Feed Research and Innovation Center's Animal Care and Use Committee (FRIC-ACUP no.17022017) and approved by the Roslin Institute Animal Welfare and Ethics Committee (RI-AWA-24). The sample size was determined based on a preliminary study of gut microbiota affected by different feed ingredients and anti-coccidial

vaccination performed earlier at the sponsor's facilities in Thailand with advice from the Roslin Institute's statistician.

3.3 Materials and Methods

3.3.1 Animal management

Fertilised eggs from the Ross 308 broiler breeder unit of the Feed Research and Innovation Center, Chonburi province, Thailand, were incubated and routinely processed at the hatchery unit of the center. Vaccination against Newcastle disease, infectious bronchitis and infectious bursal disease were given to the chicken as a routine preventive strategy of the research center. After vaccination and sex determination were performed, a total of 1,680 male chicks hatched on 1st of December 2017 were randomly selected and allocated into 4 treatments in a 2 by 2 factorial arrangement as shown in **Table 3.1**. The given factors were anti-coccidial vaccination and exogenous enzyme supplementation.

Table 3.1: Treatments with 2 by 2 factorial arrangement.

Treatment	Anti-coccidial vaccination	Exogenous enzymes
1	Vaccinated	Without
2	Vaccinated	Added
3	Non-vaccinated	Without
4	Non-vaccinated	Added

Each of the 4 treatments had 10 replicates and 42 chickens per replicate (one chicken pen per replicate). Treatments were allocated in a randomised complete block design. Chickens were reared on the floor pen with new rice hull bedding in a negative pressure tunnel-ventilating system house. A solid plastic wall was used to minimise contact between chickens, bedding material or faecal matter to the adjacent pen and prevent chickens from crossing between pens. A schematic diagram of the animal house and treatment allocation are shown in **Figure 3.1**. Birds from all treatments were reared on both sides of the house to minimise confounding factors related to location within the house. At the front end of the house, vaccinated and non-vaccinated chickens were allocated to opposite sides of the house to prevent cross-contamination while at the rear end, they were switched to another side of the house with one empty pen between them to minimise cross-contamination as shown in **Figure 3.1**. The dimensions of the pens were 1.5 by 2.8 metres (or 4.2 square metres) with the stocking density at 26 kilogrammes per square metre (estimated body weight at 2.6 kilogrammes per chicken). A sample photo of a chicken pen is shown in **Figure 3.2**. Daily routine husbandry or research intervention by animal husbandry or researchers were performed using shoes provided in front of each pen to prevent any carry-over from pen to pen. After routine work with the non-vaccinated chickens was completed, routine works with the vaccinated chickens were performed without returning to the non-vaccinated chicken pens within that day. Chickens were reared to 35 days of age which was the standard age of slaughter in Thailand.

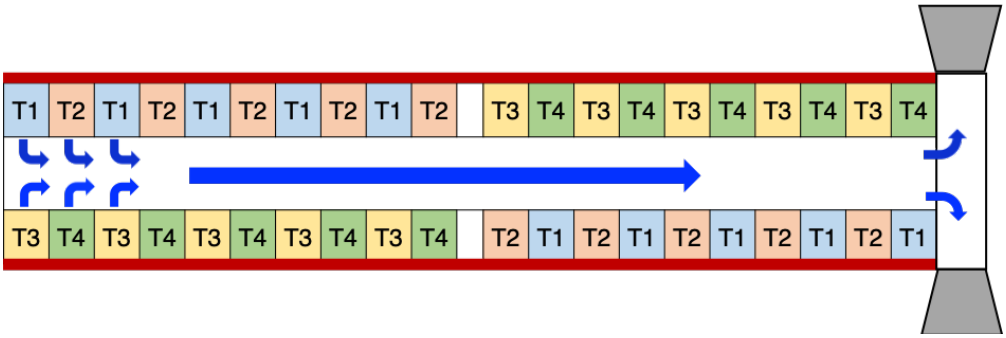


Figure 3.1: A schematic diagram showing treatment allocations of the chickens within the animal house. Vaccinated (T1 and T2) and non-vaccinated (T3 and T4) chickens were reared on the different side of the house and were switched at the rear end of the house to minimise confounding factors from the pen location. Every pen received fresh air flow through the water-cooling evaporative pads (red) which were located at the side along the house. The air (dark blue) went through the pen to the main corridor and left the house through the exhaust fans (grey).

Figure 3.2: Chicken rearing pen with rice hull bedding.

3.3.2 Diets

The wheat-maize soya-bean meal diets were formulated according to the phases and nutritional requirements from the Ross 308 nutritional specification version 2014. Diets given to the chickens were based on the specific nutrient requirement differences between chicken ages. Starter diets were given from day 1 to day 10 of age (starter phase), grower diets were given from day 11 to day 24 of age (grower phase), and finisher diets were given from day 25 to day 35 of age (finishing phase). The percentage of available phosphorus in the diet at each phase was decreased by 0.15% from the breed recommendation as phytase enzymes (Ronozyme® HiPhos GT, DSM Nutritional Products, Kaiseraugst, Switzerland) were included at 100 ppm (1,000 phytase units/kg of diet) in all diets. In exogenous enzyme supplemented diets (treatment 2 and 4), xylanase plus beta-glucanase enzymes (Ronozyme® Multigrain, DSM Nutritional Products, Kaiseraugst, Switzerland) were included at 100 ppm (endo-1,4-beta-xylanase: 270 unit/kg of diet, endo-1,3(4)-beta-glucanase: 70 unit/kg of diet, endo-1,4-beta-glucanase: 80 unit/kg of diet) and protease enzymes (Ronozyme® ProAct, DSM Nutritional Products, Kaiseraugst, Switzerland) were included at 200 ppm (15,000 protease units/ kg of diet). These enzymes were selected at the suggestion of DSM in order to maximise the potential of the use of multiple exogenous enzymes to help lessen the impact of anti-coccidial vaccination on chicken growth performance. Moreover, these enzymes are commercially available in the sponsor's country and used regularly (both individual or multiple) in the sponsor's practice. The

compositions of the experimental diets at each phase were as shown in **Table S6**, and their calculated nutritional values were as shown in **Table S7**. Diets were provided as crumbled pellets during the starter phase and as pellets during the grower and finisher phases. Diets and water were given *ad libitum*.

3.3.3 Anti-coccidial vaccine administration

Fortegra® vaccine (MSD Animal Health, Madison, NJ, USA) was diluted with sterile normal saline at the manufacturer's recommended dosage. Each dosage contains live oocysts of the following species of coccidian: at least 600 oocysts of *Eimeria acervulina*, at least 200 oocysts of *Eimeria maxima*, at least 100 oocysts of *Eimeria maxima MFP*, at least 400 oocysts of *Eimeria mivati* and at least 200 oocysts of *Eimeria tenella*. These *Eimeria* species could cause mild infection across all small intestinal regions and caeca as shown in **Table 1.1**. The inclusion of the highly pathogenic strain, *Eimeria maxima MFP*, could cause a rapid immune response which is suitable for fast-growing broiler chickens (information from retailer's representative). *Eimeria mivati* is a species which is commonly confused with *Eimieria mitis* from gross pathology, however, molecular diagnostics is able to distinguished between them (Edgar and Seibold, 1964; Schwarz *et al.*, 2009). The vaccine was given to the chicken at 3 days of age via oral gavage at 0.2 millilitres per chicken (**Figure 3.3**). Chickens in non-vaccinated groups were orally gavaged with sterile normal saline at the same volume. Two researchers administered the saline solution to the non-vaccinated chickens before administering the vaccine to the vaccinated chickens.

Figure 3.3: Anti-coccidial vaccine adiministered via oral gavage.

3.3.4 Chicken performance data collection

Total body weight and feed residue of each pen were collected at the end of each phase. Chickens were observed daily and dead chickens were removed and recorded for bodyweight immediately when observed. Bodyweight, feed consumption (subtraction of the amount of feed residue from feed given), feed conversion ratio (FCR or total weight of feed divide by chicken bodyweight) and mortality rate were calculated at the end of each phase. The European Production Efficiency Factor (EPEF) was calculated at the end of the grower and finisher phases by using the following formula.

EPEF = (Average Daily Gain X Survival rate) / (Feed Conversion Ratio x 10)

3.3.5 Sample collection

At the end of each phase (10, 24 and 35 days of chicken age), one chicken per pen was randomly selected for intestinal content and tissue collection for gut bacteria and intestinal gene expression analysis. Chickens were euthanised by cervical dislocation, followed by exsanguination to confirm death. Samples were collected and processed for further analysis which will be described in the following chapters.

3.3.6 Coccidiosis lesion score evaluation

Coccidiosis lesion score was evaluated at every sample collection. The aim of the lesion score evaluation was to confirm the success of anti-coccidial vaccine administration and to monitor the status of the vaccine or disease among treatments; therefore, no statistical comparison was performed on the coccidiosis lesion score. Duodena, ilea and caeca were evaluated with a score from 0 to 4, as these gut regions are colonised by different *Eimeria* species in the vaccine. (Johnson and Reid, 1970; Raman *et al.*, 2011). The duodenum is colonised by *Eimeria acervulina,* and *Eimeria mivati*, the ileum is colonised by *Eimeria maxima* and *Eimeria mivati* and the caecum is colonised by *Eimeria tenella* (Johnson and Reid, 1970). Different scoring systems at each intestinal region are shown in **Table 3.2**.

The presence of *Eimeria* protozoa in the intestinal tissue was confirmed by histopathological analysis. The intestinal lumen was opened longitudinally with clean dissecting scissors before preservation in 0.4% formalin solution. The

sample was shipped to the Veterinary Diagnostic Lab of Charoen Pokphand Group after sample collection for histopathological diagnosis using Hematoxylin and Eosin staining. The vaccination and coccidiosis lesion score of the samples were blinded to the veterinary pathologist.

Table 3.2: Coccidiosis lesion scoring system at duodenum, ileum and caecum of the chicken*.

Score	Duodenum	Ileum	Caecum
0	No visible lesion	No visible lesion	No visible lesion
1	Scattered white plague-like lesions (< 5 lesions per square centimetres)	Small red petechiaes appearing on the serosal side of intestine	Few scatter bleedings on the mucosal surface with no thickening
2	Closer lesions but not coalescent. (6-10 lesions per square centimetres)	More numerous red petechiae on the serosal surface with orange mucous filled lumen	Normal caecal content present with blood. Thickened caecal wall
3	Lesions are numerous enough to cause coalescent. Intestinal wall thickening. (11 - 15 lesions per square centimetres)	Ballooned and thickened intestinal wall with orange and traces of blood in the mucous	Large amount of blood with greatly thickened caecal wall
4	Greyish mucosal wall. Lesions fused together createing coalesced colonies	Significant ballooning with blood clots. Orange mucous and watery content with putrid odor	Massive hemorrhage. Reddish or brownish mucous contents

*Summarised from Johnson and Reid, (1970) and Raman et al. (2011).

3.3.7 Statistical analysis

The effects of anticoccidial vaccination and enzyme supplementation and their interactions on chicken growth performance (average chicken body weight, feed consumption, FCR, mortality rate and EPEF) were analysed by 2-way analysis of variance (ANOVA), as a 2 by 2 factorial treatment arrangement with R version 3.5.3. The chicken growth performance was analysed at each phase and from hatch to the end of each phase. A significant difference was defined as a P-value < 0.05. The pen was defined as an experimental unit throughout the statistical analysis of growth performance (n = 40 pens).

The effect of enzyme supplementation on different chicken gut health status parameters was analysed. A growth performance data comparison between gut damaged chickens (T1 vs T2) and between non-vaccinated chickens (T3 vs T4) was performed at the end of each phase using the pairwise *t*-test in R version 3.5.3. A significant difference was defined as a P-value <0.05.

3.4 Results

3.4.1 Chicken growth performance

During anti-coccidial vaccine administration, no ill effects due to the oral gavage procedure such as gagging, coughing or asphyxation were observed; therefore, the mortality rate at the end of each phase was not a direct result of the administration technique.

At the end of the starter phase, vaccination factor significantly affected the average chicken body weight and feed consumption but did not significantly

affect the FCR and mortality rate as shown in **Table 3.3**. In contrast, enzyme supplementation significantly decreased feed consumption and improved FCR.

The growth performance from hatch to the end of the grower phase (1-24 days of chicken age) is shown in **Table 3.4**. During this period, enzyme supplementation significantly decreased the FCR by 0.036 without significantly affecting the average body weight and feed consumption. Conversely, the average body weight of vaccinated chickens was significantly lower than non-vaccinated chickens, at 139 grammes per bird, with significantly lower feed consumption at 1,708 grammes per bird compared to 1,784 grammes per bird in non-vaccinated chickens. Therefore, the FCR of vaccinated chickens was significantly higher than non-vaccinated chicken at 0.072. The effects from anti-coccidial vaccination and enzyme supplementation in the grower phase during 11 to 24 days of chicken age (**Table S8**) also showed a similar trend to the starter-grower phase during days 1 to 24 of chicken age (**Table 3.4**).

At the end of the experiment on day 35 of chicken age, the final average body weight of the chickens in the vaccinated group was significantly lower at 98 grammes per bird, with a significantly higher FCR of 0.037, than the non-vaccinated group (**Table 3.5**). Conversely, the inclusion of enzymes in the diet led to significant improvements in FCR and EPEF as shown in **Table 3.5**. When considering growth performance at the finisher phase (during day 25 to 35 of chicken age), the vaccinated chickens had significantly higher weight gain and lower FCR than non-vaccinated chickens (**Table S9**). This effect was

due to a coccidiosis infection or cross-contamination from the vaccinated chickens to the non-vaccinated chickens. This was confirmed by the coccidiosis lesion score determined from the intestines of non-vaccinated chickens (**Figure 3.4**) and the histopathological diagnosis results from duodenum tissues (**Figure S8** and **Figure S9**). Despite the coccidiosis infection or cross-contamination to the non-vaccinated chickens, the enzymes still showed positive effects on FCR and EPEF at the end of the finishing phase as shown in **Table 3.5**.

Although a significant interaction between exogenous enzymes and anti-coccidial vaccination was not observed in this study, the results still showed the benefits of feeding the anti-coccidial vaccinated chickens with the exogenous enzyme supplemented diet. In vaccinated chickens, the FCRs of the enzyme supplemented chickens were significantly lower than non-supplemented chickens at all phases, as shown in **Figure 3.5** (P-value = 0.001, 0.045 and 0.022 at 10-, 24- and 35-days of chicken age). Enzyme supplementation also significantly reduced the FCRs of the non-vaccinated chickens at 10- and 35-days of chicken age (P-value = 0.015 and 0.026 respectively).

Table 3.3: Summary of chicken growth performance (mean ± SD) from hatch to the end of the starter phase (day 1 - 10 of chicken age)

Factors	Average body weight (g)	Cumulative feed consumption (g/bird)	FCR	Mortality rate (%)
Enzyme supplement				
Without	287 ± 8.6	278 ± 6.9 †	0.969 ± 0.014 †	0.595 ± 1.3
Added	288 ± 5.8	274 ± 4.5 †	0.950 ± 0.014 †	1.190 ± 2.3
Vaccination				
Non-vaccinated	291 ± 6.4 ‡	278 ± 5.9 ‡	0.955 ± 0.016	1.071 ± 2.0
Vaccinated	284 ± 6.6 ‡	274 ± 6.1 ‡	0.963 ± 0.016	0.714 ± 1.7
P-value				
Enzyme supplement	0.600	0.014	< 0.001	0.875
Vaccination	0.001	0.020	0.06	0.432
Interaction	0.054	0.072	0.59	0.432

†, ‡ Represents statisticaly significant difference between groups of the same symbol within column (P-value <0.05)

Table 3.4: Summary of chicken growth performance (mean ± SD) from hatch to the end of the grower phase (1 - 24 days of chicken age); EPEF = European Production Efficiency Factor.

Factors	Average body weight (g)	Cumulative feed consumption (g/bird)	FCR	Mortality rate (%)	EPEF
Enzyme supplement					
Without	1373 ± 80	1762 ± 82	1.286 ± 0.062 †	1.67 ± 2.4	436 ± 41 †
Added	1386 ± 71	1730 ± 60	1.250 ± 0.039 †	1.90 ± 2.3	451 ± 37 †
Vaccination					
Non-vaccinated	1449 ± 22 ‡	1784 ± 50 ‡	1.232 ± 0.031 ‡	2.02 ± 2.6	476 ± 20 ‡
Vaccinated	1310 ± 32 ‡	1708 ± 72 ‡	1.304 ± 0.048 ‡	1.55 ± 2.1	411 ± 21 ‡
P-value					
Enzymes supplement	0.158	0.104	0.004	0.749	0.015
Vaccination	< 0.001	< 0.001	< 0.001	0.523	< 0.001
Interaction	0.252	0.821	0.490	0.523	0.911

†, ‡ Represents statisticaly significant difference between groups of the same symbol within column (P-value <0.05)

Table 3.5: Summary of chicken growth performance (mean ± SD) from hatch to the end of the finisher phase (1 - 35 days of chicken age); EPEF = European Production Efficiency Factor.

Factor	Average body weight (g)	Cumulative feed consumption (g/bird)	FCR	Mortality rate (%)	EPEF
Enzyme supplement					
Without	2705 ± 74	4221 ± 109	1.561 ± 0.041 †	3.10 ± 3.2	471 ± 30 †
Added	2727 ± 75	4160 ± 96	1.526 ± 0.031 †	2.86 ± 2.9	490 ± 23 †
Vaccination					
Non-vaccinated	2765 ± 46 ‡	4216 ± 94	1.525 ± 0.035 ‡	3.21 ± 3.8	494 ± 29 ‡
Vaccinated	2667 ± 64 ‡	4165 ± 114	1.562 ± 0.037 ‡	2.74 ± 2.0	467 ± 20 ‡
P-value					
Enzyme supplement	0.211	0.067	0.001	0.812	0.014
Vaccination	< 0.001	0.117	< 0.001	0.635	< 0.001
Interaction	0.494	0.602	0.874	1.000	0.672

†, ‡ Represents statisticaly significant difference between groups of the same symbol within column (P-value <0.05)

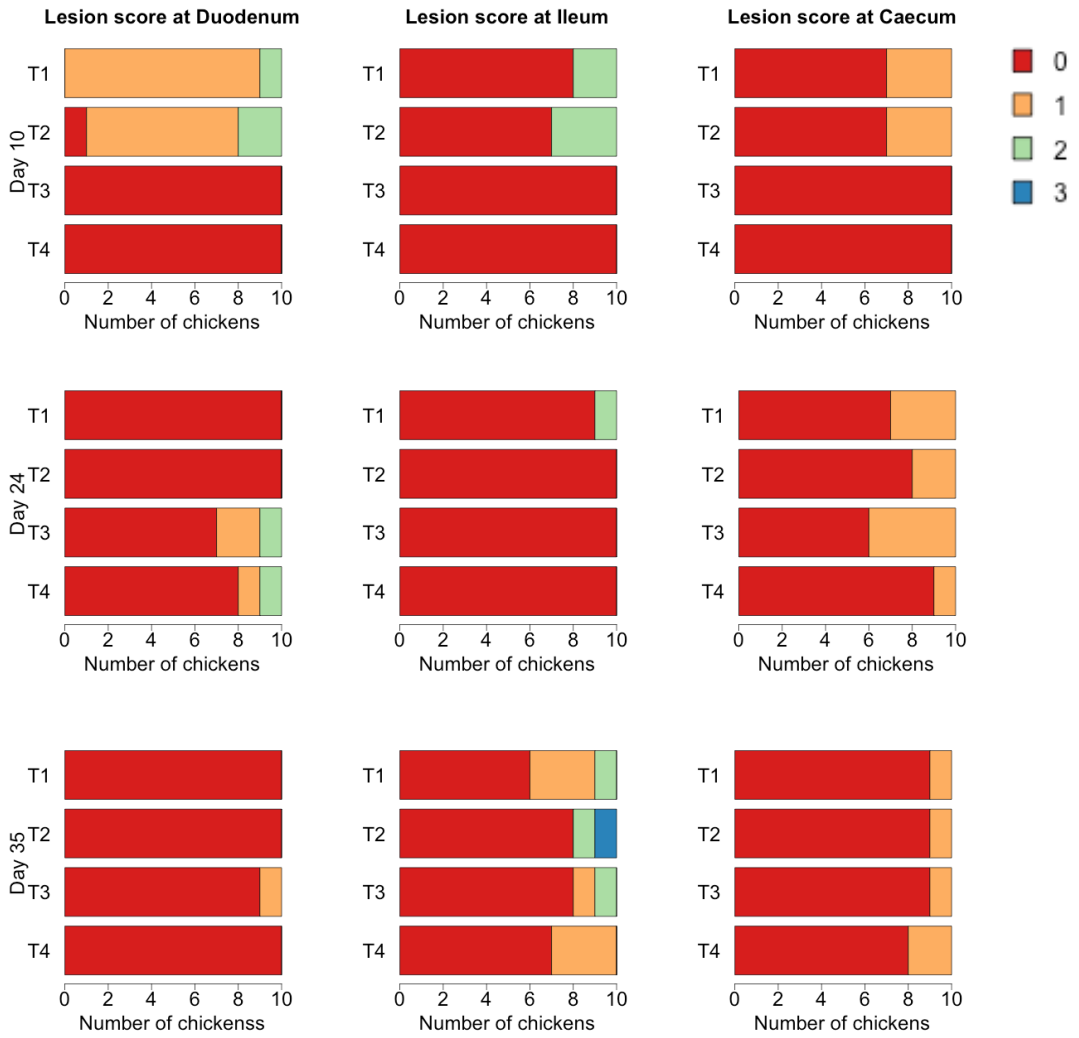


Figure 3.4: The count of coccidiosis lesion scores at 3 intestinal sections (duodenum, jejunum and caecum) of 10 chickens per treatment on each sample collection (10-, 24- and 35-day-old chickens). The lesion scores ranged from 0 to 4. (T1 = vaccinated + non-enzymes supplemented chickens, T2 = vaccinated + enzymes supplemented chickens, T3 = non-vaccinated + non-enzymes supplemented chickens and T4 = non-vaccinated + enzymes supplemented chickens,)

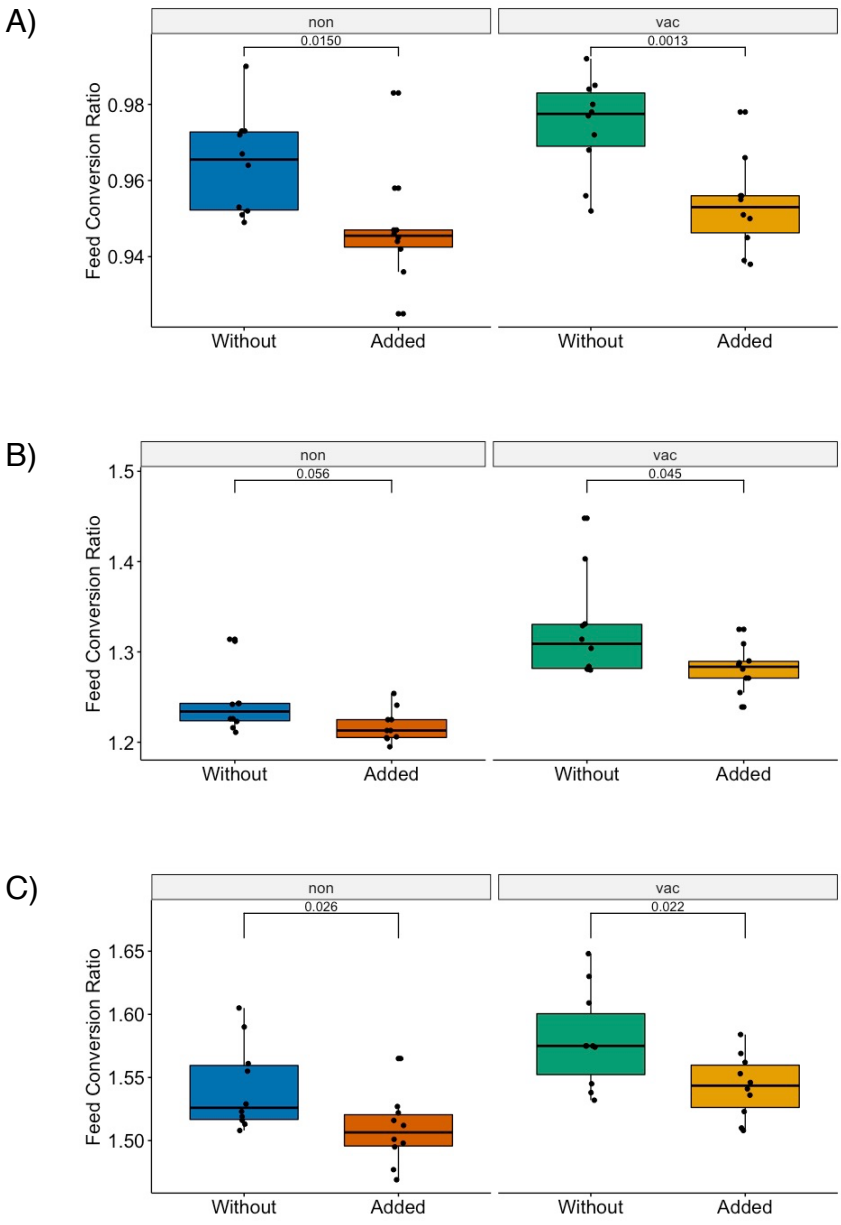


Figure 3.5: Feed Conversion Ratio of each treatment at; A) 10 days, B) 24 days and C) 35 days of chicken age with P-value generated by a pairwise t-test. (non; non-vaccinated chicken, vac; vaccinated chicken, without; non enzyme supplement and added; enzymes supplement).

3.4.2 Coccidiosis lesion score

The coccidiosis lesion score was evaluated on individual chickens, and the score was counted and summarised as shown in **Figure 3.4**. At 10 days old, 90% of the vaccinated chickens had coccidiosis lesions at duodenum, while approximately 30% of them were observed at ileum and caecum (**Figure 3.4**). In contrast, none of the chickens in the non-vaccinated group showed visible coccidiosis lesions at 10 days of age.

At 24 days old, the coccidial lesions were observed and scored in all intestinal regions in the non-vaccinated chickens (**Figure 3.4**). Visible coccidiosis lesions were not observed at the duodenum of vaccinated chickens, but lesions were still observed at the jejunum and caecum. The finding of these visible lesions in the non-vaccinated group with the decrease growth performance during finisher phase (25 to 35 days of chicken age) as shown in **Table S6** may be evidence of cross-contamination from the vaccinated to the non-vaccinated group.

As suggested by a veterinarian, duodenal samples were sent to the diagnostic lab for histopathological diagnosis: two samples with visible coccidiosis lesions from non-vaccinated chickens and one sample without visible coccidiosis lesion from a vaccinated chicken. The histopathological diagnostic results showed the presence of gametocyte and oocyst stages of coccidia in the duodenal tissues of the two sampled non-vaccinated chickens (**Figure S8**). Interestingly, there was no presence of coccidia in the duodenal tissues of the sampled vaccinated chicken (**Figure S9**). These findings confirmed that there

was a coccidiosis infection or cross-contamination from vaccinated chickens to non-vaccinated chickens.

At of 35 days of chicken age, coccidiosis lesions were observed in the caecum of chickens in all treatments at low incidence and score (**Figure 3.4**). Lesions were found at the duodenum of one chicken from treatment 3 and at the ileum of at least 2 chickens in every treatment.

3.5 Discussion

As coccidiosis lesions are the result of gut epithelial damage caused by the developmental stages of the protozoa, it could not be confirmed whether lesions were the results of vaccination or infection from wild-type protozoa. However, there was no report of a coccidiosis outbreak or infection in the other chicken houses at the research center during the same period of this experiment.

At 10 days of chicken age, coccidiosis lesions were observed in the duodena, ilea and caeca of vaccinated chickens. The presence of the coccidiosis lesions with a low score (score 1-2) in the anti-coccidial vaccinated chicken was similar to previous observations (Chapman, 2000; Williams, 2002). The major site of coccidiosis lesions of 10-day-old chickens was the duodenum, as almost all vaccinated chickens showed positive lesion scores. Interestingly, the majority of the 10-day-old vaccinated chickens (70%) showed no lesions in the ileum and caecum. This finding might be explained by the previous report that

lesions caused by *Eimeria acervulina* were expected to be found earlier than other *Eimeria* species (Williams and Andrews, 2001).

In this study, coccidiosis lesion score at the duodenum, ileum and caecum were observed at the end of starter phase or 7 days post-vaccination. The previous study reported that the first observation of lesion formation was 5 days post-vaccination, and the peak observation was at approximately 12-18 days post-vaccination when using live-attenuated anti-coccidial vaccine (Williams and Andrews, 2001). As the vaccine was given on day 3 of chicken age, the time from vaccination to the end of the starter phase (day 10 of chicken age) was closely related to the first complete *Eimeria* lifecycle and the beginning of the second cycle (Williams and Andrews, 2001; Tewari and Maharana, 2011; Chapman, 2014). Therefore, the damage of the epithelium from the development of the protozoa should occur during the starter phase and may have resulted in the negative growth performance observed at the end of the starter phase. The negative correlation between growth performance and the presence of the coccidiosis lesion score was also observed in a previous study (Conway, McKenzie and Dayton, 1990).

Interestingly, the presence of lesions in the duodenum at the end of the starter phase was observed in 95% of vaccinated chicken samples. The damage of the duodenal epithelium may be related to the significantly lower bodyweight of the chickens, as the duodenum is known as a location that plays a pivotal role in nutrient digestion and absorption (Russell and Ruff, 1978; Chapman, 2014). In contrast, a previous study showed that the coccidiosis lesion scores

observed after the inoculation of *Eimeria acervulina* (10^6 oocysts/bird)k which targets the duodenal region, did not significantly relate with the bodyweight of the chicken at 5-days post-inoculation (Conway, McKenzie and Dayton, 1990). A negative relationship between coccidiosis lesion score and bodyweight has been observed in chickens inoculated with *Eimeria maxima* and *Eimeria tenella* (Conway, McKenzie and Dayton, 1990). However, in this study, the coccidiosis lesion scores at the target gastrointestinal regions of *Eimeria maxima* and *Eimeria tenella* (ileum and caecum respectively) range from scores 1-2 and were observed in approximately 30% of the vaccinated chicken. These findings collectively show that the coccidiosis lesion score could be used as evidence of a gut-damaged chicken related to protozoal infection. However, the relationship between score and growth performance cannot be compared between studies, as the vaccine, strains of protozoa, observation period, chicken breed and environment were different from study to study.

Although the coccidiosis lesions were observed in non-vaccinated chickens at the end of the grower phase (24 days of chicken age), these chickens still had significantly higher body weights and lower FCRs than the vaccinated chickens. The vaccinated chickens showed a poor growth performance as the grower phase (11-24 days of chicken age or 8-21 days post-vaccination) was the peak period of the development of the immune response to the *Eimeria* infection which has been reported as 9 to 20 days post-infection (Trees *et al.*, 1989; Wallach, 2010). To develop the immune response against the pathogen,

chickens need to sacrifice a significant amount of the nutrients used for growth to support the immune system (Iseri and Klasing, 2014; Dersjant-Li *et al.*, 2016). Iseri and Klasing, 2014, reported that the amount of lysine in the immune system of healthy chicken was equivalent to the amount of lysine in 5.4% of a pectoralis muscle (Iseri and Klasing, 2014). With a limited supply of nutrients due to due to poor digestion and absorption, increasing demand from the immune system could result in insufficient nutrients for the growth of the animal (Iseri and Klasing, 2014; Dersjant-Li *et al.*, 2016). Increased energy usage to support the immune system and growth has also been observed in chickens challenged with high-dose of anti-coccidial vaccination (Dersjant-Li *et al.*, 2016). Therefore, during the estimated peak period of the immune response to the anti-coccidial vaccination both the poor absorption of nutrients from the damaged gut and also the immune response may have led to the decrease in growth performance of the vaccinated chickens when compared to the non-vaccinated chickens at the grower phase. Further analysis on the nutrient availability or an *in vivo* digestiblity analysis could help explain the different absorption status between non-vaccinated and vaccinated chickens. However, this analysis was not performed in this study.

During the finisher phase, after the cross-contamination or coccidiosis infection in non-vaccinated chickens, the vaccinated chickens had higher average body weight and lower FCR than the non-vaccinated chickens. This compensatory growth of the vaccinated chickens might be the results of the protection from the vaccine to the infection, similar to many other previous

observations (Li *et al.*, 2005; Lehman, Moran and Hess, 2009; J. T. Lee *et al.*, 2011; Ritzi *et al.*, 2016). From histopathological diagnosis, the absence of the gametocytes at the duodenum of vaccinated chicken could be a result of an effective immune repsonse against the protozoa due to the anti-coccidial vaccination. As previous findings have shown that the vaccine can significantly reduce the coccidiosis lesion score caused by *Eimeria* protozoa at 7-days post-infection (Williams, 2003), the effective protection against protozoa could reduce the damage from re-infection of the protozoa and result in better performance of the vaccinated chickens compared to the non-vaccinated chickens at the finisher phase. Conversely, as the non-vaccinated chickens started developing intestinal lesions around 24 days of age, their poor growth performance during the finisher phase could be a result of the poor nutrient utilization of damaged gut and the development of immune responses against protozoa similar to the starter and grower phase of the vaccinated chickens.

For the exogenous enzyme supplementation, the enzymes significantly improved growth performance at all phases. Enzymes-supplemented chickens had lower FCR than non-supplemented chickens; 0.019, 0.039 and 0.034 at the end of grower, starter and finisher phase respectively. These findings resemble those of several studies on the effects of exogenous enzymes on chicken performance (Cowieson and Adeola, 2005; Józefiak *et al.*, 2006; Angel *et al.*, 2011; Kalmendal and Tauson, 2012; Munyaka *et al.*, 2015; Stefanello *et al.*, 2015; Pasquali *et al.*, 2016; Erdaw, Wu and Iji, 2017; Mohammadigheisar and Kim, 2018). Supplementation of multiple enzymes

improved the growth performance of chickens similar to previous reviews and studies (Selle *et al.*, 2003; Cowieson and Adeola, 2005; Juanpere *et al.*, 2005). However, it cannot be concluded whether it was the protease or carbohydrase enzymes alone or in combination that improved chicken feed efficiency. Recent research shows that exogenous enzymes improved feed efficiency at different chicken ages. Protease enzymes significantly improved the FCR of the chicken at 21 days of age, while xylanase improved the FCR of the chicken at 42 days of age (dos Santos *et al.*, 2017). Therefore, in this study, a combination of multiple exogenous enzymes gave a beneficial effect on feed efficiency in all phases of chicken growth.

The effects of exogenous enzyme supplementation on anti-coccidial vaccinated, or coccidial infected chickens contrasts across studies. A previous study showed that protease enzymes alleviated the adverse effects of coccidiosis of chickens during 10-16 days of age (Peek *et al.*, 2009). In contrast, other researchers found no significant difference of enzyme supplementation on feed efficiency in anti-coccidial vaccinated chickens (Parker *et al.*, 2007; Walk *et al.*, 2011; Dersjant-Li *et al.*, 2016). Dersjant-Li et al. (2019), found that, although feed efficiency was not improved, there was an interaction effect between direct-fed microbial plus exogenous protease enzymes on the reduction of the inflammatory response to a high dosage of anti-coccidial vaccination (Dersjant-Li *et al.*, 2016). In this study, no significant difference was observed in the interaction between exogenous enzyme supplementation and anti-coccidial vaccination. No observed interaction

suggested that there was no significant difference in the response to the exogenous enzymes supplementation between vaccinated and non-vaccinated chickens. However, when analysing only the results of vaccinated chickens, the exogenous enzyme decreased the FCR of the vaccinated chickens at all phases. These results provide evidence that the exogenous enzymes help chickens overcome the adverse effects on growth performance of the anti-coccidial vaccination. Although their growth performance parameters were still not equal to those of non-vaccinated chickens, the improvement in feed efficiency showed that the exogenous enzymes supplementation to the vaccinated chickens could be an alternative solution to the use of anti-coccidial drugs in coccidiosis prevention. Moreover, the cost of the enzyme supplementation is considerably small when compares to the return from improved growth performance. This shows that enzyme supplementation to vaccinated chicken is a cost effective solution to the poultry producer. Better nutrient utilisation in the vaccinated chicken by the supplementation of enzymes could also help reduce the impact from the nutrient waste and drug residue while the chicken is immunised to the disease.

In summary, the use of anti-coccidial vaccination with exogenous enzyme supplementation for coccidiosis prevention could provide a better sustainable chicken production when compare to the use anti-coccidial drugs. However, it is still a challenge to find the best solution to the adverse effects of the anti-coccidial vaccination on growth performance. Further studies to find the best dosage and species mixture of the vaccine, which induces enough of an

immune response to protect the chicken from *Eimeria* protozoa, are still required. Novel enzymes, new combinations of multiple exogenous enzymes or other feed additives that help vaccinated chickens overcome the decreased growth performance are still needed. Once a solution is found, a ban of anti-coccidial drugs could result in better and sustainable way to produce chicken to feed the world population.

Chapter 4

Intestinal microbiome study of exogenous enzyme supplementation and anti-coccidial vaccination in chickens

4.1 Introduction and Aims

In **Chapter 3**, it was shown that anti-coccidial vaccination significantly decreased growth performance of the chicken and exogenous enzymes supplementation significantly increased feed efficiency, but no interaction was found between vaccination and supplementation. It was also shown that exogenous enzymes improved FCR of the vaccinated chickens. In this Chapter, gut microbiome analysis was performed to study the effects of anti-coccidial vaccination and exogenous enzymes on the gut microbiota.

The chicken gut microbiome was compared between anti-coccidial vaccination and exogenous enzymes supplementation using quantitative 16S rRNA metabarcoding analysis. In **Chapter 2**, Microbiome Helper was found to be the most accurate pipeline for 16S rRNA metabarcoding analysis, so Microbiome Helper v1 was used for this analysis in the current Chapter. Effects of the anti-coccidial vaccination and exogenous enzymes supplementation on gut microbial diversity were analysed and compared. Bacterial OTU richness within individual chickens (alpha diversity) were calculated and compared between anti-coccidial vaccination and enzyme supplementation (Goodrich *et al.*, 2014). The effects of the anti-coccidial vaccination and exogenous enzymes supplementation on the difference in bacterial taxonomic abundance between chickens given these factors (beta-diversity) were analysed (Goodrich *et al.*, 2014). Functional analysis of gut microbiota was performed using shotgun metagenomics. Using the Carbohydrate Active enZYmes (CAZy) database, the abundance of the carbohydrate-active enzyme

(CAZyme)-related genes in the chicken gut bacterial genome was compared between given factors (Cantarel *et al.*, 2009). As the primary function of the caecum is bacterial fermentation, the micro-organisms of the caecum produce a range of CAZymes such as cellulases and hemicellulases, to digest or deconstruct the recalcitrant polysaccharides such as beta-glucans, cellulose and arabinoxylans found in grain-type diets (Clench and Mathias, 1995; Sergeant *et al.*, 2014). The main product from the bacterial fermentation of these polysaccharides in the caecum is short-chain fatty acids which are absorbed by the chicken as a nutrient and also regulate the caecal micro-organisms (Sergeant *et al.*, 2014). Comparison of the abundance of CAZymes was performed in order to study the effects of the anti-coccidial vaccination and enzyme supplementation on the functional properties of the gut bacteria.

Previous studies reported the effects of anti-coccidial vaccination and exogenous enzymes supplementation on the chicken gut microbiome (Munyaka *et al.*, 2015; Park *et al.*, 2017). However, the interaction effects between vaccination and exogenous enzymes supplementation have not been reported. In this Chapter, the effects of the anti-coccidial vaccination and exogenous enzymes supplementation on the gut microbiome will be analysed. Ileal and caecal contents collected at the end of each phase were collected and processed for 16S rRNA metabarcoding and shotgun metagenomics analyses.

4.2 Materials and Methods

4.2.1 Sample collection

One chicken per pen (total 40 samples with 10 chickens per treatment) was randomly selected at the end of each phase (10, 24 and 35 days of chicken age) as described in **Chapter 3**, section 3.3.5. Caecal digesta were collected on all sample collections while ileal digesta were only collected on 24 and 35 days of chicken age as the preliminary study showed poor DNA quality from the ileum of young chickens. This finding might be due to the several reasons such as different DNA extraction kit used in the preliminary study, high amount of mucous with low amount of ileal content of young chicken or the role of bile salt as inhibitor in downstream process (Pollock *et al.*, 2018). Therefore, the ileal digesta of the 10-day-old chickens was not collected for the analysis. After euthanisation, the chicken abdomen was opened with surgical scissors and forceps to prevent contamination from the researcher's gloves to the sample. Ileum (from Merkel's diverticulum to the opening of caecum) and caeca were cut and placed on separate sterile petri dishes. Intestinal content was gently squeezed with forceps into a sterile 2 millilitre tube and kept on ice to the end of sample collection. Surgical equipment was cleaned with alcohol spray and tissue paper and then sterilised with an alcohol burner for 10 seconds and left for 3 minutes before the sample collection of the next chicken to minimise carry-over contamination. The sterile petri dish was discarded after a single-use. The sample collection of each time point was finished within 4 hours. All samples were transferred to the laboratory for DNA extraction.

4.2.2 DNA extraction

DNA extraction with DNeasy Powersoil Kit (Qiagen, Valencia, CA, USA) was performed immediately after the samples arrived (within 30 minutes after sample collection). Contents from both caeca of each chicken were mixed with a sterile pipette tip before DNA extraction. The intestinal content sample was weighed at 250 milligrammes and transferred to Power bead tube (garnet, 0.7 millimetres) with DNA Powersoil Kit solution c1 and was heated at 65 °C for 10 minutes. A bead beating step was performed using Qiagen TissueLyser LT (Qiagen, Valencia, CA, USA) at 50 hertz for 2 minutes. After this step, DNA extraction was carried out according to the manufacturer's instruction. Solution c6 of the DNA Powersoil Kit (250 microlitres) was used as a template for negative, reagent-only control samples, which were included in every DNA extraction batch. A ZymoBIOMICS Microbial Community Standard (Zymo Research, Irvine, CA, USA) which contains cells from 8 and 2 species of bacteria and yeasts respectively, was used as a positive mock community control for DNA extraction. DNA samples were stored at -80 °C in Thailand. All samples were then shipped on dry ice to the Roslin Institute, United Kingdom for library preparation and further analysis. The samples reached the laboratory within 96 hours after departure and were in frozen condition on dry ice.

4.2.3 16S metabarcoding analysis

4.2.3.1 Library preparation and sequencing

The hypervariable 4 (V4) region of the 16S bacterial rRNA gene was amplified using dual-indexed primers 515f (5'- GTGCCAGCMGCCGCGGTAA-3') and 806r (5'-GGACTACHVGGGTWTCTAAT-3') with Illumina Truseq adapters and barcodes (**Table S10** and **Table S11**) (Caporaso *et al.*, 2011; Kozich *et al.*, 2013). Primers were manufactured and purified with Trugrade technology from Integrated DNA Technologies (Integrated DNA Technologies, USA). Primers and barcodes were randomly selected for each sample. High-Fidelity 2x Master Mix (New England BioLabs, Beverly, MA, USA) was used for 16S rRNA V4 region amplicon amplification. The PCR amplification program was as follows: 95 °C for 2 minutes, followed by 30 cycles of 95 °C for 20 seconds, 55 °C for 15 seconds and 72 °C for 5 minutes, followed by 72 °C for 10 minutes. A ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA) was used as a positive control sample while PCR-grade distilled water was used as a negative control sample for PCR amplification. The Ampure XP PCR purification system (Beckman Coulter, La Brea, CA, USA) was used for amplicon purification following the manufacturer's instructions. The concentration of purified amplicons was then measured using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Hemel Hempstead, UK). Each sample was pooled into a single library at a similar concentration (8 nanomolar) and added to the pooled library at 10 microlitres per sample. The concentration of all negative control samples was lower than 8 nanomolar;

therefore, they were added directly at 10 microlitres to the pooled library. In total, 214 samples were pooled into the final library (75 ileal samples, 119 caecal samples, 2 mock community micro-organisms standard, 1 mock community DNA standard, 15 reagent-only negative control samples and 2 negative PCR control samples). The pooled library was sequenced using paired-end 250 bp reads on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using v2 chemistry (Edinburgh Genomics, Edinburgh, United Kingdom). Sequencing of the pooled library was performed twice in order to increase the number of reads per sample. Results from both runs were combined at the individual sample level.

4.2.3.2 Bioinformatics analysis

Bioinformatics analysis was performed with Microbiome Helper v1 as described in **Chapter 2**, section 2.2.3.1.

4.2.3.3 Statistical analysis

Alpha-diversity analysis was performed using R version 3.5.3. OTU richness (the number of observed OTUs) and the inverse Simpson diversity index were calculated with the *phyloseq* package (McMurdie and Holmes, 2013). An increase in value of inverse Simpson diversity index reflects an increase in diversity (Simpson, 1949). OTU richness and inverse Simpson diversity index were statistically compared between treatments using a 2-way ANOVA, and visualised as boxplots using the *ggpubr* package in R version 3.5.3 (McMurdie and Holmes, 2013; Kassambara, 2018). Non-Metric Multidimensional Scaling

(NMDS) plots using Bray-Curtis dissimilarity method between treatments were constructed with the *ggplot2* package. Statistical analysis using the adonis test (multi-variate analysis of variance based on dissimilarities) in the *vegan* package was performed to identify the significant differences between groups (Dixon, 2003). Percentage abundance of OTUs was analysed and visualised as barplots at phylum and family level using the *ggplot2* package. Differential OTU abundance comparisons between anti-coccidial vaccination and enzymes supplementation were performed using a negative binomial Wald test with the *DESeq2* package in R version 3.5.3 (Love, Huber and Anders, 2014). Significantly differentially abundant OTUs were identified using the Benjamini-Hochberg adjusted P-value (adjusted P-value < 0.05).

4.2.4 Shotgun metagenomics

4.2.4.1 Library preparation and sequencing

After submission of DNA for 16S metabarcoding analysis, gel electrophoresis (1% agarose gel) was performed on every caecal and ileal sample collected from 24-day-old chickens. To remove short DNA fragments, agarose gel with the band of genomic DNA above 10 kilobases in length was cut and kept in 2 millilitre tubes. Agarose gel with genomic DNA was purified using the Monarch DNA Gel Extraction Kit (New England BioLabs, Beverly, MA, USA). The concentration of purified DNA was quantified using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Hemel Hempstead, UK). In total, 20 DNA samples (5 samples per treatments or 10 samples per factor) from caecal

digesta and 12 DNA samples from ileal digesta were submitted to Edinburgh Genomics (Edinburgh, UK) for further quality control and library preparation. The DNA library was prepared using the Truseq Nano 350 bp gel-free protocol (Illumina, San Diego, CA, USA) and was sequenced on an Illumina NovaSeq 6000 system (Illumina, San Diego, CA, USA) by Edinburgh Genomics.

4.2.4.2 Bioinformatics analysis

Unless otherwise stated, all parameters used were the default. Illumina adapters were removed from the sequencing reads with trimmomatic version 0.38 (Bolger, Lohse and Usadel, 2014). The chicken reference genome (galGal6) obtained from Ensembl release 96 (ftp://ftp.ensembl.org/pub/release-96/gtf/gallus_gallus/Gallus_gallus.GRCg6a.96.gtf.gz) was used as a reference database for mapping trimmed reads with the Burrow-Wheeler Aligner tool (BWA-MEM) version 0.7.15 (Li and Durbin, 2010). Trimmed sequencing reads mapped to the chicken genome were removed from the sample using SAMtools version 1.3.1 (Li *et al.*, 2009). Single-sample assemblies of host-genome-free samples were performed using idba-ud version 1.1.3 with options '--num_threads 16 --pre_correction --min_contig 300' (Peng *et al.*, 2012). Single-sample assembly was used as reference for mapping the trimmed sequencing reads with BWA-MEM. Mapped results were converted to BAM format using SAMtools version 1.3.1. Metagenomics binning was performed with single-sample assemblies using MetaBAT2 version 2.12.1 with option '-m 2000'. The quality of genome bins was assessed with CheckM version 1.0.13 (Parks *et al.*, 2015). All filtered bins were

dereplicated with dRep version 2.2.3 using the CheckM completeness and contamination score at minimum 80% completeness and maximum 10% contamination with option 'dereplicate_wf -p 16 -comp 80 -con 10 -str 100 -strW 0' (Olm *et al.*, 2017). GTDB-tk version 0.2.2 was used to assign taxonomic classification to the dereplicated metagenome-assembled genomes (MAGs) (Parks *et al.*, 2018). Genome Taxonomy Database (GTDB) was used as a reference as it is more comprehensive and thorough when compared to The National Center of Biotechnology Information (NCBI) database (Parks *et al.*, 2018; Mendler *et al.*, 2019). The MAGpy pipeline (Stewart *et al.*, 2018) was used to construct taxonomic trees, using the PhyloPhlan tool version 0.99 (Segata *et al.*, 2013). GraPhlan version 1.1.3-1 was used for taxonomic tree visualisation (Asnicar *et al.*, 2015).

For quantitative analysis, dereplicated MAGs were concatenated into a single FASTA file and used as a reference for the mapping of host-free sequencing reads with BWA-MEM version 0.7.15. Mapped results were converted to BAM format using SAMtools version 1.3.1. Script *jgi_summarize_bam_contigs_depth* with options '--minContigLength 2000 --minContigDepth 2' from MetaBAT2 version 2.12.1 was used to calculate the mean average coverage depth of MAGs in the sample. Protein prediction using Prodigal software (Hyatt *et al.*, 2010) was performed within the MAGpy pipeline. The predicted proteins from each MAG were aggregated and used as a reference for mapping and counting proteins from each MAG using DIAMOND version 0.9.21 (Buchfink, Xie and Huson, 2014). Carbohydrate active enzyme families were assigned to

the predicted proteins with dbCAN2 version 7 (Zhang *et al.*, 2018) using the CAZy database (31st July 2018) as reference (Cantarel *et al.*, 2009). The coverage (abundance) of MAG CAZyme families of each chicken were grouped into 5 CAZyme classes (Auxillary Activity or AA; Carbohydrase Esterase or CE; Glycoside Hydrolase or GH; Glycosyl Transferase or GT; Polysaccharide Lyase or PL), 1 associated module (Carbohydrase-Binding Module or CBM) and 2 additional modules (cohesion and S-layer Homology domain or SLH). The average percentage abundances of CAZyme classes were visualised using stack barplot in R version 3.5.3.

4.2.4.3 Statistical analysis

The mean coverage depth of MAGs in each sample was calculated and visualised using the *ggplot2* package in R version 3.5.3. Abundance of MAGs in each sample were estimated using average coverage under the assumption of unbiased sampling. Before the analysis, rarefaction of MAGs abundance to the lowest sample coverage across the samples was performed. The dissimilarity of the abundance of MAGs between the anti-coccidial vaccination and enzyme supplementation was visualised using NMDS plots with Bray-Curtis dissimilarity method and compared using the adonis test from the *vegan* package in R version 3.5.3. Comparisons of the abundance of chicken caecal MAGs between anti-coccidial vaccination, enzymes supplementation and their interaction were performed using the *DESeq2* package in R version 3.5.3. From the statistical comparison, significant differential MAG abundance was

identified using the Benjamini-Hochberg adjusted P-value (adjusted P-value < 0.05).

For the CAZyme analysis, the abundances of MAG proteins assigned to CAZyme families were calculated and visualised using the *ggplot2* package in R version 3.5.3. The dissimilarity of the abundance of CAZyme families between the anti-coccidial vaccination and enzyme supplementation was visualised using NMDS plot with Bray-Curtis dissimilarity method and compared using the adonis test from the *vegan* package in R version 3.5.3. Abundance of CAZyme classes and families of the chicken caecal microbiome were compared between anti-coccidial vaccination, exogenous enzymes supplementation, and their interactions were performed using the *DESeq2* package in R version 3.5.3. From the statistical comparison, significantly differentially abundant CAZyme classes and families were identified using the Benjamini-Hochberg adjusted P-value (adjusted P-value < 0.05).

4.3 Results

4.3.1 16S metabarcoding analysis results

From the draft 16S rRNA metabarcoding analysis, all samples, including the mock community control samples, negative PCR control samples and negative reagents-only control samples were analysed without rarefaction to identify the bacterial composition of each sample. The bacterial composition results of mock community control samples and negative control samples are shown in **Table S12** and **Table S13**. A significant underestimate of bacteria in the

Listeriaceae family and overestimate of bacteria in the Enterobacteriaceae family were observed when compared to the expected values from the ZymoBIOMICS Microbial Community Standard. In contrast, the abundance of bacteria from the ZymoBIOMICS Microbial Community DNA Standard were close to the actual value. These findings suggest that the DNA extraction had significant influence on the OTU classification and need to be taken into account in studies that have these bacteria as a major population.

From **Table S13**, the dominant bacteria of the negative PCR control and reagents-only control samples were OTUs assigned to family Lachnospiraceae, Ruminococcaceae and Bacteroidaceae. These OTUs were also commonly found in the chicken intestinal sample in this study. These findings showed that there was possibly a low level of contamination during the library preparation or DNA extraction which could be ignored. Moreover, no contaminant from the reagents used in the DNA extraction or library preparation was found in the chicken samples. Therefore, all positive and negative samples were removed from the final 16S rRNA metabarcoding analysis. Moreover, the negative PCR control samples were removed from the final analysis due to the low number of reads (less than 150 reads per sample in the OTU classification step). In the final analysis, all bacterial OTUs found in the intestinal samples of this study were expected to originate from the intestine without the disturbance of any contaminant bacterial OTU.

Two caecal samples (from day 10 and day 24 of chicken age) were identified as outliers from the draft analysis and were removed from final analyses as

they were dominated by one single OTU and significantly affected the overall results. The caecal outlier sample from the 10-day-old chicken was dominated by an unknown species from family Bacteroidaceae (46.8%) where this OTU was rarely found in other chickens at 10 days of age (less than 0.5%). In addition, the caecal outlier sample from 24-day-old chickens was dominated by an unknown species from family Enterobacteriaceae (64.7%). Although this OTU from unknown species from family Enterobacteriaceae was found in other chickens at 24 days of age, this outlier sample was removed as it was clustered individually and separately from the rest of the samples at the similar intestinal region and age. One tube contained caecal sample from the 35-day-old chicken was damaged during shipment from Thailand to Roslin Institute. Ileal digesta was not collected from five 24-day-old chickens as there was no content in the ileum region during sample collection. One ileum sample was removed from the analysis due to the low number of reads (8,081 reads) at the OTU classification step. In summary, there were 191 samples from caecal and ileal digesta remaining for alpha- and beta-diversity analysis.

From all 191 intestinal digesta samples, the total number of raw sequencing reads was 20,774,416 reads with a mean of 108,766 $\pm$ 49,847 reads per sample. After quality filtration, including a chimaera removal step, all samples were rarefied to 13,255 reads per sample for further analysis. In total, there were 1,657 OTUs classified from all samples in this experiment.

4.3.1.1 Alpha-diversity analysis of 16S metabarcoding results

A summary of the mean average number of observed OTUs in the ileum and caecum at different ages of chicken is shown in **Table 4.1**. Anti-coccidial vaccination significantly affected the number of observed OTUs at ileum and caecum of 24- and 35-day-old chickens (**Figure 4.1A, Figure 4.1C, Figure 4.2C** and **Figure 4.2E**). Interestingly, from these significant differences, non-vaccinated chickens had a higher number of observed OTUs than the vaccinated chicken except in the ileum of 35-day-old chicken. There were 145.3 and 92.7 mean OTUs in vaccinated chickens and non-vaccinated chickens, respectively (**Figure 4.1C**). In contrast, exogenous enzymes did not significantly affect the number of observed OTUs at any ages or intestinal regions (**Figure 4.1B, Figure 4.1D, Figure 4.2B, Figure 4.2D** and **Figure 4.2F**)

Table 4.1: The mean number of observed OTUs by factor at ileum and caecum of chicken at 10, 24 and 35 days old (Non = non-vaccinated chickens; Vac = vaccinated chickens)

Intestinal region	Age of chicken (days)	Anti-coccidial vaccination		Exogenous enzymes	
		Non	Vac	Without	Added
Ileum	24	241.6 †	178.3 †	217.3	192.8
	35	92.7 ‡	145.3 ‡	118.3	118.4
Caecum	10	289.7	304.9	295.8	299.1
	24	416.7 #	350.7 #	409.3	361.1
	35	445.9 §	405.6 §	435.3	416.8

†, ‡, #, § Represent statistical significant difference between groups of the same symbol within row (P-value <0.05)

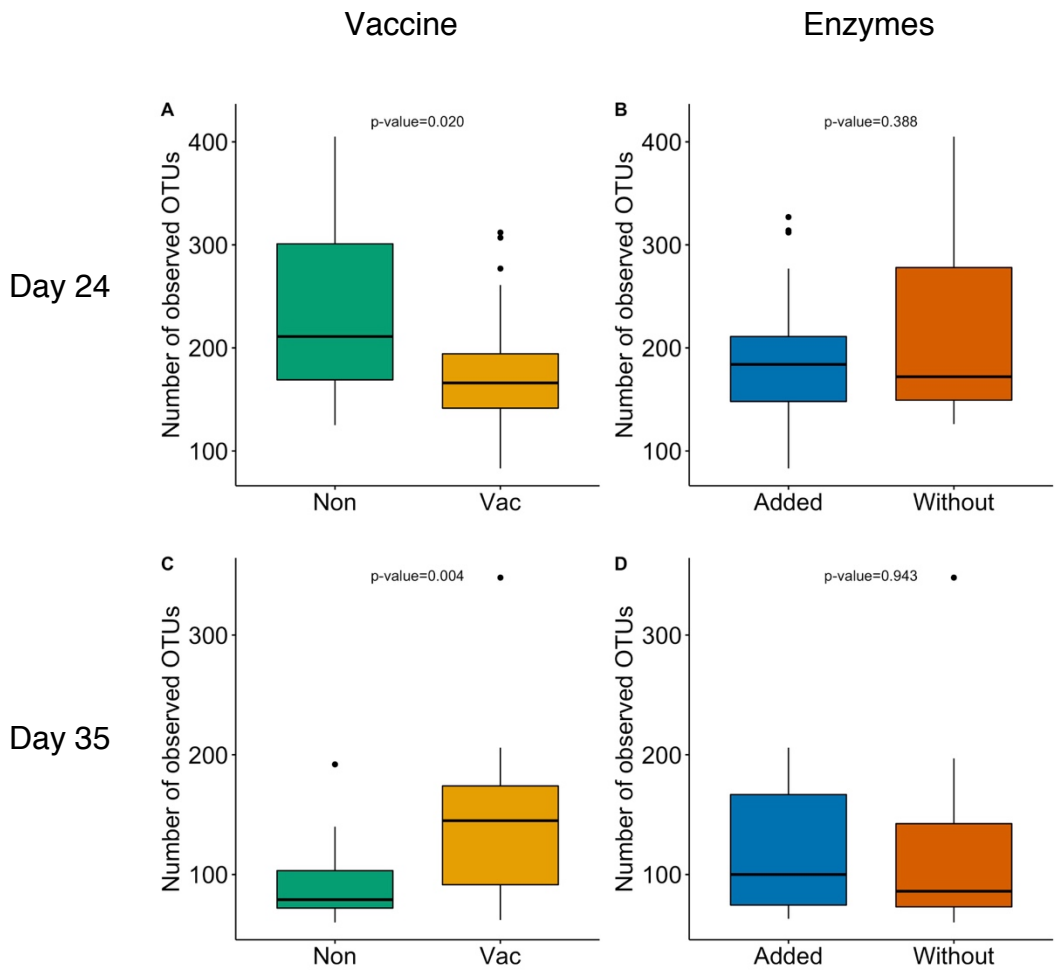


Figure 4.1: The number of observed OTUs comparison with 2-way ANOVA test (P-value < 0.05) at ileum grouped by factors at different ages. Outlier values are shown as dots; (Non; non-vaccinated chickens, Vac; vaccinated chickens)

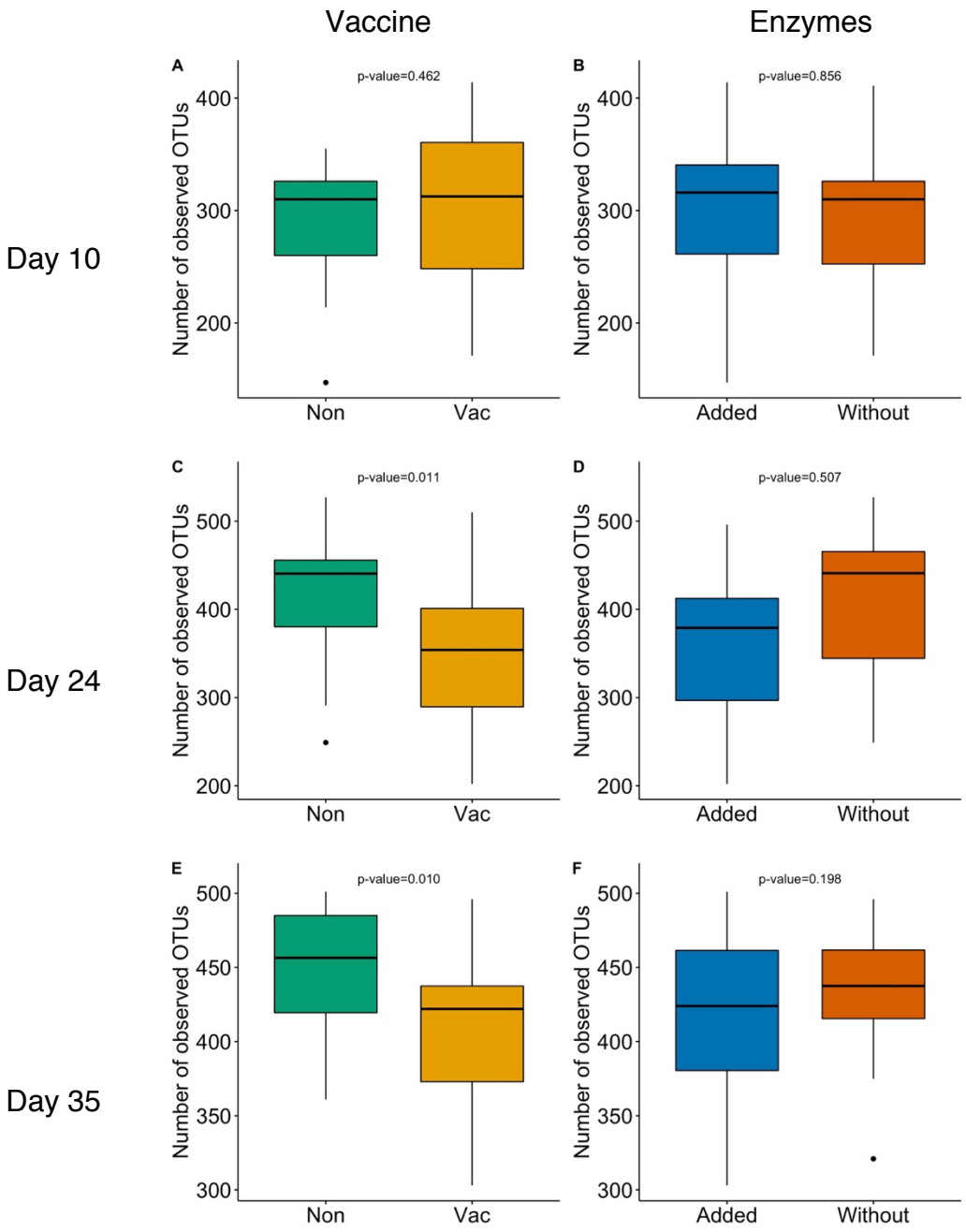


Figure 4.2: The number of observed OTUs comparison with 2-way ANOVA test (P-value < 0.05) at caecum grouped by factors at different ages. Outlier values are shown as dots; (Non; non-vaccinated chickens, Vac; vaccinated chickens)

The summary of the mean inverse Simpson diversity index values is shown in **Table 4.2**. At 24 days of chicken age, anti-coccidial vaccination significantly decreased the inverse Simpson diversity index for both the ileum and caecum (**Figure 4.3A** and **Figure 4.4C**). Exogenous enzymes did not significantly affect the inverse Simpson diversity index at any ages or intestinal regions (**Figure 4.3B, Figure 4.3D, Figure 4.4B, Figure 4.4D** and **Figure 4.4F**). The only interaction effect between anti-coccidial vaccination and exogenous enzymes on the number of observed OTUs was observed for the caecum of 35-day-old chicken (P-value = 0.04).

Table 4.2: The mean inverse Simpson diversity index by factor at ileum and caecum of chicken at 10-, 24- and 35-days of age. (Non; non-vaccinated chickens, Vac; vaccinated chickens)

Intestinal region	Age of chicken (days)	Anti-coccidial vaccination		Exogenous enzymes	
		Non	Vac	Without	Added
Ileum	24	7.79 †	3.79 †	6.33	4.63
	35	3.15	3.43	3.47	3.11
Caecum	10	19.02	21.44	19.86	20.64
	24	24.12 ‡	17.52 ‡	22.01	19.85
	35	17.32	16.36	17.61	16.06

†, ‡ Represent statistical significant difference between groups of the same symbol within row (P-value <0.05)

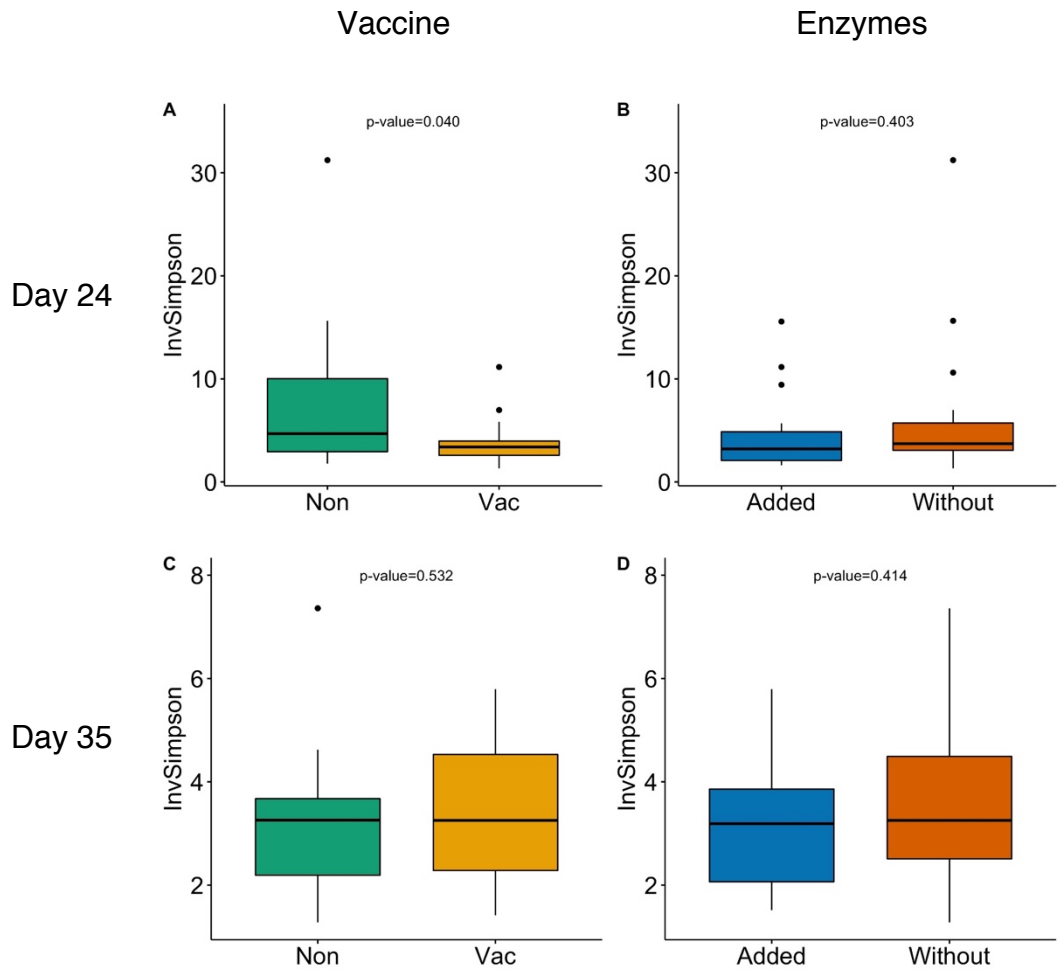


Figure 4.3: Inverse Simpson index comparison with 2-way ANOVA test (P-value < 0.05) at ileum grouped by factors at different ages. Outlier values are shown as dots; (Non; non-vaccinated chickens, Vac; vaccinated chickens)

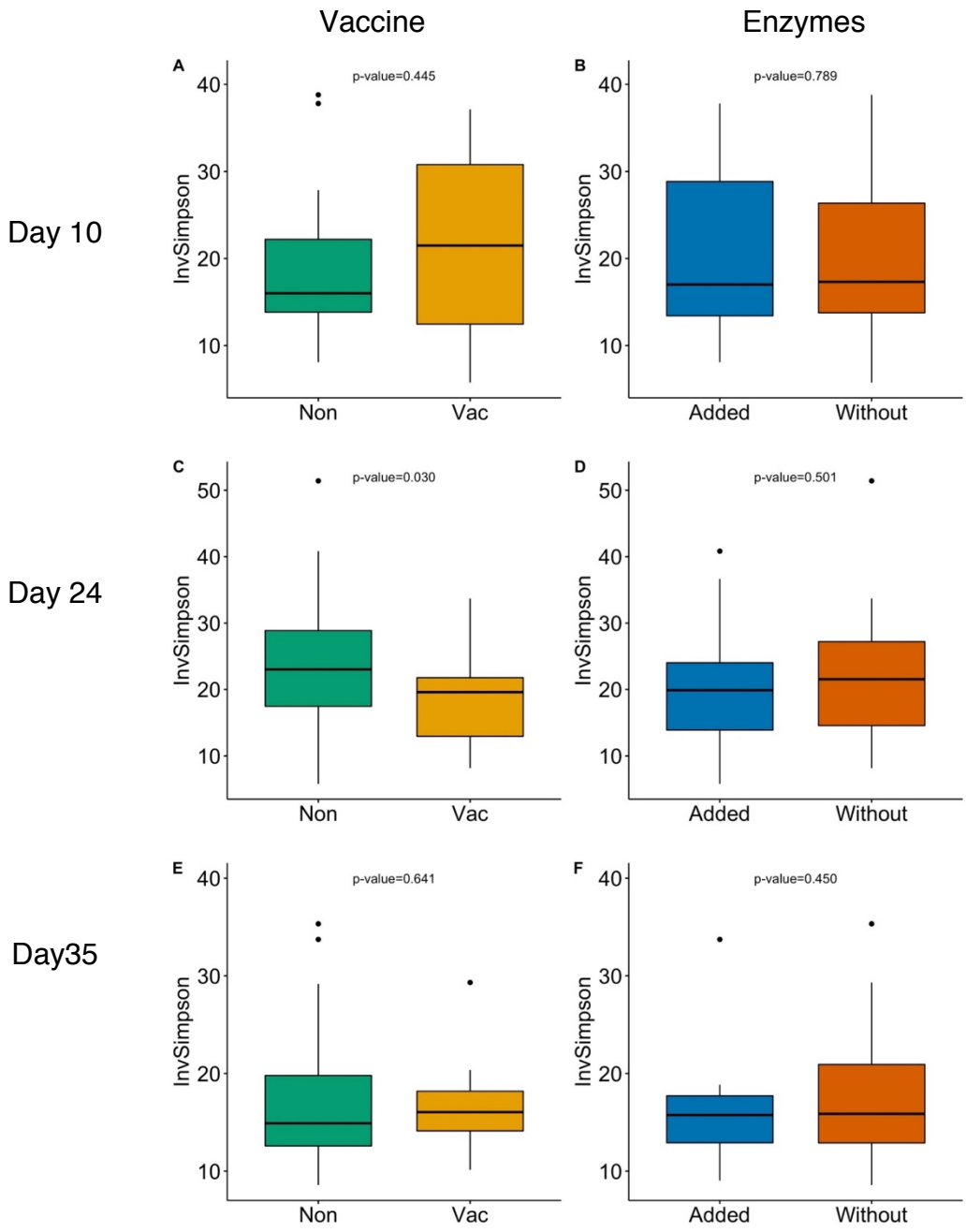


Figure 4.4: Inverse Simpson index comparison with 2-way ANOVA test (P-value < 0.05) at caecum grouped by factors at different ages. Outlier values are shown as dots; (Non; non-vaccinated chickens, Vac; vaccinated chickens)

4.3.1.2 Beta-diversity analysis of 16S metabarcoding results

NMDS plots constructed using the Bray-Curtis dissimilarity method of the total 191 samples showed that the intestinal microbiome was clustered separately by intestinal region and chicken age as shown in **Figure 4.5**. The intestinal microbiome compositions of 24- and 35-day-old chickens were clustered closely based on their intestinal region. Notably, the caecal microbiome of 10-day-old chickens clustered separately from the rest of the data. However, from the pairwise adonis test, the chicken intestinal microbiome showed significant differences between ages and intestinal regions (all P-value < 0.001).

Using the adonis test, anti-coccidial vaccination significantly affected the dissimilarity of intestinal microbiome compositions at all intestinal regions and chicken ages (**Figure 4.6A**; P-value = 0.007, **Figure 4.6B**; P-value = 0.001, **Figure 4.7A**; P-value = 0.004, **Figure 4.7B**; P-value = 0.028 and **Figure 4.7C**; P-value = 0.008). In contrast, exogenous enzymes did not show a significant effect on the clustering of microbiome communities between enzymes supplemented and non-supplemented chickens at any age. No significant interaction effect was observed between vaccination and exogenous enzymes supplementation at any age or intestinal region.

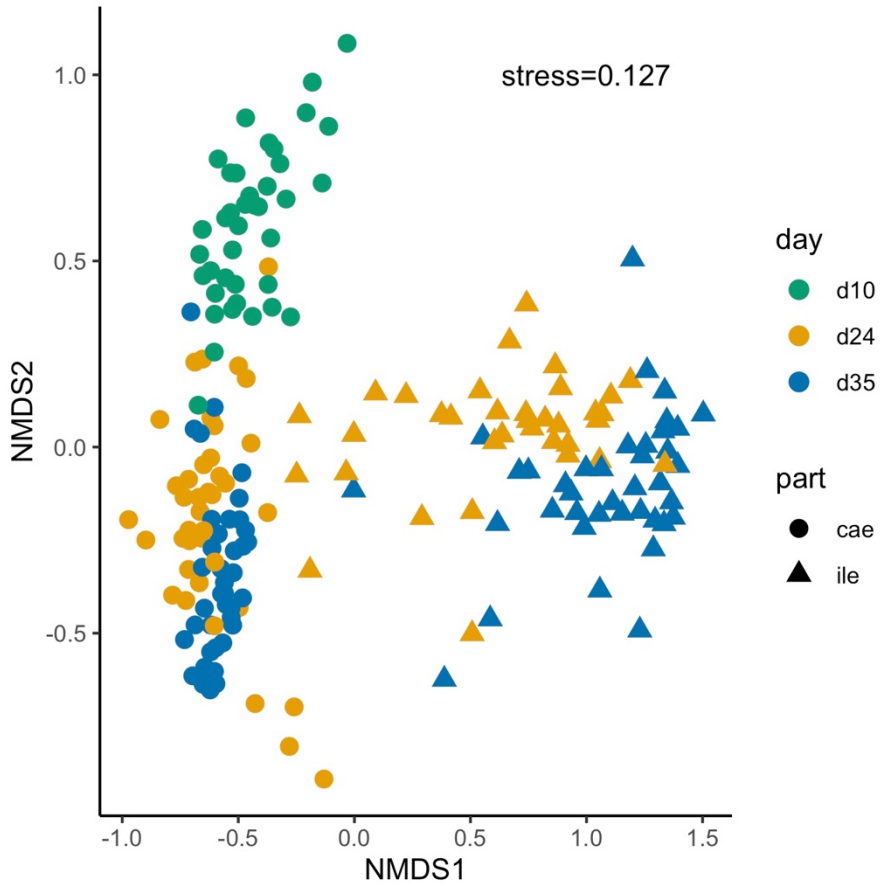
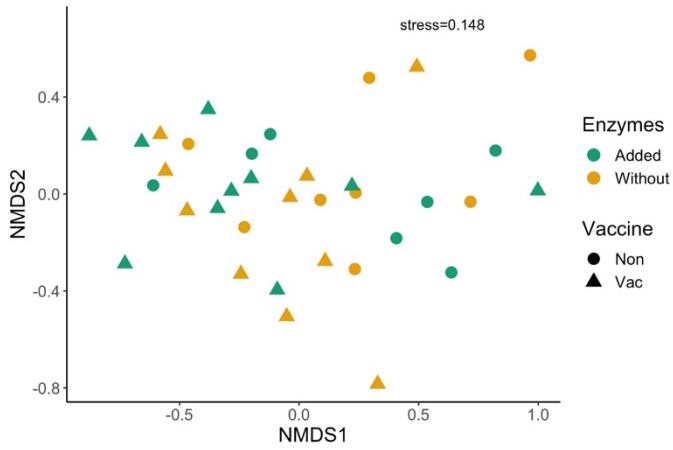


Figure 4.5: NMDS plot of the total 191 digesta samples from ileum and caecum of 10-, 24- and 35-day-old chickens with the stress value = 0.127. The intestinal microbiome showed significant differences when compared between all ages and regions (Adonis test; all P-values < 0.001). (cae = caecum; ile = ileum).

A) Day 24



B) Day 35

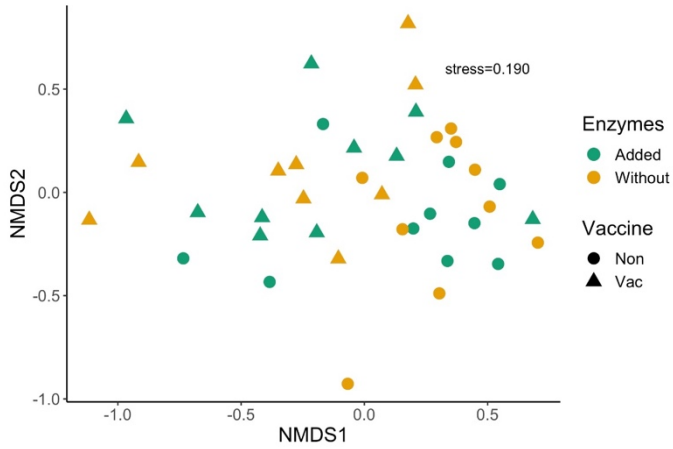
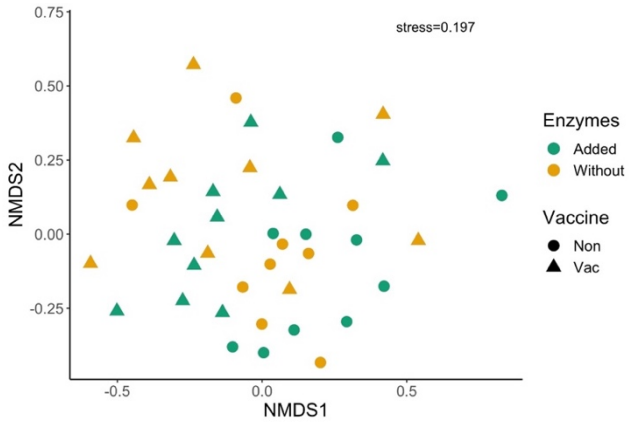
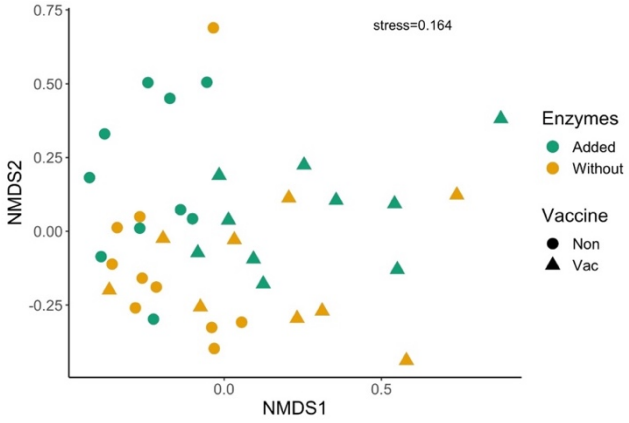


Figure 4.6: NMDS plots of ileal microbial communities at A) 24- and B) 35-days of chicken age. The ileal microbiome showed significant differences when compared between non- and vaccinated chickens (adonis test; all P-values < 0.05). (Non; non-vaccinated chickens, Vac; vaccinated chickens)

A) Day 10



B) Day 24



C) Day 35

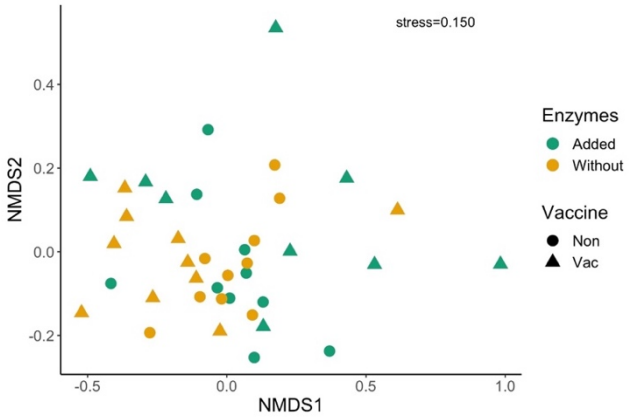


Figure 4.7: NMDS plot of caecal microbial communities at A) 10-, B) 24- and C) 35-days of chicken age. The caecal microbiome showed significant differences when compared between non- and vaccinated chickens (adonis test; all P-values < 0.05). (Non; non-vaccinated chickens, Vac; vaccinated chickens)

Overall, in the ileum, bacterial populations in both 24- and 35-day-old chicken were dominated by bacteria in the Firmicutes phylum (at mean 82.67% and 85.56% abundance, respectively) as shown in **Figure 4.8A** and **Figure 4.9A**. At family level, bacteria in the family Lactobacillaceae had the highest relative abundance at 24- and 35-days of age at 44.66% and 70.22%, respectively (**Figure 4.8B** and **Figure 4.9B**).

After performing differential abundance comparisons at the genus level, anti-coccidial vaccination was found to significantly affect the relative abundance of 11 bacterial genera in the ilea of 24-day-old chickens with adjusted P-value < 0.05 (**Figure 4.10**). Out of 11 of the significantly differentially abundant bacterial genera, 10 genera were more abundant in the non-vaccinated chickens than the anti-coccidial vaccinated chickens. Only *Rummeliibacillus spp* had a higher abundance in vaccinated chickens in comparison to non-vaccinated chickens at 0.039% to 0.012% respectively.

In contrast, no significant effect of exogenous enzymes and no interaction between vaccination and exogenous enzymes on percentage abundance of bacteria was observed in the ileum of 24-day-old chickens (adjusted P-value > 0.05). Conversely, exogenous enzymes significantly affected the relative abundance of *Brevibacterium spp* in the ileum of 35-day-old chickens with adjusted P-value = 0.023 (0.130% to 0.015% in non-enzyme and enzyme supplemented chickens respectively). No significant effect on bacterial abundance was observed from anti-coccidial vaccination and interaction

between vaccination and exogenous enzymes was found (adjusted P-value > 0.05).

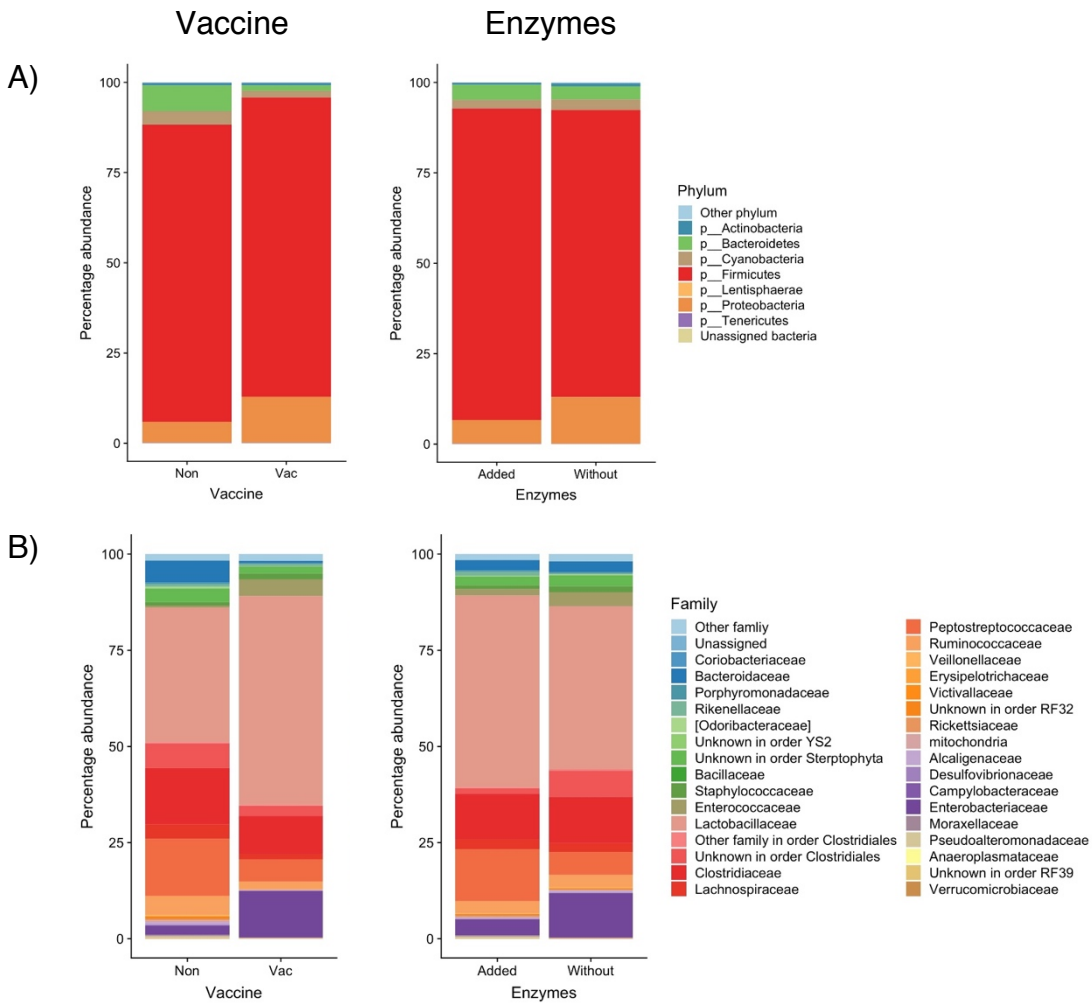


Figure 4.8: Mean percentage abundance of the ileal microbiomes of 24-days-old chickens at A) phylum level and B) family level. Major bacterial populations in the ileum of 24-day-old chicken were phylum Firmicutes and Lactobacillus family. (Non; non-vaccinated chickens, Vac; vaccinated chickens)

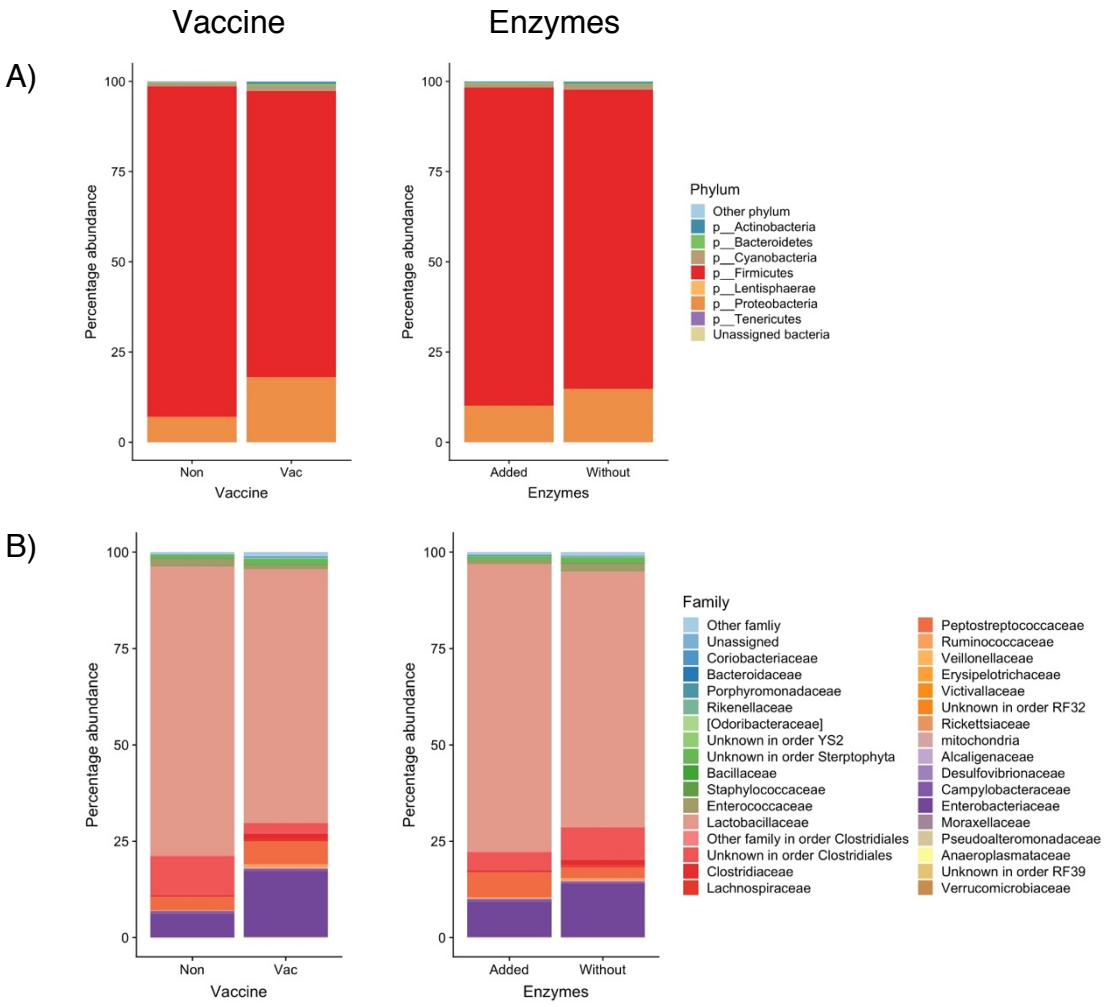


Figure 4.9: Mean percentage abundances of ileal microbiomes of 35-day-old chickens at A) phylum level and B) family level. Major bacterial populations in the ileum of 35-day-old chicken were phylum Firmicutes and Lactobacillus family. (Non; non-vaccinated chickens, Vac; vaccinated chickens)

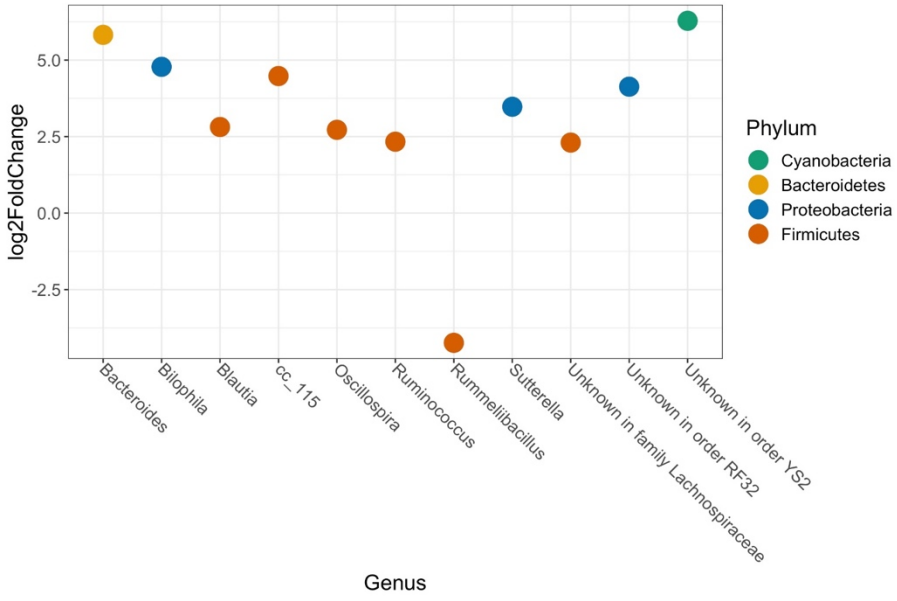


Figure 4.10: Plot of ileal OTUs that were significantly differentially abundant between 24-day-old vaccinated and non-vaccinated chickens (adjusted P-value < 0.05). Significant OTUs are represented by single data points, groups by genus on the X-axis and by colour according to which taxonomic phylum the OTU originates.

In summary, the caecal microbiome of 10-day-old chickens was dominated by bacteria in the Firmicutes phylum (mean 80.89% abundance) as shown in **Figure 4.11A**. The majority of the Firmicutes phylum at 10 days of chicken age were family Lachnospiraceae, family Ruminococcaceae and unknown family of Clostridiales order (**Figure 4.11B**). At day 24 of chicken age, approximately 80% of the caecal bacteria were members of either the Firmicutes or Bacteroidetes (mean 44.45% and 36.35% abundance, respectively) as shown in **Figure 4.12A**. At 35 days of chicken age, bacteria from Proteobacteria

became the third major population of the caecal bacteria (**Figure 4.13A**). Together, the Firmicutes, Bacteroidetes, and Proteobacteria phyla comprised 86.51% of the total caecal bacteria (mean 34.44%, 28.46% and 23.61% abundance, respectively).

Caecal bacterial abundance comparisons at the genus level showed that, at 10 days of age, vaccinated chickens had significant higher abundances of two bacterial genera (*Blautia spp* and *Anaeroplasma spp*) when compared to non-vaccinated chickens with adjusted P-value < 0.05. Non-enzyme supplemented chickens also showed increased abundance of bacterial genus *Anaeroplasma spp* than enzyme-supplemented chickens (adjusted P-value = 0.031). Moreover, there was a significant interaction effect between anti-coccidial vaccination and exogenous enzymes supplementation on *Anaeroplasma spp* (adjusted P-value = 0.005).

No significant effect of exogenous enzymes on bacterial abundance was observed in the caecum of 24-day-old chickens (adjusted P-value > 0.05). Anti-coccidial vaccination significantly affected the abundance of an unknown genus in the family Lachnospiraceae with adjusted P-value = 0.031 (3.82% and 1.75% abundance in vaccinated and non-vaccinated chickens, respectively). In contrast, non-vaccinated chickens had more abundance of *Rikenella spp* than vaccinated chickens with adjusted P-value < 0.001 at 0.28% and 0.04%, respectively. Also, a significant interaction effect between both factors was observed on the abundance of *Rikenella spp* (adjusted P-value < 0.001).

Similar to 24-day-old chickens, at 35 days of chicken age, exogenous enzymes had no significant effect on bacterial abundance in the caecum at the genus level (adjusted P-value > 0.05). Anti-coccidial vaccination significantly affected the abundance of two bacterial genera, *Megamonas spp* (mean 0.42% abundance in vaccinated and 0% in non-vaccinated chickens) and *Odoribacter spp* (mean 6.78% and 1.01% abundance in vaccinated and non-vaccinated chickens, respectively) with adjusted P-values <0.001 and 0.013, respectively. No significant interaction effect between anti-coccidial vaccination and exogenous enzyme supplementation was observed in the caecum of 35-day-old chickens (adjusted P-value > 0.05).

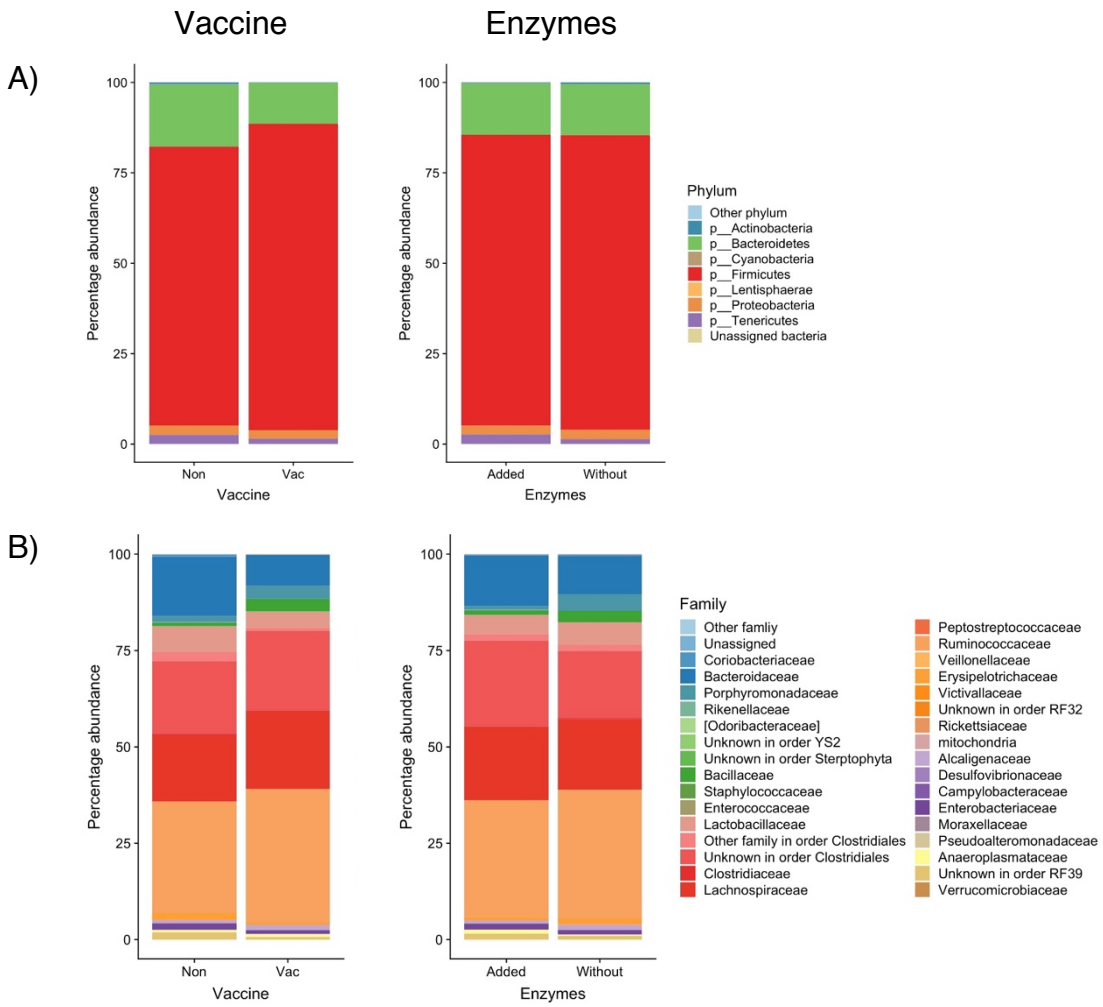


Figure 4.11: Mean percentage abundance of the caecal microbiome of 10-day-old chickens at A) phylum level and B) family level. Major bacterial populations in the caecum of 10-day-old chicken were phylum Firmicutes and families Lachnospiraceae, Ruminococcaceae and unknown family of order Clostridiales. (Non; non-vaccinated chickens, Vac; vaccinated chickens)

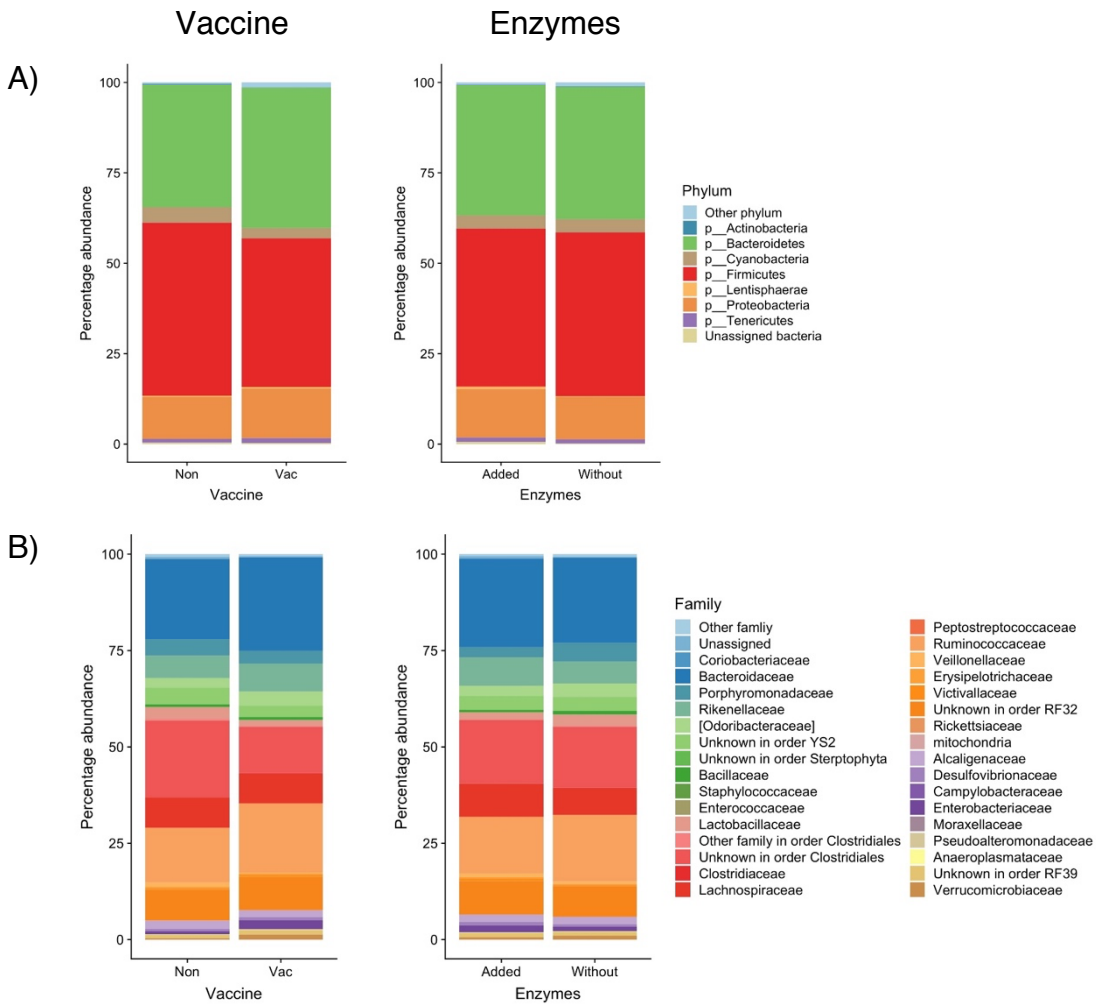


Figure 4.12: Mean percentage abundance of the caecal microbiome of 24-day-old chickens at A) phylum level and B) family level. Major bacterial populations in the caecum of 24-day-old chicken were phyla Firmicutes and Bacteroidetes. (Non; non-vaccinated chickens, Vac; vaccinated chickens)

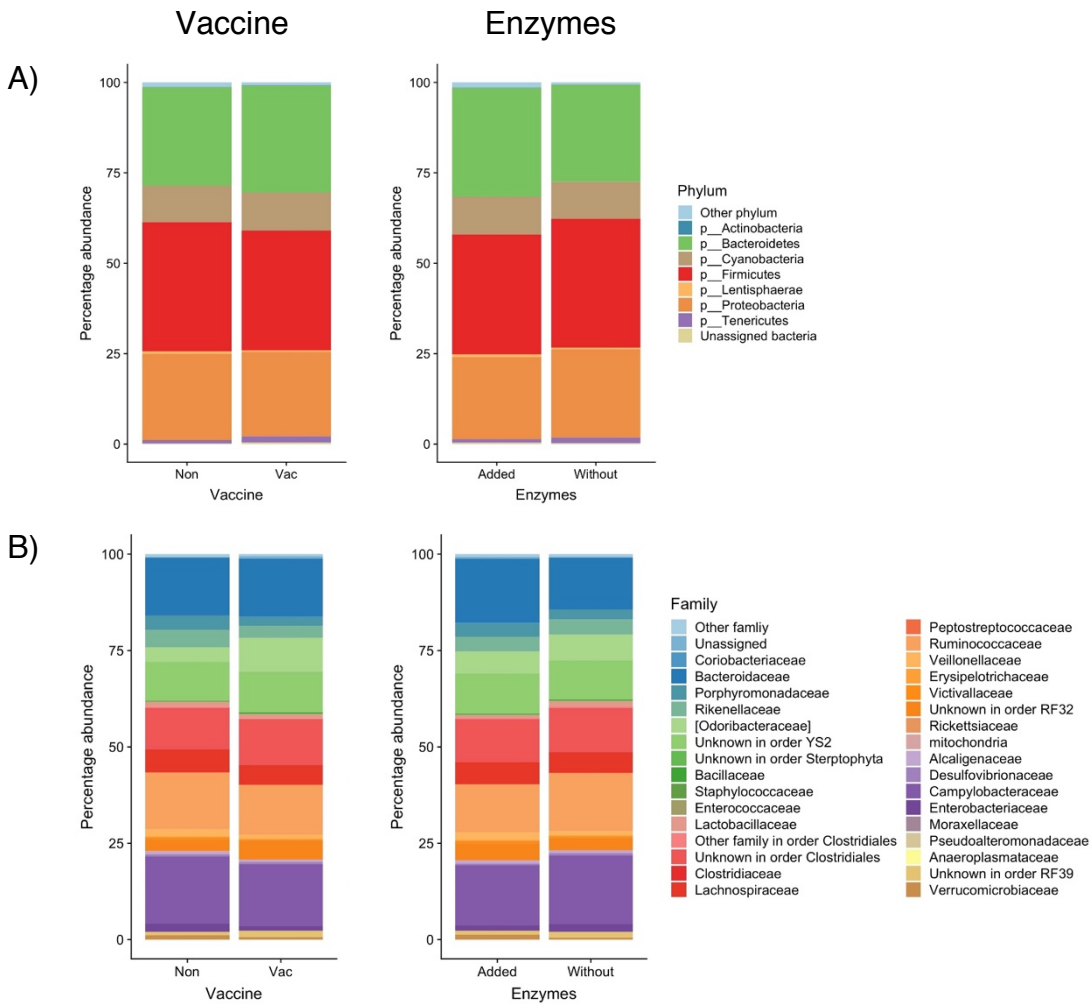


Figure 4.13: Mean percentage abundance of the caecal microbiome of 35-day-old chickens at A) phylum level and B) family level. Major bacterial populations in the caecum of 35-day-old chicken were phyla Firmicutes, Bacteroidetes and Proteobacteria. (Non; non-vaccinated chickens, Vac; vaccinated chickens)

4.3.2 Shotgun Metagenomics analysis results

From a total of 32 samples, the mean number of reads from the sequencing machine was 75,707,123.1 $\pm$ 6,594,945.2 reads per sample. From the sequencing results, 97.03 to 99.52% of sequencing reads from ileal samples were mapped to the chicken genome while the mean percentage of host genomes reads from the caecal samples was 18.49% (ranging from 1.49 to 81.03%). Therefore, all ileal samples were excluded from shotgun metagenomics quantitative analysis and abundance comparison due to the low number of reads mapped to the bacterial genome.

After binning, there were 322 MAGs from 32 intestinal samples of 24-day-old chickens with 146 unique taxonomies as shown in **Table S14**. **Figure 4.14** showed the taxonomic plot of all 322 MAGs at phylum level, and order level for the members of the Firmicutes_A phylum. Out of 322 MAGs, 256 MAGs (79.5%) were either Firmicutes, Firmicutes_A or Firmicutes_C. The sum of average abundance MAGs of each sample was rarefied to the lowest sample coverage before the beta-diversity analysis at 777 coverage abundance. NMDS plot of mean abundance of caecal samples to 322 MAGs was shown in **Figure 4.15**. From the NMDS plot, the abundance of MAGs was obviously clustered by anti-coccidial vaccination (adonis test; P-value < 0.001). When comparing the dissimilarity between non-supplemented and exogenous enzyme supplemented chickens, there was no significant difference (P-value = 0.421), and no interaction was observed between the two factors (P-value = 0.711).

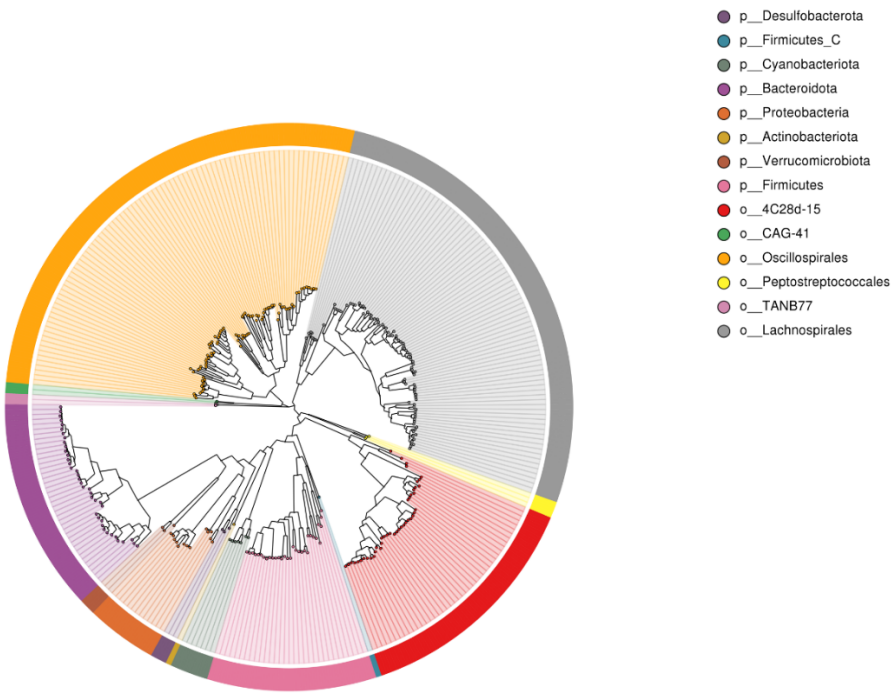


Figure 4.14: Taxonomic plot of total 322 MAGs found in ileal and caecal contents of 24-day-old chickens at phylum level, and order level for the Firmicutes_A phylum.

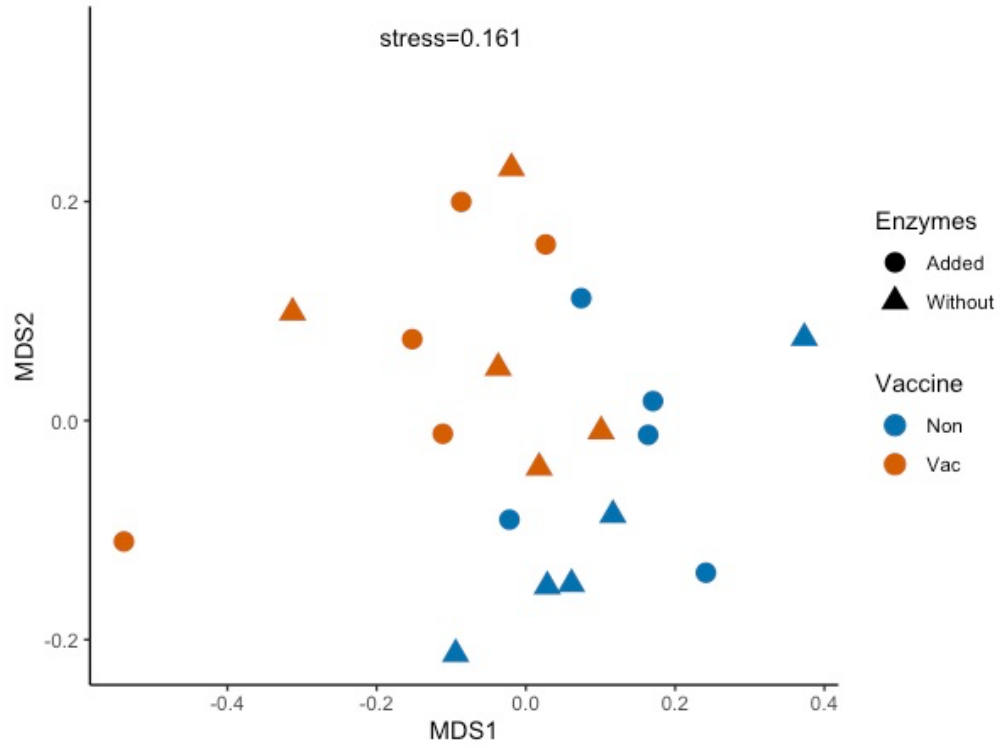


Figure 4.15: NMDS plot of mean abundance of MAGs at caecum of 24-day-old chickens. Caecal MAGs clustered separately between non-vaccinated (blue) and vaccinated chickens (red) and showed a significant difference between them (adonis test; P-value < 0.001). (Non; non-vaccinated chickens, Vac; vaccinated chickens)

From mean abundance results of MAGs from 20 chickens, caecum was dominated by bacteria in phylum Bacteroidota (41.95%) followed by the phylum Firmicutes_A (41.41%) as shown in **Figure 4.16**. The most dominant family in the caecum was Bacteroidaceae, with the mean abundance of 17.43%. At the species level, the most dominant bacteria in the caecum was *Barnesiella intestinihominis* followed by *Alistipes finegoldii* at 8.72% and 5.59% respectively.

Differential abundance comparisons between vaccinated and non-vaccinated identified 19 significantly differently abundant MAGs (**Figure 4.17** and **Table S15**-**S16**). Out of the 19 significantly different MAGs, 16 MAGs were assigned to the Firmicutes_A phylum. Vaccinated chickens had significantly higher abundances of 4 bacterial MAGs (*Bacteroides fragilis*, *Fournierella species GCF 002161595.1*, undefined species of genus *Flavonifractor* and undefined species of genus *Ruthenibacterium*) compared to non-vaccinated chickens with adjusted P-value < 0.05. However, enzymes significantly affected the abundance of only one MAGs classified as *Bacteroides clarus* with adjusted P=value = 0.021 (**Table S17**). An interaction effect between both factors was observed in the difference between the abundance of *Bacteroides clarus* with adjusted P-value = 0.028 (**Table S18**)..

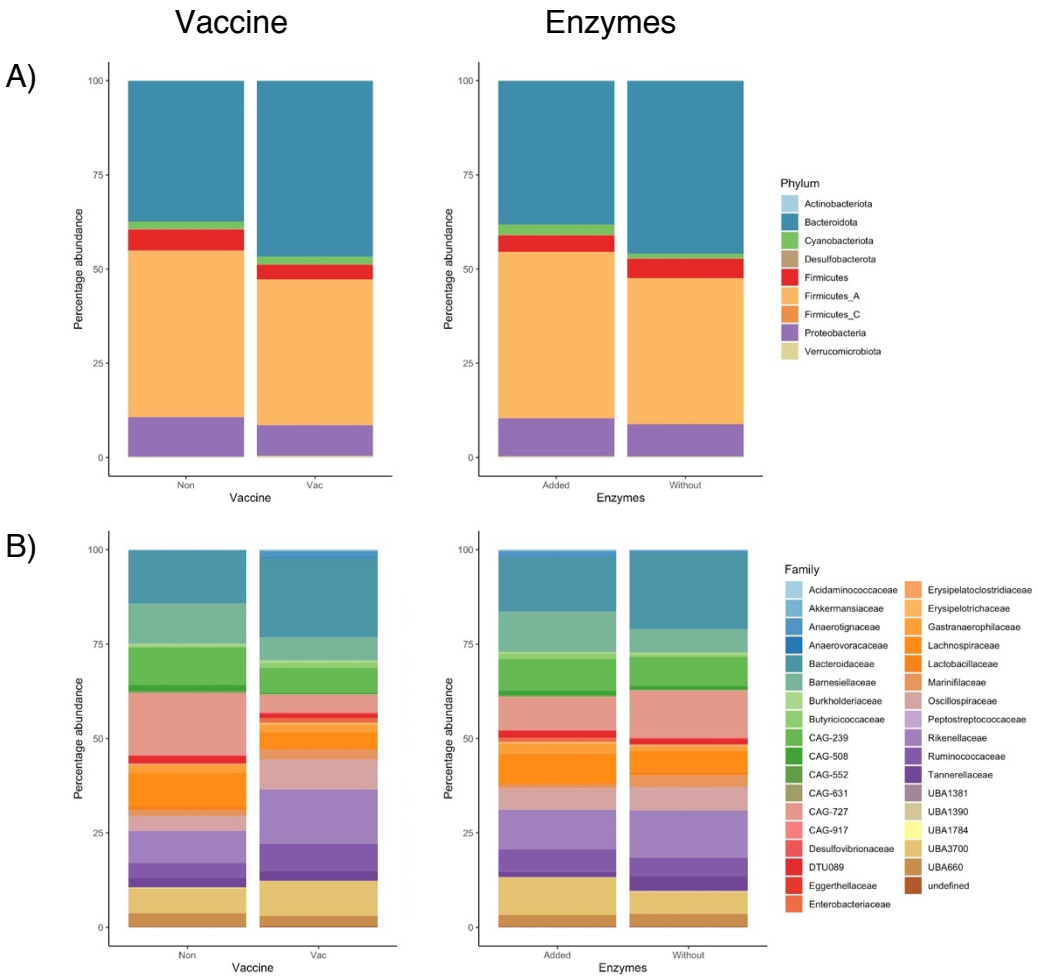


Figure 4.16: Mean MAG abundance in the caecum of 24-day-old chickens at A) phylum level and B) family level. Major bacterial populations in the caecum of 24-day-old chicken were phyla Firmicutes and Bacteroidetes. (Non; non-vaccinated chickens, Vac; vaccinated chickens)

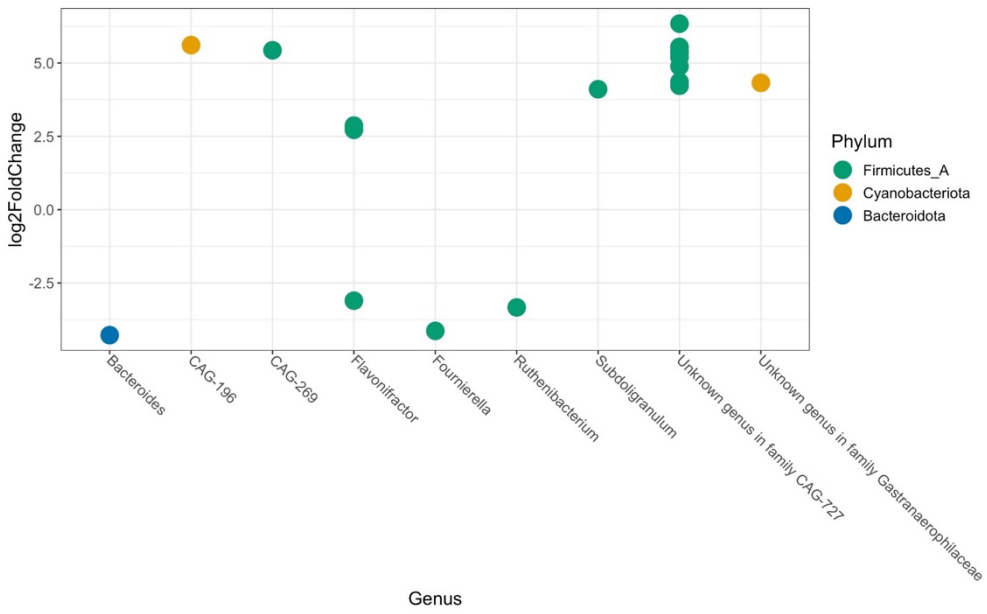


Figure 4.17: Plot of caecal MAGs that were significantly differentially abundant between 24-day-old vaccinated and non-vaccinated chickens (adjusted P-value < 0.05). Significant OTUs are represented by single data points (with some overlapping data points), groups by genus on the X-axis and by colour according to which taxonomic phylum the OTU originates.

The abundances of protein-coding gene mapped to the carbohydrate enzymes database (CAZy database) were compared between factors. In **Figure 4.18**, the abundances of CAZyme related proteins of the chicken caecal microbiome were clustered closely as shown in the NMDS plot and no significant difference between anti-coccidial vaccination, enzymes supplementation and their interaction was observed (adonis test: P-value = 0.167, 0.802 and 0.948 respectively).

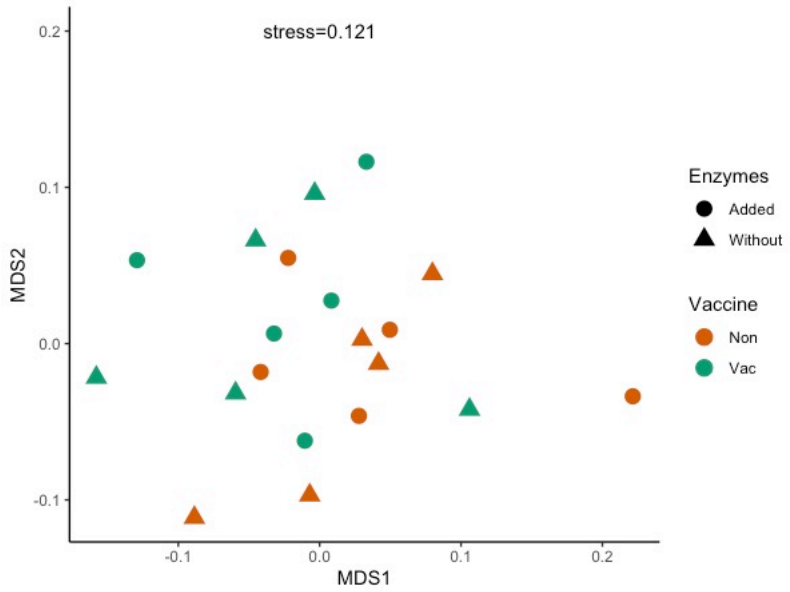


Figure 4.18: NMDS plot of abundance of protein coding genes mapped to the CAZy database, derived from caecum samples. No significant difference between anti-coccidial vaccination, enzymes supplementation and their interaction was observed (all P-value > 0.05). (Non; non-vaccinated chickens, Vac; vaccinated chickens)

The relative abundance of the CAZyme classes for anti-coccidial vaccination and exogenous enzymes supplementation factors are shown in **Figure 4.19**. From the *DESeq2* analysis, a CAZyme of the caecal microbiome showed that the abundance of proteins in the SLH class of the vaccinated chicken was higher than the non-vaccinated chickens (adjusted P-value < 0.001). In contrast, no significant effect from the exogenous enzymes supplementation and the interaction between anti-coccidial vaccination and enzymes supplementation on the average abundance of proteins assigned to CAZyme class was observed (adjusted P-value > 0.05).

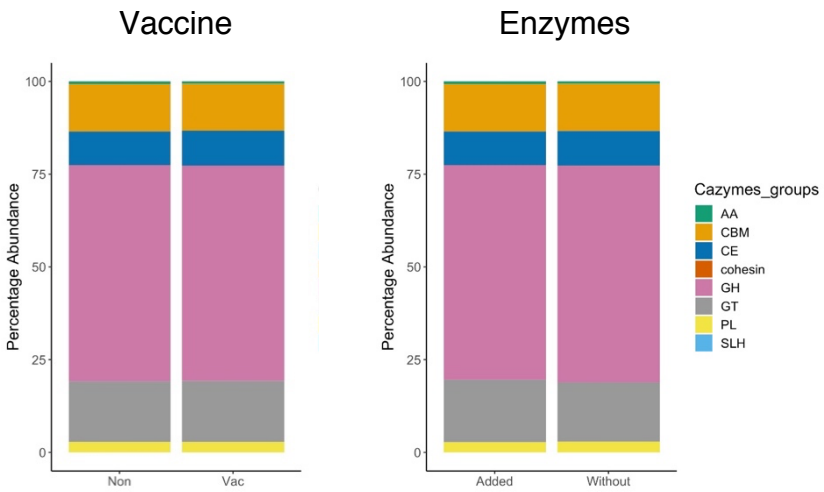


Figure 4.19: Mean abundance of gene summarised by CAZyme class at caecum of 24-day-old chickens. AA; Auxillary Activities, CBM; Carbohydrate Binding Modules, CE; Carbohydrate Esterases, GH; Glycoside Hydrolase, GT; Glycosyl Transferase, PL; Polysaccharide Lyases and SLH; S-Layer Homology. The only significant difference between non- and vaccinated chicken was observed in the abundance of the SLH class (P-value <0.001). (Non; non-vaccinated chickens, Vac; vaccinated chickens)

Anti-coccidial vaccination and exogenous enzymes had significant effects on the abundance of proteins assigned to the CAZyme families (**Table 4.3**). Anti-coccidial vaccination affected the abundance of 17 CAZyme families while exogenous enzymes supplementation affected only 1 CAZyme family (GT31 family). Significant interactions between anti-coccidial vaccination and exogenous enzymes supplementation were observed in GH43_30 and GT31 families.

Table 4.3: Significant difference abundance of proteins assigned to CAZyme family from *DEseq2* analysis. Comparison between anti-coccidial vaccination, positive log_2 fold change showed higher abundance in non-vaccinated chickens, and vice versa. Comparison between enzymes supplementation, positive log2 fold change showed higher abundance in non-enzymes supplemented chickens.

Comparison	CAZyme family	log2Fold Change	Adjusted P-value
Anti-coccidial vaccination	GT31	22.71	<0.001
	GH13_23	7.58	<0.001
	GT70	7.40	<0.001
	GH19	7.12	0.01
	CBM71	4.34	0.01
	SLH	3.69	<0.001
	GH30_6	3.19	0.02
	CBM54	2.86	<0.001
	GH111	2.80	<0.001
	GH13_18	2.79	<0.001
	PL17_1	-3.27	0.05
	PL1	-3.40	0.04
	GH13_41	-4.50	<0.001
	GH52	-7.36	<0.001
	GH43_37	-8.40	<0.001
	PL25	-15.72	0.01
	GH43_30	-22.09	<0.001
Enzyme supplementation	GT31	19.93	<0.001
Interaction	GH43_30	23.12	<0.001
	GT31	-22.28	<0.001

4.4 Discussion

From the alpha-diversity analysis of the 16S metabarcoding results, it was shown that exogenous enzymes did not affect the OTUs richness and inverse Simpson diversity index at any intestinal site at any chicken age. Conversely, anti-coccidial vaccination significantly decreased the alpha diversity of the intestinal microbiota of 24-day-old chickens, similar to the findings of previous researchers (Stanley *et al.*, 2014; Wu *et al.*, 2014). However, it was still unknown whether it was the protozoa in the vaccine, host immunity or both that limit the alpha diversity of bacteria presented at both ileum and caecum.

Interestingly, at 35 days of age where coccidial infection or cross-contamination to the non-vaccinated chicken occurred, the ileal microbiota of vaccinated chickens had a higher number of observed OTUs than non-vaccinated chickens. In contrast, anti-coccidial vaccination decreased the number of observed OTUs in the caecum of 35-day-old chicken similar to the ileum and caecum of 24-day-old chicken. The decrease in number observed OTUs at small intestine (jejunum and ileum) microbiota without affecting the number of the caecal microbiota was demonstrated in previous studies related with necrotic enteritis (Bortoluzzi *et al.*, 2019; Yang *et al.*, 2019). Necrotic enteritis was an acute infection of *Clostridium perfringens* at the chicken small intestine that produced the toxin and resulted in a significant reduction of growth performance (Paiva and McElroy, 2014). Predisposing factors such as infection of *Eimeria* protozoa or coccidiosis vaccination could worsen the negative effects of necrotic enteritis and results in sudden death (Timbermont

et al., 2011; Paiva and McElroy, 2014). In this study, the decreasing number of observed OTUs in the ileum of 35-day-old non-vaccinated chickens could be a result of early-stage of clinical or subclinical necrotic enteritis, however, necrotic enteritis clinical signs such as diarrhoea or ruffle feathers were not observed including no observation of macroscopic clinical or subclinical lesions such as distended or ballooning, necrosis and green to yellow diphtheritic membrane of the jejunum or ileum (Timbermont *et al.*, 2011; Paiva and McElroy, 2014). Moreover, no significant difference between the bacterial abundance in the ileum of vaccinated and non-vaccinated chicken at 35-days of chicken age. Therefore, the precise cause of the reduction in the observed OTUs could not be fully explained.

From beta diversity analysis, the ileal microbiota was dominated by bacteria in the phylum Firmicutes of 24- and 35-day-old chicken, similar to many studies (Gong *et al.*, 2007; Yeoman *et al.*, 2012; Xiao *et al.*, 2017). At 24 days of age, the dominant bacterial families in the ileum were Lactobacillaceae, Clostridiaceae and Peptostreptococcaceae, but the majority of the population of 35-day-old chicken was Lactobacillaceae. This dominance of Lactobacillaceae resulted in the lower number of observed OTUs and lower diversity index values observed in the ileum as the chickens grew.

The dominant bacteria in the caecum changed according to the age of the chicken as shown in previous studies (Johnson *et al.*, 2018; Ocejo, Oporto and Hurtado, 2019). In Johnson et al. (2018), *Bacteroides fragilis*, *Ruminococcus spp* and Lachnospiraceae were the core members of the caecal bacterial

populations of chickens older than 14 days. In this study, family Bacteroidaceae, family Ruminococcaceae and an unknown family in order Clostridiales were the core members of the caecal microbiome at 24 and 35 days of chicken age. This difference between studies may be the result of host genetics, diets, environment or health status (Borda-Molina, Seifert and Camarinha-Silva, 2018; Kers *et al.*, 2018; Shang *et al.*, 2018).

At 35 days of age, there was an increased abundance of the family Campylobacteriaceae in chickens in all treatments and became the most abundant bacterial population in the caecal microbiome. *Campylobacter jejuni*, a member of family Campylobacteriaceae, is one of the most important food-borne diseases and was commonly found in chickens after 21 days of age (Van Gerwe *et al.*, 2009; Awad, Hess and Hess, 2018). In the past, *Campylobacter jejuni* has been considered as a commensal bacterium which colonises the chicken wihtout infecting it (Awad, Hess and Hess, 2018). However, in recent studies, effects of *Campylobacter jejuni* colonisation in chickens were reported, such as impaired intestinal integrity, increased intestinal permeability, altered tight junctions, induction of an immune response and changes in gut microbiota (Humphrey *et al.*, 2014; Awad, Hess and Hess, 2018). Therefore, the small significant difference of caecal microbiota between treatments at 35 days of chicken age might be a confounding effect from the high abundance of family Campylobacteriaceae in all treatments.

Similar to alpha diversity, vaccination had more of an effect on the beta diversity of the intestinal microbiome than the enzyme supplementation. At 10-days of chicken age, the abundance of *Anaeroplasma spp* showed significant differences due to both factors and their interaction, in the caecum. A high abundance of *Anaeroplasma spp* has previously been related with low body weight or poor feed efficiency of the animals (Niu *et al.*, 2015; Zeng *et al.*, 2015; Tan *et al.*, 2018; Shi *et al.*, 2019). These studies provide similar results to this study where a higher abundance of *Anaeroplasma spp* was observed in poor feed efficiency (higher abundance in vaccinated chickens than non-vaccinated chicken). In heat stress chickens, the abundance of *Anaeroplasma spp* was increased when compared to the control chickens (Shi *et al.*, 2019). *Anaeroplasma spp* has also been shown to have a positive relationship with crude fibre digestibility in pigs (Niu *et al.*, 2015). In this study, a high abundance of *Anaeroplasma spp* was observed in non-enzyme supplemented chickens at 10 days old. However, no significant difference of *Anaeroplasma spp* was observed between factors at either intestinal regions in 24- and 35-day-old chickens. This result might show that the supplementation of enzymes altered the digestibility of crude fibre and related with growth performance only in the caecum of the young chickens, however, further investigation in older chickens was required to confirm this hypothesis.

In the ileum of 24-day-old chickens, 11 genera showed significant differences in abundance between non- and vaccinated chickens. Out of eleven genera, only one bacterial genus (*Rummeliibacillus spp.*) that vaccinated chickens had

significantly higher abundance than non-vaccinated chickens. First bacterial strains belong to the genus *Rummeliibacillus* were isolated from the environment in 2009 (Vaishampayan *et al.*, 2009). *Rummeliibacillus stabekisii* was characterised as a keratinolytic bacteria that digested the protein-rich materials such as bird feather into amino acids (Saarela *et al.*, 2017). Recent study demonstrated that *Rummeliibacillus stabekisii* could be used as probiotics which enhance the growth performance and immunity of the Nile tilapia (Tan, Chen and Hu, 2019). *Rummeliibacillus stabekisii* produced the enzymes xylanase and protease that may related with the nutrient digestibility and growth performance of the Nile tilapia (Tan, Chen and Hu, 2019). However, in this study, the abundance of bacteria in the genus *Rummeliibacillus* was not related with the growth performance of the chicken at 24 days old but might indicated that there was more undigested nutrients available at the intestine of the gut-damaged chickens that promote the growth of bacteria genus *Rummeliibacillus*. However, as the positive results of the use of bacteria genus *Rummellibacillus* as probiotics in the Nile tilapia was reported, more research on the role of the newly characterised genus *Rummeliibacillus* on the chicken needs to be studied in the future.

Rikenella spp was more abundant in the caecum of non-vaccinated chickens than in vaccinated chickens at 24 days old. An increase of Rikenellaceae family has previously been observed in high production performance chickens and diet-induced obesity mice (Clarke *et al.*, 2013; Zhu *et al.*, 2019). In this study, the highest abundance of *Rikenella spp* was found in the non-

vaccinated with enzyme supplementation chickens, which also had the highest body weights. However, no significant difference in *Rikenella spp* was observed in both intestinal regions of chickens at the other ages. This finding might show that the *Rikenella spp* might be related to the growth of the chicken at a specific period of the fast-growing commercial broiler chicken.

The beta-diversity results from shotgun metagenomics analysis were quite similar to the 16S metabarcoding results. The combined abundance of Firmicutes, Firmicutes_A and Firmicutes_C phyla in shotgun metagenomics were 45.83% while the Firmicutes phylum abundance from 16S metabarcoding analysis was 44.45%. The second most abundant phyla were Bacteroidota (formerly Bacteroidetes) at 42.20% from shotgun metagenomics analysis using GTDB and Bacteroidetes at 36.35% from 16S metabarcoding analysis. These similar findings at the Phylum level showed that the quantitative analysis of gut microbiome was comparable between 16S rRNA metabarcoding and shotgun metagenomics analysis. However, at the lower taxonomic level such as family level, quantitative analysis by 16S rRNA metabarcoding and shotgun metagenomics analysis showed a distinct result between them as the name of the bacterial families were different among the databases used.

The NMDS plots also showed that the factor that most affected the composition of caecal bacteria was anti-coccidial vaccination in both analyses. The abundance comparison between non-vaccinated and vaccinated chickens from shotgun metagenomics identified more significantly different genera than

the 16S metabarcoding analysis without overlapping genera between analyses (19 to 2 genera respectively). Four out of nineteen significant different bacterial MAGs overrepresented in vaccinated chickens were *Bacteroides fragilis*, *Fournierella species GCF_002161595.1*, undefined species of genus *Flavonifractor* and undefined species of genus *Ruthenibacterium*. A previous study also observed a higher abundance of *Bacteroides spp* in *Eimeria* infected chickens compared to non-infected chickens (Macdonald *et al.*, 2017). The researcher also reported that *Bacteroides spp* could prolong the survival of some pathogenic bacteria such as *Escherichia coli* and may result in more severe tissue damage (Rotstein *et al.*, 1989; Macdonald *et al.*, 2017). However, the species of bacterial genus *Bacteroides* affected by the *Eimeria* protozoa was not identified in previous study. In this study, *Bacteroides fragilis* known as an immunomodulating gut commensal bacteria in human was overrepresented in vaccinated chickens. Capsular polysaccharide (polysaccharide A) produced by *Bacteroides fragilis* related with the activation of T cell-dependent immune responses which affected the development and homeostasis of host immune response (Troy and Kasper, 2010). Moreover, novel strain of *Bacteroides fragilis* also showed the enhancement of phagocytosis by the macrophage using *in vitro* analysis (Deng *et al.*, 2016). These findings showed that the overrepresented of *Bacteroides fragilis* in vaccinated chickens may directly related with the homeostasis of the host immune response.

In contrast, *Flavonifractor spp* and *Ruthenibacterium spp* may indirectly related with the host immune response as they have previously been found to produce short-chain fatty acids in the animal and human intestine (Eeckhaut *et al.*, 2011; Levine *et al.*, 2013; Shkoporov *et al.*, 2016). Short-chain fatty acids such as butyrate or acetate have been shown to have positive effects on host immunity and gut health (Sunkara *et al.*, 2011; Arpaia *et al.*, 2013; Kim, 2014). Previous reports demonstrated that the amount of short-chain fatty acids was increased in *Eimeria*-infected chicken (Stanley *et al.*, 2014; Leung *et al.*, 2019). The possible explanations of the increased abundance of these short-chain fatty acid producing bacteria could be; (1) the altered nutrients in the caecum that was a result of damaged small intestine; (2) the cross-talk between host and bacteria in order to control the pH of the caecum; and (3) the increase demand of the energy from the host that results in the increase production of short chain fatty acid (Sunkara *et al.*, 2011; Arpaia *et al.*, 2013; Kim, 2014). Therefore, the abundance of these short chain fatty acid producing bacteria may directly or indirectly related with anti-coccidial vaccination or immune system of the chicken.

In addition, the poor performance of the vaccinated chicken may also cause by the negative effects of the unabsorbed nutrients by the damaged small intestine caused by the *Eimeria* protozoa as shown in the growth performance and the presence of coccidiosis lesions (Chapman, 2014; Apajalahti and Vienola, 2016). As the loss of proteins into the intestine is a well-known circumstance that associated with the poor or loss of intestinal barrier integrity

(Gilani *et al.*, 2017; Yang, Li and Balthasar, 2017), the combination of undigested or unabsorbed proteins with the endogenous protein could lead to the increase of putrefaction in the caecum (Apajalahti and Vienola, 2016). Putrefaction or protein fermentation by the bacteria produced toxic end-products such as amines, indoles, cresol and ammonia (Apajalahti and Vienola, 2016). These molecules were known to cause the detrimental effects on the host such as the altering intestinal epithelial morphology and DNA synthesis by the ammonia in human or the inhibition of oxidative phosphorylation at the human colon by the indoles (Hughes, Magee and Bingham, 2000; Chimerel *et al.*, 2013). From the *in vitro* study using human faecal bacteria, *Clostridium spp*, *Clostridium perfringens*, *Enterococcus spp*, *Shigella spp* and *Escherichia coli* were identified as hyper-ammonia-producing bacteria (Richardson, McKain and Wallace, 2013). However, in this study, the abundances of these bacteria in the gut were not significantly different between non- and vaccinated chickens. Moreover, the measurement of the putrefaction end-product was not performed in this study; therefore, further study on the protein fermentation could provide insightful information and knowledge on the relationship between gut bacteria and protein digestibility of the chicken including their effects on gut health.

On the contrary, *Bacteroides clarus* was significantly overrepresented in enzymes-supplemented chickens than non-supplemented chickens. In addition, interaction effect between anti-coccidial vaccination and exogenous enzymes was observed on the abundance of *Bacteroides clarus. Bacteroides*

clarus was a newly identified bacteria from human faeces (Watanabe *et al.*, 2010). In chicken, *Bacteroides clarus* was recently identified by shotgun metagenomics and was able to colonise in the young chicks intestine (Medvecky *et al.*, 2018; Kubasova *et al.*, 2019). However, with less information about the role and function of *Bacteroides clarus* in the intestine up to date, the effect of enzyme-supplementation including interaction effect between exogenous enzymes and anti-coccidial vaccination on this bacterium could not be concluded. Further investigation on this bacterium could be studied in the future to identify its role in the chicken gut. Moreover, this finding also emphasise the benefit of shotgun metagenomics analysis over the 16S metabarcoding analysis where bacterial species or strain of the bacteria can be classified and used in further in-depth study.

The most abundant CAZyme classes found in the caecal microbiome was GH class at 58.8%, followed by GT class at 16.4%. This proportion was close to those found in the cattle rumen microbiome (GH at 56-57% and GT at 18-20%) (Wang *et al.*, 2019), but less similar to mouse and human faeces where GH was approximately at 40%, and GT was approximately at 30% (Xiao *et al.*, 2015). This difference in the relative abundance of the GH family might be a result of a high proportion of plant fibre in the chicken and cattle diets, as several GH families were involved with the metabolism of polysaccharides such as xylans, cellulose and starch (Wang *et al.*, 2019).

From the comparison of the abundance of protein in CAZyme class, the abundance of protein in SLH or S-layer homology domain class of the caecal

microbiome were significantly different between non-vaccinated and vaccinated chickens. SLH was shown to be related to the binding of the S-layer proteins to the plant cell walls (Mesnage *et al.*, 2000). S-layer proteins are glycoproteins that overlay the cell surface of several species of micro-organisms and are in direct contact with the environment and may be involved in many surface properties including adherence to various substrates, mucins, aggregation and coaggregation with other bacteria (Gerbino *et al.*, 2015). S-layer also played an important role in the interaction with the host immune system in the modulation of the cytokine gene expression (Gerbino *et al.*, 2015). Therefore, increased abundance of the protein in the SLH class of the vaccinated chicken may be related to changes in the interaction between the bacteria and the nutritional environment, other bacteria and host immune response.

Anti-coccidial vaccination also significantly affected the abundance of 17 CAZyme families. The abundance of 7 CAZyme families in a combination of GH and PL families of vaccinated chickens were significantly higher than non-vaccinated chickens. In contrast, 10 CAZyme families in a combination of CBM, GT, GH and SLH of vaccinated chickens were lower than non-vaccinated chickens. The differences between the abundance of these carbohydrate-active enzymes or fibre-degrading enzymes between non-vaccinated and vaccinated chickens could be related with the ability of the microbiota to extract nutrients and energy from the un-absorbed particles (Kiarie, Romero and Nyachoti, 2013). Unfortunately, as the measurement of

the carbohydrate molecules such as xylose or arabinose was not performed in this study, it could not be concluded whether it was the amount ofnutrients that caused the significant differences in these CAZyme families in the chicken caecum.

Vaccinated chickens had a significantly higher abundance of CAZyme family GH43 subfamily 30 and 37 than non-vaccinated chickens. GH43 family was considered as the most abundant CAZyme from studies of the human gut microbiome (Mewis *et al.*, 2016). This CAZyme family contains several debranching enzymes for the degradation of hemicelluloses, such as arabinoxylans and pectin (Mewis *et al.*, 2016). A higher abundance of proteins in CAZyme family GH43 might indicate that the nutrients in the caecum of the non-vaccinated and vaccinated chickens were different. Moreover, the interaction effect of anti-coccidial vaccination and exogenous enzymes supplementation was also observed on the abundance of proteins in CAZyme family GH43 subfamily 30. Increased abundance of the proteins in CAZyme family GH43 subfamily 30 were observed in vaccinated chickens without enzyme supplementation. This interaction finding may suggest that vaccinated chickens have undigested nutrients that could enter the caecum where bacteria could produce more enzymes to utilise them. In addition, enzyme supplemented chickens might absorb these small molecules that were digested by the exogenous enzymes, therefore, vaccinated chickens without enzyme supplementation had more of these nutrients for the bacteria in the caecum to digest. However, this finding needs to be confirmed by further study.

In contrast, non-vaccinated chickens had a significantly higher abundance of CAZyme family GT31 than vaccinated chickens. The main function of the enzymes in the GT class is to catalyse the transfer of a sugar compound onto saccharide or non-saccharide acceptors (Sinnott, 1990; Coutinho *et al.*, 2003). Moreover, the galactosyltransferase function of the GT31 family relates to the synthesis of *N*- and *O*-glycans on glycoprotein (Egelund *et al.*, 2010). The *O*-glycans were demonstrated to be related to the mucous layer of the intestinal epithelium which maintains the homeostasis between the host and gut bacteria (Bergstrom and Xia, 2013; Yamada *et al.*, 2019). The increased abundance of this galactosyltransferase protein family in the caecal bacteria that is involved with the *O*-glycans synthesis could be related with the mimicry function of the bacteria to evade host immune response (Comstock and Kasper, 2006; Varki, 2017). These findings may suggest that the communication between host mucous layer and bacteria of the vaccinated chickens could be disrupted due to the unbalanced nutrients and elevated immune status of the chickens.

Although the xylanase and glucanase enzymes are members of the GH class (Nguyen *et al.*, 2018), no significant difference in the abundance of CAZyme families belong to the GH class was observed when compared between non-supplemented and enzymes-supplemented chickens. Non enzymes-supplemented chickens had a significantly higher abundance of the CAZyme family GT31 than enzymes supplemented chicken. The CAZyme familes in the glycosyl transferase class may relate to the glycosylation of the gut bacteria which regulates homeostasis between host and gut microbiome (Latousakis

and Juge, 2018). However, further study on the role of the GT31 CAZyme family in chicken gut bacteria needs to be studied to further explain the role of the CAZyme family GT31 in the chicken gut.

From the results of the 16S rRNA metabarcoding and shotgun metagenomics analysis on the chicken gut microbiome study using the anti-coccidial vaccination and exogenous enzymes as an experimental model, the results showed that exogenous enzymes had small effects on the gut microbial population and function on using CAZymes analysis. These findings could be explained by the nutritional role of the caecum and the role of enzymes on the digesta. The nutrient particles entering the caecum were expected to be undigested starch and protein as well as fibre that were finely-ground particles, low-molecular-weight and non-viscous molecules (Kiarie, Romero and Nyachoti, 2013; Svihus, Choct and Classen, 2013). The inclusion of exogenous enzymes such as xylanase which cleave the large molecule of arabinoxylan could be related to the entry of the nutrient into the caecum (Svihus, Choct and Classen, 2013). Exogenous enzymes were shown to have an effect on the nutrients that entering the caecum in previous studies. An increasing amount of arabinose and xylose in the caecum were observed in the chicken fed with diets pre-treated with xylanase (Denstadli *et al.*, 2010).

Conversely, no significant change in the caecal fermentation was observed in the chicken fed with beta-glucanase inclusion diet as the small effects on the viscosity between negative control chickens and enzymes supplemented chickens were observed (Józefiak *et al.*, 2006). These findings demonstrated

that the effects on the caecal function might depend on other aspects of the digesta, such as quality and quantity of the nutrients that enter the caecum and digesta viscosity. Unfortunately, in this study, no measurement of the digesta viscosity, caecal fermentation or the quantity of the nutrients in the digesta was performed. Therefore, it could not be concluded whether the small effect of the exogenous enzymes supplementation on the gut microbiome in this study was the results of the quality and quantity aspects of the digesta.

As the function property of the gut bacteria and bacterial population were not affected by the exogenous enzymes, supplementation of the enzymes might affect the quantity of the gut bacteria. Using the qPCR technology or the flow cytometry technique to measure the number of bacteria in the sample could provide more information on the effects of the exogenous enzymes on the quantity of gut microbiota. However, the measurement of the quantity of the gut microbiota was not performed in this study. In addition, another possibility was that the significant beneficial effects of the exogenous enzymes on the FCR not on the feed intake and body weight of the chickens were insufficient for the ability of the sequencing technology and bioinformatics analysis up to date to detect the differences. With the advance of technology in the future such as improved quality and quantity of sequencing reads, better taxonomic databases for the classification of bacteria, and better database for bacterial functionetc., it might be possible to detect these small effects of exogenous enzymes on the gut microbiota.

In summary, this chapter showed the effects of a host health intervention and a dietary intervention on the chicken intestinal microbiota. The factors that most influenced the intestinal bacteria were age and intestinal region. Anti-coccidial vaccination affected both bacterial composition and the function of the gut microbiota, while exogenous enzymes showed less of an effect on bacterial composition but still affected the function of the gut microbiota. No bacteria were found to be consistently significantly differentially abundant between factors at all chicken ages. This finding indicated that no strong relationship between factors and specific bacteria was found in the whole production period as the bacteria that related with the immune status and absorption changed at the different age of the chicken were demonstrated in this study. However, the small changes in the ileal and caecal microbiome analysis could be a result of the cross-contamination or infection of the *Eimeria* protozoa in non-vaccinated chickens. As the coccidiosis lesion score was observed in non-vaccinated chickens at 24 days of age, the microbial population and metabolic function of the gut microbiome may have been affected by the protozoa. The coccidiosis lesion score results suggested that the cross-contamination or infection to non-vaccinated chickens might have occurred a few days before the sample collection (before 24 days of chicken age). Therefore, performing the microbiome analysis at the early stage of immune response in the non-vaccinated chicken and the latter stage of immune response in the vaccinated chicken could have led to small

differences between them. Further transcriptomic analyses in the following chapter could support this viewpoint.

Chapter 5

Intestinal transcriptomic analysis of exogenous enzyme supplementation and anti-coccidial vaccination in chickens

5.1 Introduction and Aims

In **Chapter 4**, it was shown that exogenous enzymes had a smaller effect on the gut microbiome in comparison to anti-coccidial vaccination. From the 16S rRNA metabarcoding analysis and shotgun metagenomics analysis, the gut bacterial population was shown to be affected mainly by the anti-coccidial vaccination. Functional analysis of the bacteria using CAZymes analysis also showed a larger effect of the anti-coccidial vaccination in comparison to exogenous enzyme supplementation. As the exogenous enzyme supplement improved growth performance, it is hypothesised that there was an effect on the host response related to the nutrient absorption in enzyme-supplemented chicken. Moreover, identification of the host gene expression could also lead to further study about the interaction between host and gut microbiota.

Differential gene expression analysis has been performed by many researchers to observe the effects of the interventions on the host response in the intestine (Schena *et al.*, 1998; VanGuilder, Vrana and Freeman, 2008; Lovén *et al.*, 2012). The expression of immune-related genes has been studied in order to evaluate the effect of nutrients or pathogens on the host immune response (Parreira *et al.*, 2016; Truong *et al.*, 2017; Li *et al.*, 2019). Nutrient transporter related genes have also been studied by using an intestinal gene expression analysis to identify genes related to the host phenotypes and diets (Gal-Garber *et al.*, 2000; Mott *et al.*, 2008; Gilbert *et al.*, 2010). Although these intestinal gene expression analyses were performed using conventional PCR methods, comprehensive studies using more advanced sequencing

technologies should provide more information about the effects of anti-coccidial vaccination and enzyme supplementation on the chicken digestive system. Therefore, using RNA-seq analysis, intestinal gene expression in response to the anti-coccidial vaccination and enzyme supplementation could be used to compare gut-damaged and control chickens, and the host response to nutrient manipulation with the exogenous enzymes.

In this chapter, a transcriptomic analysis was performed with the ileal and caecal samples of 24-day-old chickens in order to study the effects of anti-coccidial vaccination and exogenous enzymes on the host response. Although the coccidiosis lesion score was observed in 24-day-old non-vaccinated chickens, the growth performance at the end of the starter phase (24 days of chicken age) still maintained the expected results from the experimental design of the comparison between gut damaged and control chickens. Moreover, previous reports demonstrated that the peak observation of the coccidiosis lesion score after the administration of anti-coccidial vaccine was 12-18 days (Williams and Andrews, 2001); therefore, sample collection from 24-day-old chickens or 21 days post-vaccination was expected to show the effects of the vaccination on the gut microbiota and intestinal gene expression between gut-damaged and control chickens. Differential gene expression analysis between these factors was compared statistically. The enrichment of the gene sets was analysed and statistically compared between factors to identify significantly enriched pathways or functions.

5.2 Materials and Methods

5.2.1 Sample Collection

Intestinal samples were collected from chickens as described in **Chapter 3**, section 3.3.5. Intestinal tissue was washed with sterile normal saline to remove intestinal contents. Tissue was cut with a sterile surgical blade and forceps into approximately 0.5 x 0.5 x 0.5 centimetres in size sections on the sterile petri-dish. Tissue was stored overnight at 4 °C in RNA*later* solution (Thermo Fisher Scientific, Waltham, MA, USA) in 2 millilitres sterile tube before being transferred to the -80 °C freezer in Thailand.

5.2.2 RNA extraction

Caecal and ileal tissue samples of 20 chickens (similar to the samples in Shotgun Metagenomics analysis) were selected for RNA extraction (total 40 samples). RNA extraction was performed with the RNeasy mini kit (Qiagen, Valencia, CA, USA). In brief, 20 mg of intestinal tissue was homogenised with Qiagen stainless steel beads (diameter = 5 millimetres) using the Qiagen TissueLyser LT (Qiagen, Valencia, CA, USA) at 50 Hertz for 3 minutes. After this step, RNA extraction was carried out according to the manufacturer's protocol. RNA samples were kept at -80 °C in Thailand and were shipped on dry ice to the Roslin Institute for library preparation and further analysis. The samples reached the laboratory within 96 hours after departure and were in frozen condition on dry ice.

5.2.3 mRNA sequencing

RNA samples were submitted to Edinburgh Genomics (Edinburgh, UK) for quality control and sequencing. A mRNA library was constructed using a TruSeq stranded mRNA-seq library prep kit (Illumina, San Diego, CA, USA). Paired-end reads of 100 base pairs in length were generated from this library, sequenced on the NovaSeq 6000 system (Illumina, San Diego, CA, USA) by Edinburgh Genomics.

5.2.4 Bioinformatics analysis

Illumina adapters were removed from the sequencing reads with trimmomatic version 0.38 (Bolger, Lohse and Usadel, 2014). The chicken reference transcriptome (galGal6) obtained from Ensembl release 96 was used as a reference database (ftp://ftp.ensembl.org/pub/release-96/fasta/gallus_gallus/cdna/Gallus_gallus.GRCg6a.cdna.all.fa.gz). An index of the chicken reference genome was created by Kallisto version 0.44.0 (Bray *et al.*, 2016). Kallisto uses a pseudo-alignment without mapping the nucleotide base to the reference genome. This alignment-free method improves the speed and computer resources from the alignment method such as Tophat-HTSeq or STAR-HTSeq with similar accuracy (Bray *et al.*, 2016; Everaert *et al.*, 2017).Transcripts mapped to the reference were quantified as transcripts per million (TPM) with Kallisto version 0.44.0 and were imported to R version 3.5.3 for differential gene expression analysis. Transcripts were annotated using gene annotation reference from GTF file of galGal6 obtained from Ensembl

release 96 (ftp://ftp.ensembl.org/pub/release-96/gtf/gallus_gallus/Gallus_gallus.GRCg6a.96.gtf.gz) using the *DESeq2* package in R version 3.5.3. The TPM counts were normalised using the *DESeq2* package in R version 3.5.3 before differential gene expression analysis was performed. PCA graphs using TPM counts at the ileum and caecum were plotted and compared between factors with the adonis test from the *vegan* package in R version 3.5.3. Differential gene expression analysis was compared based on a 2 by 2 factorial design and performed using the *DESeq2* package in R version 3.5.3. Significant differential gene expression between anti-coccidial vaccination and enzyme supplementation and their interaction was classified by absolute $\log_2$ fold change (≥ 2) and the Benjamini-Hochberg adjusted P-value (adjusted P-value < 0.05). Gene Set Enrichment Analysis (GSEA) was performed using chicken Gene Ontology (GO) term version April 2015 from GO2MSIG as a reference, and comparisons between factors were performed using the *fgsea* package in R version 3.5.3 (http://www.go2msig.org/go2msig/collections/Gallus_gallus_GSEA_GO_sets_all_symbols_April_2015.gmt.zip) (Subramanian *et al.*, 2005; Powell, 2014; Sergushichev, 2016). From the statistical comparison, significant enriched gene set was identified, using the Benjamini-Hochberg adjusted P-value to control for multiple tests (adjusted P-value < 0.05). Data were plotted and visualised using the *gplots* and *ggplot2* packages in R version 3.5.3.

5.3 Results

A mean average of 64,529,658 ± 16,353,876 RNA reads per sample were generated from sequencing. After the quantification by Kallisto, reads were pseudo-aligned at an average of 74.62% per sample (ranging from 71.2% to 77.3%).

The PCA plots of the normalised TPM results of ileal and caecal samples are shown in **Figure 5.1A** and **Figure 5.1B**, respectively. From dissimilarity comparisons using the adonis test, the only factor that significantly affected overall intestinal gene expression was anti-coccidial vaccination (P-value < 0.001 in both the ileum and caecum). No significant difference was observed due to enzyme supplementation with no interaction between factors in either the ileum or caecum.

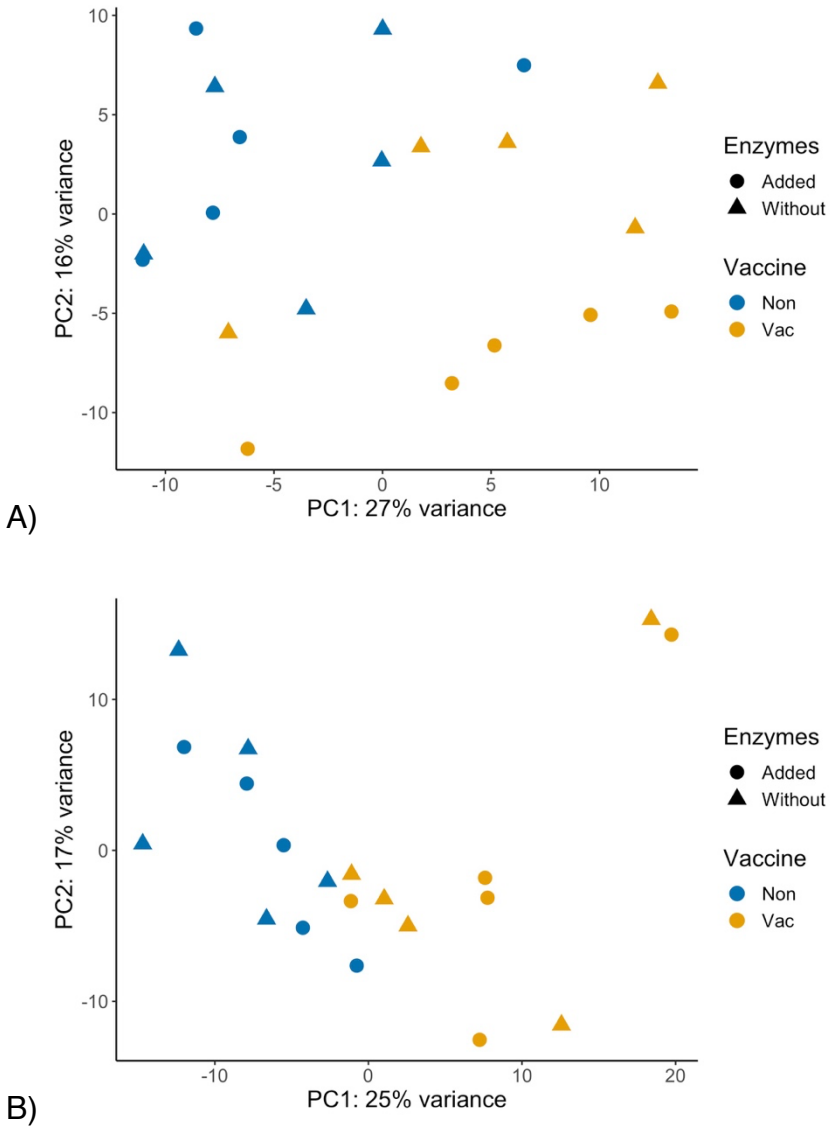


Figure 5.1: PCA plot of gene expression in the A) ileum and B) caecum of 24-day-old chickens. Using the adonis test, gene expression at both regions showed significant different when compared between non-vaccinated and vaccinated chickens (both P-value < 0.001) while the comparison of non-supplemented and enzyme-supplemented chickens showed no significant difference (P-value > 0.05). (Non = non-vaccinated chickens; Vac = vaccinated chickens)

In the ileum, there were 4 differentially expressed genes when compared between non-supplemented and enzyme-supplemented chickens (**Table 5.1** and **Table S19-S20**). Two out of four were unannotated genes, while the *NEU4* and *GAL3ST2* genes were up-regulated in enzyme-supplemented chickens. There were 96 differentially expressed genes between non-vaccinated and vaccinated chickens (**Figure 5.2** and **Table S21-S22**). Interestingly, several up-regulated genes in vaccinated chickens related to the immune system, e.g. *CCL1*, *CCL4*, *CCL26*, *IFNG*, *ACKR4* and *IL4I1*. In contrast, many up-regulated genes in non-vaccinated chickens were members of solute carrier family related genes such as *SLC4A9, SLC5A12, SLC26A3* and *SLC26A4*. In addition, 11 genes showed a significant interaction between both factors (**Table S23**). Interestingly, the interaction effect showed that enzyme-supplementation significantly up-regulated solute carrier family related genes such as *SLC4A9* and *SLC26A4* in non-vaccinated chickens when compared to the vaccinated chickens. In addition, none of the unannotated genes at the ileum had a high confident orthologue to human, mouse or other avian species.

Table 5.1: Number of significantly differently expressed genes from each factor comparison and their interaction using *DESeq2* analysis (log_2 fold change < -2 or > 2 and adjusted P-value < 0.05)

Factor comparison	Differential expression	Ileum	Caecum
Vaccinated	Up-regulated in vaccinated chickens	51	33
	Up-regulated in non-vaccinated chickens	45	13
Enzymes supplementation	Up-regulated in non-enzyme supplemented chickens	1	1
	Up-regulated in enzyme supplemented chickens	3	2
Interaction		11	0

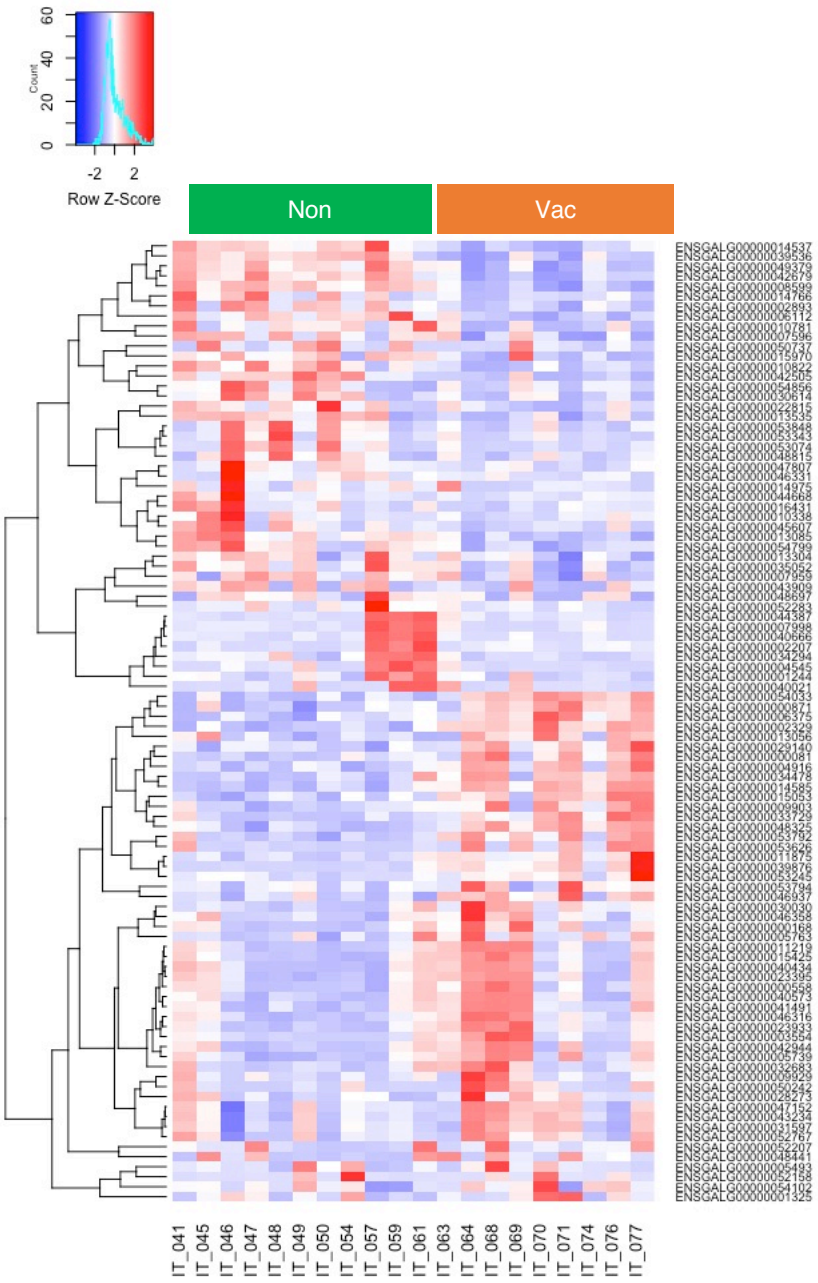


Figure 5.2: Heatmap showing significantly differentially expressed genes between non-vaccinated and vaccinated chickens in the ileum (absolute log_2 fold change > 2 and adjusted P-value < 0.05). (Non = non-vaccinated chickens; Vac = vaccinated chickens)

For the enzyme supplementation comparison in the caecum, two unannotated genes were up-regulated in enzyme supplemented chickens, while *CES1L2* was up-regulated in non-enzyme supplemented chickens (**Table S24-S25**). There were 46 differentially expressed genes when compared between non-vaccinated and vaccinated chickens (**Table 5.1** and **Figure 5.3**). Lists of 46 differentially expressed genes are shown in **Table S26-S27**. Several up-regulated genes in vaccinated chickens were related to membrane transport (e.g. *TMEM252* and *GPNMB*) and the immune system (e.g. *GZMA*, *GZMK*, *CCL1*, *CCL4*, *CIITA* and *GNLY*). Some up-regulated genes in non-vaccinated chickens were classified as sodium channel family related genes (*SCNN1B* and *SCNN1G*) and oligopeptide transporter gene belonged to the *SLC15* family such as *SLC15A5*. Moreover, no significant interaction between both factors was observed. In addition, none of the unannotated genes at the caecum had a high confident orthologue to human, mouse or other avian species.

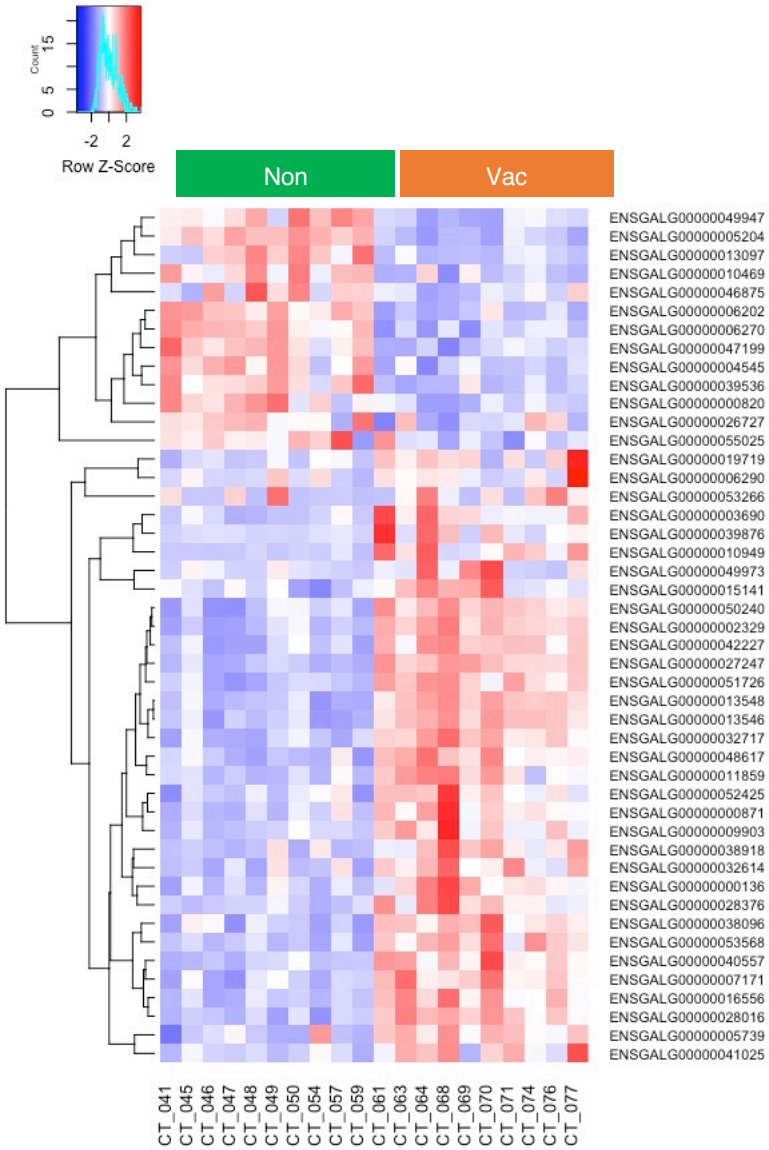


Figure 5.3: Heatmap showing significantly differentially expressed genes between non-vaccinated and vaccinated chickens in the caecum (absolute $\log_2$ fold change > 2 and adjusted P-value < 0.05). (Non = non-vaccinated chickens; Vac = vaccinated chickens)

The GSEA results for the ileum showed no significantly enriched GO terms when compared between anti-coccidial vaccination and enzyme supplementation. In the caecum, no GO term was significantly enriched when compared between non-supplemented and enzyme-supplemented chickens. Conversely, 173 GO terms showed significantly different enrichment between non-vaccinated and vaccinated chickens in the caecum (132 and 41 GO terms were enriched in vaccinated chickens and non-vaccinated chickens respectively). From the top 50 GO terms, several GO terms enriched in non-vaccinated chickens were related with transmembrane transport such as hydrogen transport & proton transport, hydrogen ion transmembrane transport and cation transmembrane transporter (shown as positive normalised enrichment scores in **Figure 5.4**). In contrast, multiple GO terms enriched in vaccinated chickens were related to the immune system; such as leukocyte differentiation, T-cell differentiation, immune system development, adaptive immune response, lymphocyte differentiation and many more (shown as negative normalised enrichment scores in **Figure 5.4**).

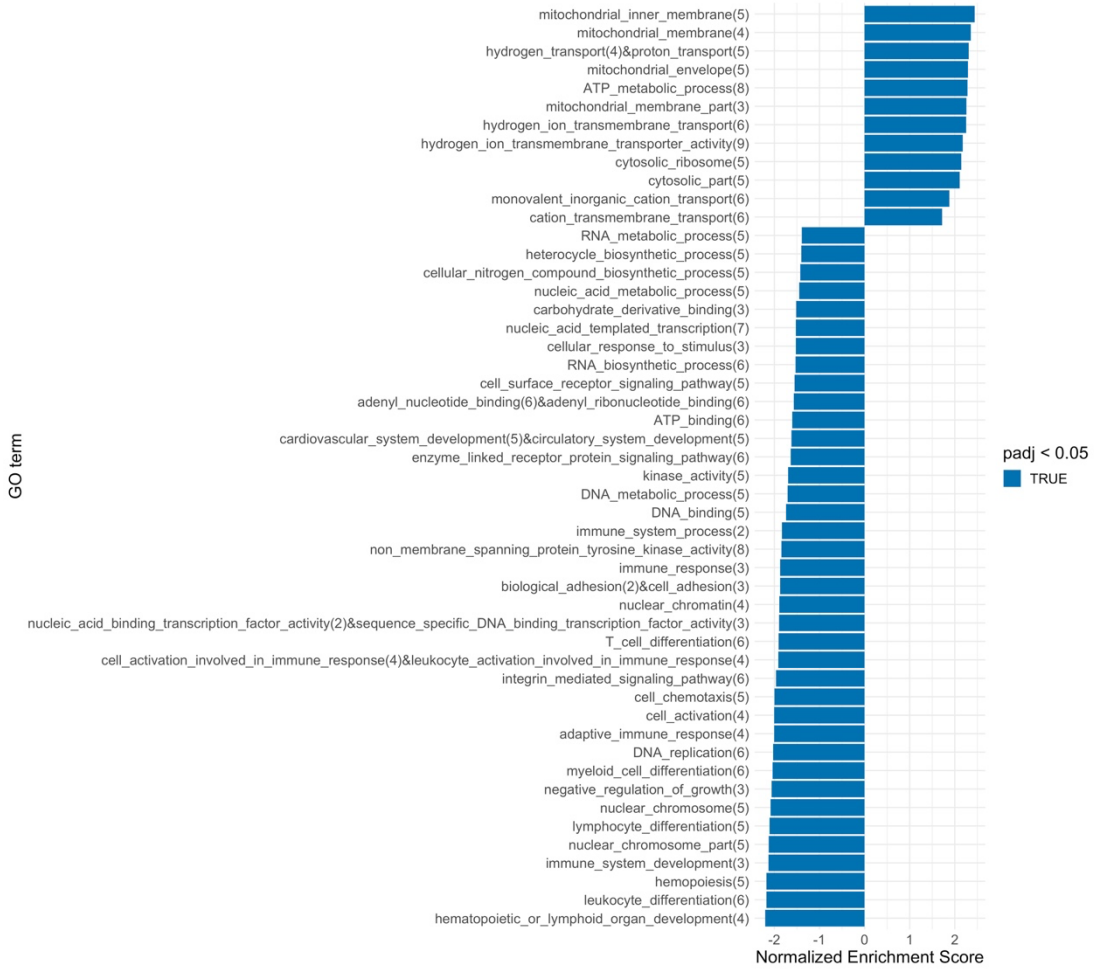


Figure 5.4: Top 50 significantly enriched GO terms from Gene Set Enrichment Analysis (GSEA) in the caecum of 24-day-old chickens (adjusted P-value < 0.05). Gene sets enriched in anti-coccidial vaccinated chickens had a negative normalised enrichment score while non-vaccinated chickens had a positive enrichment score. The shortest distance between GO term to the root term (such as cellular component, biological process or molecular function) was shown in the parentheses behind each GO term.

5.4 Discussion

Similar to the results of the 16S rRNA metabarcoding and shotgun metagenomics analysis in **Chapter 4**, section 4.3, in 24-day-old chickens, anti-coccidial vaccination had more of an effect on intestinal gene expression than the exogenous enzyme supplementation. The number of significantly differentially expressed genes when compared between vaccinated and non-vaccinated groups was higher than when comparing groups which had or had not been given exogenous enzyme supplementation, in both the ileum and caecum. However, the number of differentially expressed genes in this study was far lower than in previous research, there are many possible reasons for this (Kyung Kim *et al.*, 2011; Truong *et al.*, 2017; Li *et al.*, 2019).

As discussed in Chapter 4, section 4.4, the evidence of coccidiosis lesion score in 24-day-old non-vaccinated chickens could indicate that the chicken developed an early stage immune response to the protozoa. When compared to the latter stage of the immune response in vaccinated chickens, the number of significantly differentially expressed genes between non-vaccinated and vaccinated chickens could be minimised by these differing stages of the immune response. Another possible reason might be due to the mild infection caused by the low number of oocysts in the vaccine when compared to the high-number of *Eimeria* oocysts in infection studies. Another possibility is that the samples in this study were collected at 21 days post-vaccination, whereas almost all previous infection studies collected samples around 6-10 days post-infection. Moreover, the fold change cutoff value of the differential gene

expression analysis in this study was higher than those previous studies. This cutoff value was selected in order to focus on the high significantly differentially expressed genes for further gene-micorobiota interaction analysis. Therefore, the number of significantly differentially expressed genes in this study might be low due to mild infection from the vaccination or different downstream bioinformatics analysis.

Anti-coccidial vaccination significantly altered the expression of genes related to the immune system of the chickens, with some differences between the ileum and caecum. Out of 7 genes that were significantly differentiallly expressed in both the ileum and caecum, 4 genes annotated as the C-C motif chemokine ligand family (*CCL1* and *CCL4*), Stearoyl-coA Desaturase (*SCD*), ADP-ribotransferase 1 (*ART1*) and Interferon-gamma (*IFNG*) were highly expressed in vaccinated chickens while WD repeated domain 72 (WDR72) was highly expressed in non-vaccinated chickens. Both *CCL1* and *CCL4* are related to the innate immune response of the chicken. *CCL1* has been shown to activate macrophages and T-lymphocytes in chickens with necrotic enteritis (Iellem *et al.*, 2002; Truong, Hong and Lillehoj, 2015). *CCL4* has also been related with infection or stress-induction in many studies in chickens, both *in vivo* and *in vitro* (Ciraci *et al.*, 2010; Slawinska *et al.*, 2016; Kim, Lillehoj and Min, 2017; Schilling *et al.*, 2019). The *SCD* gene has previously been related to fat metabolism, inflammation and positively linked with bodyweight of the chicken (Lefevre *et al.*, 2001; Dridi *et al.*, 2007; Liu, Strable and Ntambi, 2011; Resnyk *et al.*, 2017). Although the increased expression of *SCD* gene was

observed in vaccinated chickens which had poor growth performance, this gene might be involved in the inflammatory response of the chicken. From the *in vitro* study, *SCD* gene was upregulated in the mouse macrophages treated with the inflammation inducing protein, beta-amyloid, but the underlying mechanisms remained unknown (Uryu, Tokuhiro and Oda, 2003). Interferon gamma is a major cytokine with anti-coccidial effects which inhibit the invasion of *Eimeria* protozoa (Kim, Chaudhari and Lillehoj, 2019). Interferon gamma is produced predominantly by T-cells at the site of *Eimeria* infection and by splenocytes (Rothwell, Muir and Kaiser, 2000). Interferon gamma is a cytokine that moderates cell-mediated immunity (CMI), and it is upregulated in *Eimeria* infected chickens (Yun, Lillehoj and Choi, 2000). Exogenous injection of interferon-gamma has also been shown to decrease oocyst counts and increase the bodyweight of *Eimeria* infected chickens (Lillehoj and Choi, 1998). The increased expression of these genes in vaccinated chickens might show that these genes were directly affected by the vaccination at the systemic level or, in contrast, these genes might be involved in a systemic response to the mild infection or inflammation caused by the anti-coccidial vaccination.

CCL1, *CCL4* and *IFNG* are cytokines which are produced by T-lymphocytes and are associated with the Th1 pathway (Hsu *et al.*, 2013; Kishi *et al.*, 2016; Kim, Chaudhari and Lillehoj, 2019). These Th1-type cytokines produce the responses related to the killing of intracellular parasites including *Eimeria* (Berger, 2000; Kim, Chaudhari and Lillehoj, 2019). Therefore, the upregulation of *CCL1*, *CCL4* and *IFNG* could indicate that vaccination against *Eimeria*

protozoa induced the immune response to the protozoa in vaccinated chickens.

Rothwell et al., (2000) also reported that the immunised chickens were able to kill or remove approximately 90% of the protozoa from the challenge in the caeca. Therefore, the developed immune response of the 24-day-old vaccinated chicken could help the chickens clear the protozoa, which resulted in the lower number of the coccidiosis lesion score in the duodenum, ileum and caecum when compared to 10-day-old chickens (**Figure 3.4**). In addition, from the histo-pathological analysis (**Figure S2**), the absence of *Eimeria* gametocyte at the duodenum of the vaccinated chickens might indicate that the chickens were protected by the vaccine and the protozoa had been removed from the duodenum. Unfortunately, the histopathology was not performed in the ileal and caecal tissues of vaccinated chickens.

On the other hand, the down-regulation of IFN-gamma in non-vaccinated chicken in the ileum and caecum when compared to the vaccinated chickens could be due to the early stage of infection. A previous study found that interferon-gamma production in the caecum was detected following infection after 3 days of *Eimeria tenella* challenge (Rothwell, Muir and Kaiser, 2000). However, the earliest observation of coccidiosis lesion scores in the caecum caused by *Eimeria tenella* was at 3.8 days (Williams and Andrews, 2001). From this evidence, IFN-gamma in the caecum of non-vaccinated chickens might be produced but it is still lower than in the vaccinated chickens as the coccidiosis lesions in the caecum were already observed in some chickens.

In vaccinated chickens, in the ileum, highly expressed genes related with the immune system were *ACKR4* and *IL4I1,* while highly expressed genes in the caecum were *GZMA*, *GZMK* and *CIITA.* Previous research has also shown that *ACKR4* or Atypical Chemokine Receptor 4 limits the proliferation of activated B cells which reduces their subsequent differentiation (Kara *et al.*, 2018). Reducing B cell counts might lead to decreased activity of the humoral immune system, which supports previous evidence that the cell-mediated immune system is the major factor in resistance to coccidiosis (Yun, Lillehoj and Lillehoj, 2000). *IL4I1* or Interleukin-4 induced gene-1 is the regulator of T cells that promotes the iTreg and Th17 cells which control the potentially damaging effects of the inflammatory response (Romagnani, 2016). In the caecum, *GZMA* and *GZMK* or Granzyme A and Granzyme K respectively, are serine proteases found in cytotoxic T cells and natural killer cells that are associated with the clearance of antigen (Hameed *et al.*, 1988; Carter *et al.*, 1996; Santiago *et al.*, 2017). *CIITA* or Major Histocompatibility complex (MHC) class II transactivator is a critical transcriptional factor of the MHC class II genes which triggers the adaptive immune response (Steimle, Siegrist and Mottet, 1994; Accolla *et al.*, 2001; Chang, 2004). These results show that vaccinated chickens established an immune response to the protozoa infection, though the host defence mechanisms in the ileum and caecum were different.

In contrast, vaccinated chickens had significantly decreased expression of genes related to the absorption system, in both the ileum and caecum. In the

ileum, the expression of several solute carrier family genes, such as *SLC4A9, SLC5A12*, *SLC26A3* and *SLC26A4*, were decreased. *SLC4A9* or Solute Carrier Family 4, Member 9 is a Sodium Bicarbonate Transporter that is responsible for bicarbonate secretion (Kiela and Ghishan, 2016). Down-regulation of *SLC4A9* in vaccinated chickens may cause changes in the luminal pH which could affect mucin viscosity and mucosal ulcerations, which can subsequently lead to poor growth performance (Ghishan and Kiela, 2012). *SLC5A12* or Solute Carrier Family 5, Member 12 is a sodium-lactate transporter that is related to lactate absorption in humans (Gopal *et al.*, 2007). Lactate is a form of lactic acid which can be used as an energy source by the host (Bergman, 1990; Rinttilä and Apajalahti, 2013). Therefore, poor lactate absorption and pathogen control may be related to poorer performance in vaccinated chickens. *SLC26A3* and *SLC26A4* (Solute Carrier Family 26, Member 3 and Member 4 respectively) regulate anion transport, where *SLC26A3* plays a role in infectious diarrhoea and high-chloride content diarrhoea (Scott *et al.*, 1999; Schweinfest *et al.*, 2006; Das, Jayaratne and Barrett, 2018). These differentially expressed genes could demonstrated the important of the nutrient and ion transport in the ileum of the chicken that may related with growth performance difference between vaccinated and non-vaccinated chickens.

In the caecum, Sodium transporter genes such as *SCNN1B* and *SCNN1G* were highly expressed in non-vaccinated chickens. *SCNN1B* and *SCNN1G* are epithelial sodium channels that are responsible for sodium absorption

(Hummler and Beermann, 2000; Brennan, Pique and Schrijver, 2016). Decreasing absorption of sodium has been found to be the result of water and electrolytes imbalance which related with diarrhoea in human (Sandle, 1998). In *Eimeria separata* infected rats, sodium absorption was decreased at the infected regions (caecum and proximal colon) but increased in compensation at the distal colon (Cirak *et al.*, 2004). In this study, the lower expression of *SCNN1B* and *SCNN1G* genes in the caecum of vaccinated chickens when compared to the non-vaccinated chickens might be the direct effects of the *Eimeria* infection or the consequence of electrolyte imbalance caused by differentially expressed genes related with electrolytes transport in the ileum such as *SLC4A9, SLC26A3* and *SLC26A4*. From these results, vaccination seems to affect the balancing of nutrient or electrolyte transportation in non-vaccinated and vaccinated chicken, respectively, with the ileum and caecum utilizing different genes and mechanisms.

Interestingly, when comparing between non-supplemented and enzyme supplemented chickens, differentially expressed genes were related to mucin in the ileum. The function of *NEU4* or Neuraminidase 4 in mammals relates to neuronal differentiation and apoptosis (Miyagi and Yamaguchi, 2012). *NEU4* is also a sialidase enzyme that cleave sialic acid from mucin (Yamaguchi *et al.*, 2005). Sialic acid is a sugar that can be used by commensal and opportunistic bacteria in the gut (Ng *et al.*, 2013; Juge, Tailford and Owen, 2016). However, in this study, in the ileum of 24-day-old chickens, there was no significant difference in abundance of bacteria between non-supplemented

and enzyme supplemented chickens; therefore, it is unknown which bacteria were related or gained the benefit with the up-regulation of these mucin related genes by the enzyme supplemented chickens.

Another gene related to mucin production, *GAL3ST2* or Galactose-3-Sulfotransferase 2, was also up-regulated in the ileum of enzyme supplemented chickens (Ren *et al.*, 2018). *GAL3ST2* is involved in the sulphation of mucin, which is known to protect intestinal epithelial cells against infection or inflammation (Hasnain *et al.*, 2017; Ren *et al.*, 2018). In previous studies, the effects of enzyme-supplemented on the sulphation of mucin showed contrasting results. Increased sulphomucin production in the goblet cells of the intestine of xylanase supplemented chicks has also been observed (Fernandez *et al.*, 2000), while another study reported reduced sulphomucin in the ileum of xylanase supplemented chickens (Liu, Guo and Guo, 2012). From these studies, the effect of enzyme supplementation on mucin sulphation was still unclear.

From the decreased expression of mucin-related genes, *NEU4* and *GAL3ST2* genes in non-enzymes supplemented chickens, several studies have shown a relationship between exogenous enzymes and mucin production. Mucin production was suggested to be an effect of some anti-nutritional factors such as fibre or phytate presented in the intestine but these studies showed contrasting results among them (Fernandez *et al.*, 2000; Cowieson, Acamovic and Bedford, 2004; Cowieson and Bedford, 2009; Ayoola *et al.*, 2015). In turkeys, supplementation of xylanase, amylase and protease enzymes

reduced the mucosal thickness in the ileum (Ayoola *et al.*, 2015). In contrast, the thickness of the mucosal layer of the chicken intestine and growth performance were increased in protease supplemented *Eimeria*-infected chickens (Peek *et al.*, 2009). The possible explanation suggested by the authors was that the protease might initiate a higher rate of mucous production and mucosal turnover resulting in mucous layer thickness (Peek *et al.*, 2009). Increased mucin production could be beneficial to the chicken as the mucin thickness help the host in the resistance to the mucin degradation to the pathogen glycosidase enzymes such as *Clostridium perfringrens* (Roberton and Wright, 1997; Liu, Guo and Guo, 2012). In contrast, increased in the production of the protein-rich mucus layer has been shown to increase usage of endogenous amino acid (Cowieson, Acamovic and Bedford, 2004; Cowieson and Bedford, 2009). From these findings, homeostasis between host mucin production and bacteria is still poorly understood and further studies are required to increase our understanding on it.

No significantly different enrichment of GO terms was observed between exogenous enzyme supplemented and non-supplemented groups from GSEA in both the ileum and caecum. These findings might be the consequence of the low number of significant differentially expressed genes between non-supplemented and enzyme supplemented chickens. On the other hand, 173 out of 1,720 GO terms showed significantly different enrichment between non-vaccinated and vaccinated chickens in the caecum. Similar to differential gene expression, several GO terms enriched in non-vaccinated chickens were

related to transmembrane transport while GO terms enriched in vaccinated chickens were related to the immune system. Using the layer hen as an animal model, similar patterns of enrichment of immune-related gene sets in the caecum were observed in a previous study which compared the enrichment of GO terms between *Eimeria maxima* infected and control chickens (Guo *et al.*, 2013). Surprisingly, no significantly different enrichment of GO terms was observed in the ileum even though the numbers of significant differentially expressed genes were higher than in the caecum. In the ileum, 18.75% of the highly expressed genes in vaccinated chickens were unannotated, whereas this percentage in vaccinated chickens in the caecum was only 10.87%. As the unannotated genes could not be recognised in GO terms, this could lead to non-significantly different GO term enrichments in the ileum.

In conclusion, chicken intestinal gene expression was influenced by anti-coccidial vaccination more than exogenous enzyme supplementation. In both the ileum and caecum, genes related to the immune system were highly expressed in the vaccinated chickens while genes related to nutrient absorption or membrane transport were highly expressed in non-vaccinated chickens. However, the differentially expressed genes and GO terms of non-vaccinated and vaccinated chickens were not similar between the ileum and caecum. These results demonstrate that the responses, functions and maybe the microbiota related to these responses were different between the ileum and caecum. The relationship between growth performance, the intestinal

microbiome and host gene expression response to anti-coccidial vaccination and exogenous enzymes will be analysed in the following chapter.

Chapter 6

Correlation between the gut microbiome and intestinal transcriptomic analysis

6.1 Introduction and Aims

From the results in **Chapter 4** and **Chapter 5**, the analyses showed that the anti-coccidial vaccination had more impact on the chicken gut microbiome and intestinal gene expression than the exogenous enzymes supplement. In this chapter, the final aims were to identify the relationship between the chicken gut microbiome and chicken intestinal gene expression. The microbe-host-gene interactions could be highly positively or negatively correlated across all chickens in response to the anti-coccidial vaccination and exogenous enzymes supplement. Although the correlation cannot determine a causative link, it can suggest host genes that may regulate or be regulated by specific microbes. Identification of the correlation between gut bacteria and host gene expression could generate new hypotheses relating to the attempt to manipulate the gut bacteria or host response in the future.

Recent studies reported the correlation between intestinal microbiota and host gene expression of the animals using different techniques (Malmuthuge, Liang and Guan, 2019; Shah *et al.*, 2019). Using Weighted gene co-expression network analysis (WGCNA), researchers demonstrated the correlation between shotgun metagenomics results and the gene expression of the calf rumen (Malmuthuge, Liang and Guan, 2019). WGCNA clustered the gene expression results into modules before calculating the correlation of the gene modules to phenotypes or bacterial population (Langfelder and Horvath, 2008). Pathways or functions of the genes belonging to gene modules of interest could be identified further using GSEA. Therefore, more meaningful

responses of the host to the bacteria could be identified. Another approach was the direct calculation of the correlation between expressed genes and OTU counts as reported in a recent chicken study (Shah *et al.*, 2019). The positive correlations between the transcription of heat shock protein, *HSP90AA1* at the chicken ileum and bacteria in genera *Clostridium*, *Weissella* and *Bacillus* were demonstrated (Shah *et al.*, 2019). Heat shock proteins were related to many cell functions, including inflammation and oxidation (Shah *et al.*, 2019). Furthermore, the study found a negative correlation between the transcription of an anti-viral gene, *G3BP1* and bacteria in genera *Gallibacterium*, *Veillonella* and *Faecalibacterium* (Shah *et al.*, 2019). *G3BP1* gene is related to the Wnt signal transduction pathway, which is involved in inflammation in humans (Shah *et al.*, 2019). These findings showed that the correlation between gut microbiota and host intestinal immune response could reveal information on the relationship between them.

In this chapter, correlation analyses between the gut microbial population from 16S rRNA metabarcoding and intestinal gene expression from RNA-seq analysis in the ileum and caecum were performed. As the experimental design was focused on the effects of the anti-coccidial vaccination and exogenous enzymes on gut health, the relationship between gene expression and gut microbiome was focused mainly on the immune-related and nutrient-absorption related genes.

6.2 Materials and Methods

6.2.1 Correlation analysis between intestinal gene co-expression network and microbiota in the ileum

Ileal microbiome results from the 16S rRNA metabarcoding reported in **Chapter 4**, Section 4.3.1.2 and the ileal gene expression results reported in **Chapter 5**, Section 5.3 were used for this correlation study. WGCNA (Langfelder and Horvath, 2008) was performed with *WGCNA* package (version 1.68) in R version 3.5.3 to display the link between intestinal gene expression and gut microbiome results (16S rRNA metabarcoding) in the ileum. Bacterial OTU results were pre-processed by removing the low abundance OTUs from the OTU table. Criteria for removing low abundance OTUs were: (1) OTUs with less than 0.05% of the sum of the reads from all of the samples (fewer than 132 reads from a total of 265,100 reads from 20 ileal samples); (2) OTUs where more than half of the samples have no reads. Outlier values of OTU abundance and TPM counts were defined by using the 1.5 interquartile range below the first quartile or above the third quartile value and these were removed from the analysis. The 16S rRNA metabarcoding results were selected from the 20 chickens matched with the gene expression results. Co-expression networks were constructed and clustered into modules using a hierarchical clustering approach. Module detection functions were performed with a minimum module size of 30 genes per module. Module eigengenes or first principal component of modules were calculated. The Spearman

correlation between eigengene of the modules and bacterial OTUs were calculated and plotted into heatmap in R version 3.5.3. Significant Spearman correlation was classified using Benjamini-Hochberg adjusted P-value of less than 0.05. Functions related to the genes belonging to the gene co-expression network modules were analysed using GSEA. Enrichment of gene ontology was performed using R package *anRichment* in order to identify the enriched GO term related to the genes in each module (https://horvath.genetics.ucla.edu/coexpressionnetwork/). Chicken GO term version April 2015 from GO2MSIG was used as reference (http://www.go2msig.org/go2msig/collections/Gallus_gallus_GSEA_GO_sets_all_symbols_April_2015.gmt.zip) (Powell, 2014). Significantly enriched GO terms were identified using the Benjamini-Hochberg adjusted P-value at less than 0.05.

6.2.2 Correlation analysis between intestinal gene co-expression network and microbiota in the caecum

WGCNA between caecal gene expression results and caecal microbiome results from 16S rRNA metabarcoding was performed as described in section 6.2.1. Data pre-processing steps were performed using the same criteria to remove low abundance OTUs, outliers OTUs and outliers TPM counts as described in section 6.2.1.

6.2.3 Correlation between gut microbiota and differentially expressed genes in the ileum and caecum

From the differential gene expression analysis in **Chapter 5**, Section 5.3, normalised TPM counts of significantly differentially expressed genes in the ileum and caecum were selected for correlation analysis. From **Table 5.1**, duplicated significantly differentially expressed genes at each intestinal region were removed from the TPM count tables. In total, 99 and 49 significantly differentially expressed genes the from ileum and caecum respectively were used for correlation analysis. Outlier TPM counts were removed before further analysis using the same approach as described in section 6.2.1. OTU tables of ileum and caecum were pre-processed in the same way as the WGCNA analysis. The 16S rRNA metabarcoding results were selected from the 20 chickens matched with the gene expression results. Correlation between differentially expressed genes and bacterial OTUs were calculated using Spearman correlation analysis in R version 3.5.3. The significant Spearman correlation was identified using Benjamini-Hochberg adjusted P-value at less than 0.05. Significant correlations between OTUs and significantly differentially expressed gene were visualised as scatterplot using *ggpubr* package in R version 3.5.3.

6.3 Results

6.3.1 Correlation results between ileal 16S gut microbiome and ileal co-expressed gene network

From the gene network and module construction, 30 modules were generated from the WGCNA analysis at the ileum. After the removal of outliers and low abundance OTUss, there were 98 OTUs from the total 1,657 OTUs for WGCNA analysis. A heatmap of the Spearman correlation coefficient between 30 modules and 98 bacterial OTUs at the ileum is shown in **Figure 6.1**. No gene co-expressed modules showed significant Spearman correlation with any bacterial OTU in the ileum.

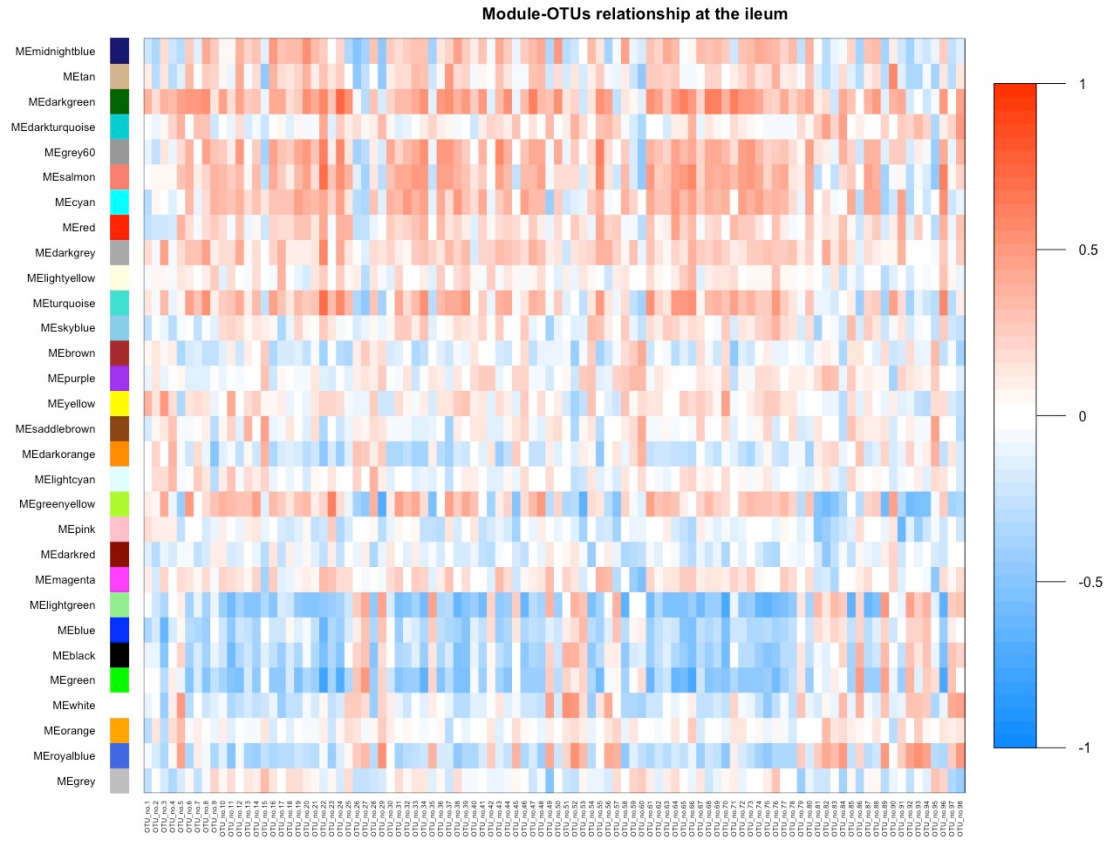


Figure 6.1 Heatmap of Spearman correlation between ileal gene expression modules and ileal 16S gut microbiome results at the OTU level using WGCNA analysis. Spearman correlation coefficient are shown ranging from -1 (blue) to 1 (red).

6.3.2 Correlation results between caecal 16S gut microbiome and caecal co-expressed gene network

From the gene network and module construction, 20 modules were generated from the WGCNA analysis on the chicken caecum. After the pre-processing step, there were 146 OTUs from the total 1,657 OTUs for WGCNA analysis. A heatmap of the Spearman correlation coefficient between 20 modules and 146 bacterial OTUs at the caecum is shown in **Figure 6.2**. Two modules that showed significant Spearman correlation with bacterial OTUs at the caecum were MEred and MEpurple. MEpurple significantly correlated with 2 OTUs classified as *Oscillospira spp* (Spearman correlation coefficient = -0.78, adjusted P-value = 0.048) and unknown genus in Ruminococcaceae family (Spearman correlation coefficient = 0.79, adjusted P-value = 0.048). MEred significantly correlated with one unknown genus in order Clostridiales (Spearman correlation coefficient = 0.82, adjusted P-value = 0.024).

Top 10 GO terms enriched by MEpurple and MEred are shown in **Table 6.1** and **Table 6.2**. Several GO terms from these modules were related with immune response of the host, however these were not significant.

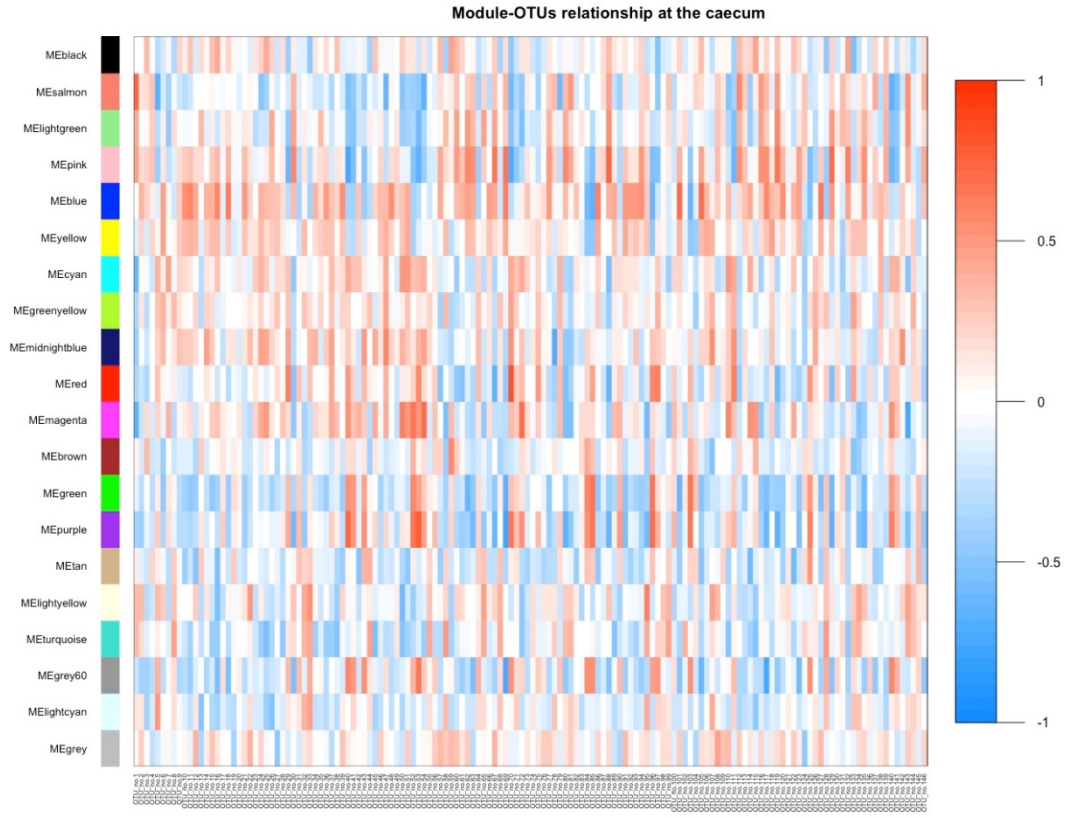


Figure 6.2: Heatmap of Spearman correlation between caecal gene expression modules and caecal 16S gut microbiome results at the OTU level using WGCNA analysis. Spearman correlation coefficient are shown ranging from -1 (blue) to 1 (red).

Table 6.1: Top 10 enriched GO terms of MEpurple module from WGCNA analysis between gene expression and bacterial OTUs in the caecum of 24-day-old chickens; (BP = Biological process and MF = Molecular function)

GO term ID	GO term	Ontology	Adjusted P-value
GO:0046631	alpha-beta T cell activation	BP	0.591
GO:0043047	single-stranded telomeric DNA binding	MF	0.824
GO:0098505	G-rich strand telomeric DNA binding	MF	0.824
GO:0098847	sequence-specific single stranded DNA binding	MF	0.824
GO:0046634	regulation of alpha-beta T cell activation	BP	1.000
GO:0002684	positive regulation of immune system process	BP	1.000
GO:0050663	cytokine secretion	BP	1.000
GO:0002532	production of molecular mediator involved in inflammatory response	BP	1.000
GO:0006760	folic acid-containing compound metabolic process	BP	1.000
GO:0016742	hydroxymethyl-, formyl- and related transferase activity	MF	1.000

Table 6.2 : Top 10 enriched GO terms of MEred module from WGCNA analysis between gene expression and bacterial OTUs in the caecum of 24-day-old chickens; (BP = Biological process and CC = Cellular component)

GO term ID	GO term	Ontology	Adjusted P-value
GO:0002376	immune system process	BP	0.128
GO:0002764	immune response-regulating signaling pathway	BP	0.156
GO:0002429	immune response-activating cell surface receptor signaling pathway	BP	0.225
GO:0002253	activation of immune response	BP	0.234
GO:0006955	immune response	BP	0.242
GO:0002768	immune response-regulating cell surface receptor signaling pathway	BP	0.303
GO:0002757	immune response-activating signal transduction	BP	0.429
GO:0050778	positive regulation of immune response	BP	0.481
GO:0050776	regulation of immune response	BP	0.503
GO:0005886	plasma membrane	CC	0.533

6.3.3 Correlation results between gut microbiota and differentially expressed genes in the ileum and caecum

Spearman correlation between 99 significantly differentially expressed gene and 98 OTUs at the ileum, and 49 significantly differentially expressed gene and 146 OTUs at the caecum were calculated. From the correlation analysis, 84 significant Spearman correlations (adjusted P-value < 0.05) were identified in the ileum (**Table S28**). From the total of 84 significant correlations, 34 unique ENSEMBL IDs from these correlations were identifiable genes whereas 8 out of 34 ENSEMBL IDs were unannotated genes. In addition, there were 34 unique bacterial OTUs in these correlations as shown in **Table S28**.

Several genes described in **Chapter 5** showed significant negative correlations with the abundance of multiple ileal OTUs. The gene that had significant correlations with the most ileal OTUs was *CCL1* gene (**Table S28**). *CCL1* gene negatively correlated with 11 bacterial OTUs from the ileum which were classified as *Ruminococcus spp, Coprococcus spp, Oscillospira spp, Sutterella spp*, *Bacteroides spp*, an unknown family in order RF32, an unknown family in order Clostridiales, an unknown genus in family Ruminococcaceae, and an unknown genus in family Erysipelothichaceae. Another immune related gene, *CCL4* showed a significant negative correlation with one bacterial OTU classified as *Coprococcus spp*, which was also negatively correlated with the *CCL1* gene (**Figure 6.3A-B**).

Interestingly, one bacterial OTU classified as *Coprococcus spp* had significant correlation with 12 genes that were significantly differentially expressed between vaccinated and non-vaccinated chickens in the ileum. This finding suggests that this bacterial OTU may play some roles in the chicken immune response to the anti-coccidial vaccine. However, the mean relative abundance of this OTU classified as *Coprococcus spp* in the ileum of all 24-day-old chickens was just 0.27%.

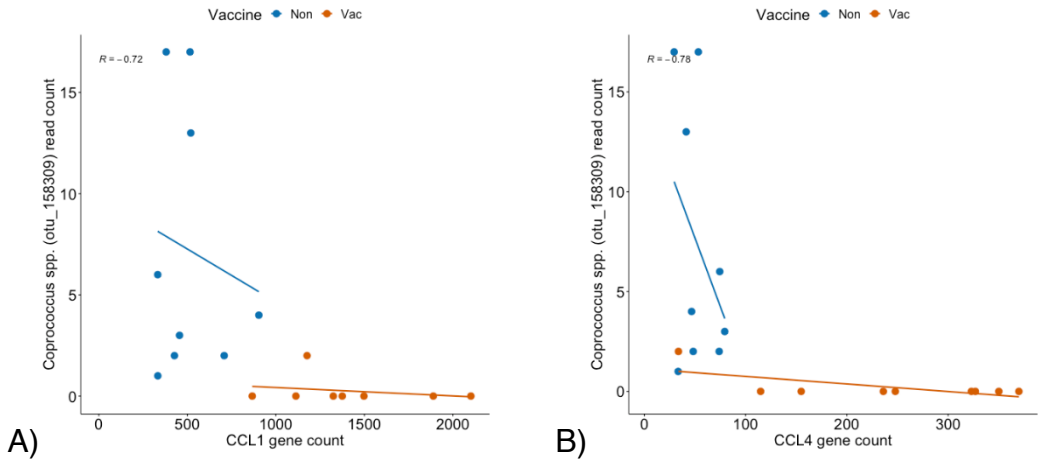


Figure 6.3: Significant Spearman correlation plots between genes and one bacterial OTU classified as *Coprococcus spp* in the ileum (adjusted P-value <0.05): A) *CCL1* gene and B) *CCL4* gene. (Non = non-vaccinated chickens, Vac = vaccinated chickens, R = Spearman correlation coefficient)

At the caecum, 191 significant Spearman correlations were identified from the correlation analysis (**Table S29**). From a total of 191 significant correlations, 44 unique ENSEMBL IDs present in these correlations were identifiable whereas 10 out of 44 ENSEMBL IDs were unannotated genes. A total of 43 unique bacterial OTUs found in these correlations as shown in **Table S29**.

Similarly to the ileum, a gene that had significant correlations with the most caecal OTUs was the *CCL1* gene. *CCL1* positively correlated with 6 OTUs and negatively correlated with 5 OTUs. Interestingly, OTU_129401 and OTU_369097, both classified as *Oscillospira spp* had significant correlations with several immune-related and sodium absorption related genes in the opposite direction. OTU_129401 had negative correlations with *CCL1, CCL4, CIITA, GZMA* and *GZMK* genes while OTU_369097 had positive correlations with these genes (**Figure 6.4**, **Figure 6.5** and **Figure 6.6A-B**). Moreover, OTU_369097 also showed significant positive correlation with *IFNG* (**Figure 6.6C**) but OTU_129401 did not showed any significant correlation with this gene. Conversely, *SCNN1B* and *SCNN1G* had positive and negative correlations with OTU_129401 and OTU_369097 respectively (**Figure 6.7**). The *SCD* gene showed positive correlations with 4 different bacterial OTUs, 3 out of these 4 OTUs were classified as *Oscillospira spp*.

The bacterial OTU that had the most correlations with the caecal genes was OTU_369097 classified as *Oscillospira spp,* which was correlated with 26 genes. Moreover, out of 191 significant correlation between bacterial OTUs and gene expression in the caecum, OTUs classified as *Oscillospira spp* was

observed in 68 correlations. This result suggests that *Oscillospira spp,* which had a mean relative abundance of 4.09%, may directly or indirectly play a major role in influencing the gene expression in the caecum of the 24-day-old chicken.

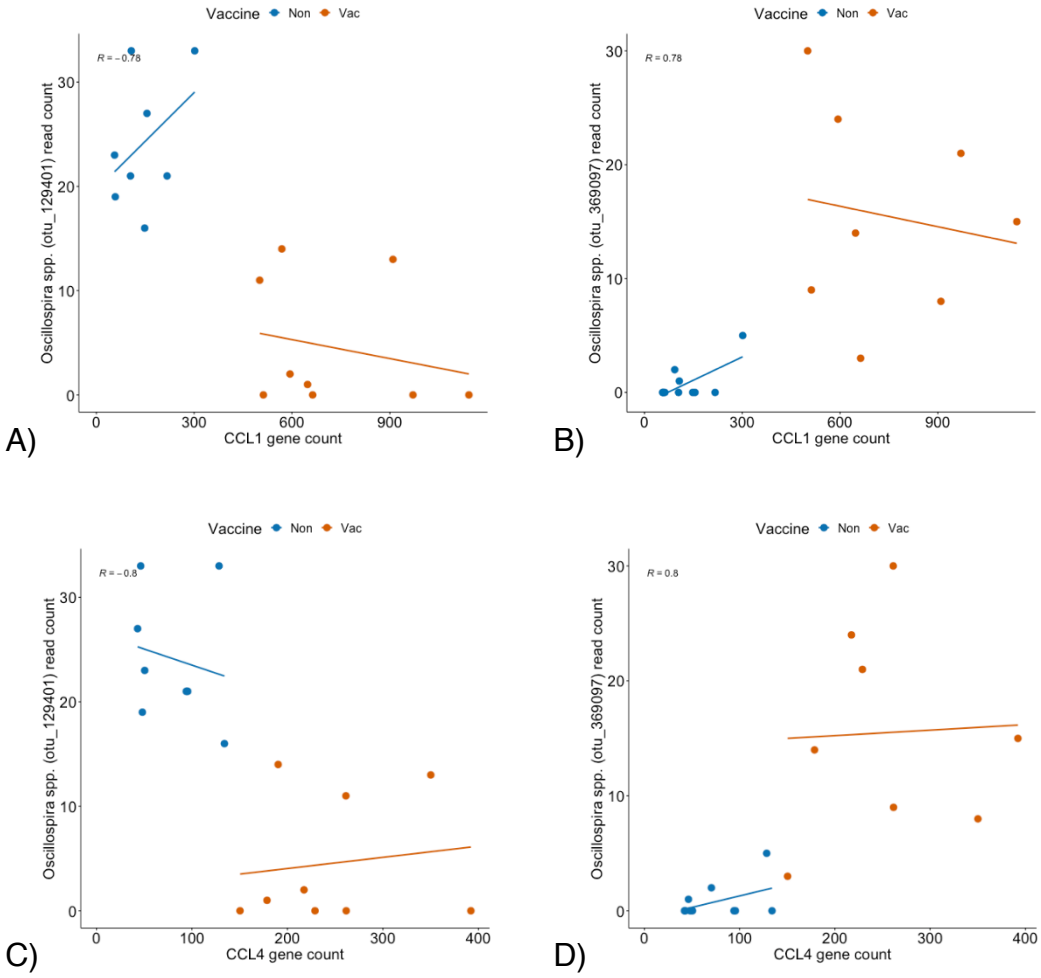


Figure 6.4: Significant Spearman correlation plots between genes and one bacterial OTU classified as *Oscillospira spp* in the caecum (adjusted P-value <0.05): A) *CCL1* gene and OTU_129401; B) *CCL1* gene *and* OTU_369097 gene; C) *CCL4* gene and OTU_129401; D) *CCL4* gene *and* OTU_369097. (Non = non-vaccinated chickens, Vac = vaccinated chickens, R = Spearman correlation coefficient)

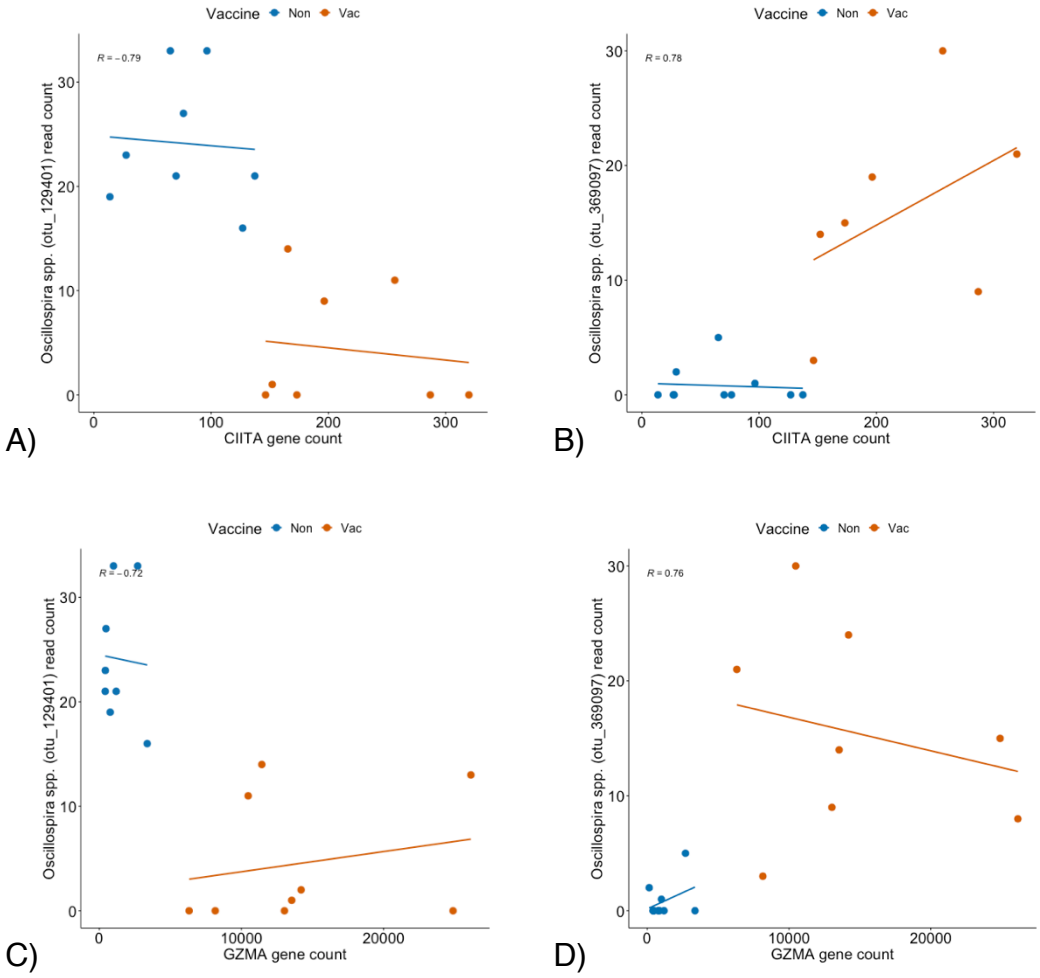


Figure 6.5: Significant Spearman correlation plots between genes and one bacterial OTU classified as *Oscillospira spp* in the caecum (adjusted P-value <0.05): A) *CIITA* gene and OTU_129401; B) *CIITA* gene *and* OTU_369097 gene; C) *GZMA* gene and OTU_129401; D) *GZMA* gene *and* OTU_369097. (Non = non-vaccinated chickens, Vac = vaccinated chickens, R = Spearman correlation coefficient)

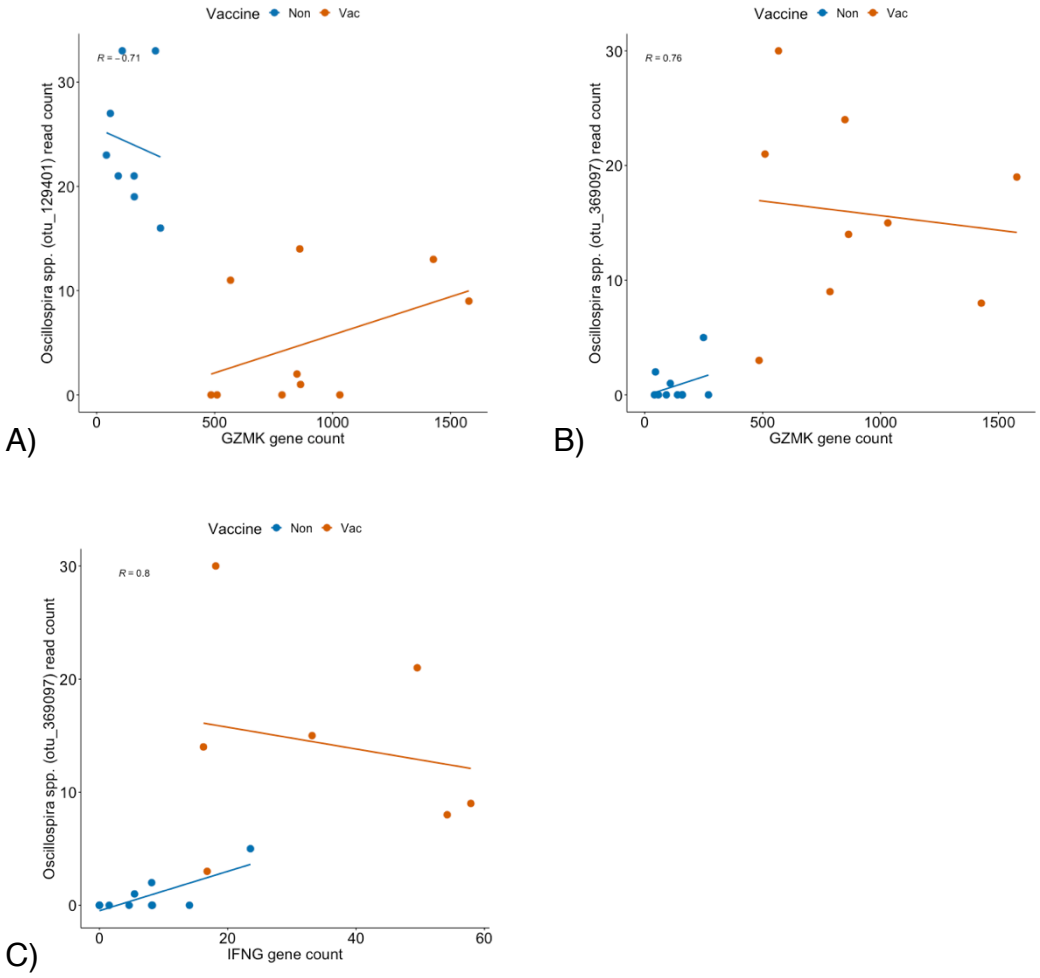


Figure 6.6: Significant Spearman correlation plots between genes and one bacterial OTU classified as *Oscillospira spp* in the caecum (adjusted P-value <0.05): A) *GZMK* gene and OTU_129401; B) *GZMK* gene *and* OTU_369097 gene: and C) *IFNG* gene *and* OTU_369097. (Non = non-vaccinated chickens, Vac = vaccinated chickens, R = Spearman correlation coefficient)

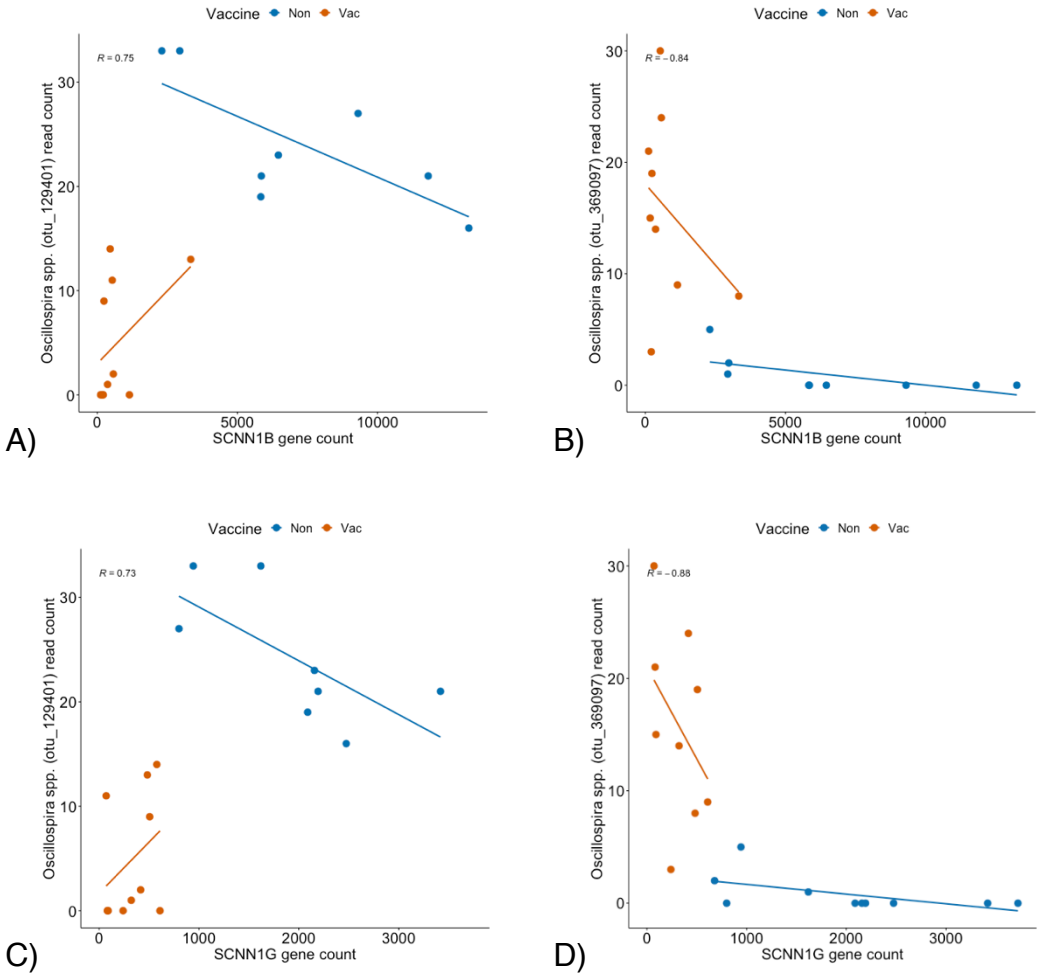


Figure 6.7: Significant Spearman correlation plots between genes and one bacterial OTU classified as *Oscillospira spp* in the caecum (adjusted P-value <0.05): A) *SCNN1B* gene and OTU_129401; B) *SCNN1B* gene *and* OTU_369097 gene; C) *SCNN1G* gene and OTU_129401; D) *SCNN1G* gene *and* OTU_369097. (Non = non-vaccinated chickens, Vac = vaccinated chickens, R = Spearman correlation coefficient)

6.4 Discussion

In the ileum, no gene co-expressed module showed significant correlation with the ileal microbiota from WGCNA analysis. This non-significant finding might be due to the homogeneity of the RNA-seq results, which showed the low number of significant differentially expressed genes when compared to other gene expression studies as mentioned in section 5.4. Moreover, as the gene co-expression analysis was the analysis of multiple samples, the low sample size could lead to non-biologically meaningful results as suggested in the WGCNA manual. In this study, 20 samples per intestinal region were used in the WGCNA which is the smallest sample size recommended by the WGCNA developers, therefore, increasing the sample size could lead to more meaningful and less variation results of the WGCNA and GO term enrichment analysis. As no significant correlation was observed; unfortunately, it could not be concluded whether the abundance of OTUs had significant relationships with any function of the ileum.

From the WGCNA analysis, 2 co-expressed modules were significantly correlated with several bacterial OTUs in the caecum. Among the 3 bacterial OTUs that significantly correlated with MEpurple and MEred modules (*Oscillospira spp*, unknown genus in Ruminococcaceae family and unknown genus in order Clostridiales), a bacterial OTU classified as *Oscillospira spp* demonstrated to be related to intestinal inflammation. *Oscillospira spp* negatively correlated with MEpurple module which several top GO terms related with immune system. The abundance of *Oscillospira spp* is significantly

reduced in several human diseases such as Crohn's disease and non-alcoholic steatohepatitis (Walters, Xu and Knight, 2014; Konikoff and Gophna, 2016). *Oscillospira* has been classified a butyrate-producing bacteria which play a key role in maintaining gut health (Gophna, Konikoff and Nielsen, 2017). Induction of differentiation of regulatory T-cells and down-regulation of the expression of pro-inflammatory cytokine genes have been reported to be caused by butyrate (Furusawa *et al.*, 2013; Kim, 2014; Gophna, Konikoff and Nielsen, 2017). In this study, the negative correlation found could show that the role of the genus *Oscillospira* on the immune system of the chicken could occur through the role of butyrate.

From the GSEA analysis, several GO terms related to immune response were correlated with the MEpurple and MEred module of caecal gene expression. Interestingly, some members of these GO terms were immune-related genes such as *IL18*, *IRF1* and *TLR2A* in MEpurple and *NFKB2* and *CD28* in MEred. *IL18*, is a cytokine produced by macrophages and dendritic cells which induces interferon-gamma secretion and plays important role in T helper cells dominated immune response (Kohno *et al.*, 1997; Marshall *et al.*, 2006). In previous studies, increased expression of *IL18* gene in chickens were observed after inoculation of pathogens such *Eimeria* protozoa and *Salmonella* Typhimurium (Hong *et al.*, 2006; Dar *et al.*, 2019). *IL18,* has also been used as a vaccine adjuvant to improve the immune response against infectious diseases in chicken (Lim *et al.*, 2012; Wang *et al.*, 2015). *IRF1* (interferon regulatory factor 1) has an important role in MHC class I, interferon

expression, development of $CD8^+$ T-cells, induction T helper differentiation and natural killer development (Nguyen, Hiscott and Pitha, 1997; Schwartz, Shajahan and Clarke, 2011). *TLR2A* (toll-like receptor 2A) is a member of toll-like receptor (TLR) which is transmembrane pathogen recognition receptor that detects the pathogen and then activates immune response which is mediated by nuclear factor kappa B (NF-kB) to initiate expression of immune-related genes and cytokines (Downing *et al.*, 2010; Kogut *et al.*, 2013). *NFKB2* or nuclear factor kappa B subunit 2 which related with the NF-kB complex which related with TLR (Kogut *et al.*, 2012, 2013), was a member of several GO terms in MEred module. *CD28* is a protein receptor on the surface of T-cells which stimulated during contact with antigen presenting cells (Linsley and Ledbetter, 1993). Stimulation of CD28 receptor affected the T-cell activation and amplification of signals in response to antigen recognition (Beyersdorf, Kerkau and Hünig, 2015). Interestingly, in the caecum, these immune-related genes were not significantly differentially expressed when compared between vaccinated and non-vaccinated chickens. Therefore, significant correlations of MEpurple and MEred modules with several bacterial OTUs may suggest that these bacteria were directly or indirectly involved with the inflammation or infection process of the chicken through the co-expression of these genes. However, more investigations on the correlation between these individual genes and the bacteria are still required in order to identify their in-depth relationship and their roles on chicken gut health.

Significant correlations between bacterial OTUs and gene expression were identified with Spearman correlation analysis using the differentially expressed genes list from **Chapter 5**. Several interesting findings on the correlation of bacterial OTUs and differentially expressed genes were observed in the ileum of the chicken. Although 11 OTUs negatively correlated with the *CCL1* gene, only an OTU classified as *Coprococcus spp* had negative correlations with both *CCL1* and *CCL4* in the ileum. The decreased abundance of bacteria in the genus *Coprococcus* has been previously reported as having an association with inflammatory bowel disease inhumans (Gevers *et al.*, 2014; Shaw *et al.*, 2016). Gevers et al., 2014 also suggested that the agglutination antibodies for *Coprococcus* could be used as a biomarker for inflammatory bowel diseases screening (Gevers *et al.*, 2014). *Coprococcus* is also known to be a butyrate-producing bacteria in the human colon and was considered normal flora in the chicken intestine (Louis and Flint, 2009; Oakley *et al.*, 2014). The negative correlation between butyrate-producing bacteria and immune-related genes could be an indirect relationship between them that were the outcome of inflammation of the intestines or through the role of butyrate.

Among the 11 OTUs with negative correlation with the *CCL1* gene in the ileum, 3 bacterial OTUs were also showed to have significant changes in abundance between non-vaccinated and vaccinated chickens in the 16S rRNA metabarcoding analysis discussed in **Chapter 4**. The abundance of *Sutterella spp*, *Bacteroides spp* and unknown family in order RF32 were significantly increased in non-vaccinated chickens when compared to vaccinated chickens.

As most of these bacteria were commonly found and considered core gut microbiota of in the chicken (Oakley *et al.*, 2014; J. Wang *et al.*, 2017), decreased abundance in the vaccinated chicken may suggest that the homeostasis of gut health was disrupted by the vaccination or damaged gut epithelium. As this study cannot show that the effects of immune response on the other normal gut flora are caused by the expression of the *CCL1* gene, further study on the relationship between *CCL1* and these bacteria is still required.

Bacteria that correlate with the expression of several genes in the caecum of the chickens were *Oscillospira spp*. The most interesting OTUs were OTU_129401 and OTU_369097, which were both classified as *Oscillospira spp*. OTU_129401 negatively correlated with immune-related genes (*CCL1, CCL4*, *CIITA, GZMA and GZMK*) but positively correlated with sodium absorption-related genes (*SCNN1B* and *SCNN1G*). In contrast, OTU_369097 were positively correlated with immune related genes (*CCL1, CCL4*, *CIITA, GZMA*, *GZMK* and *IFNG*) and negatively correlated with sodium absorption related genes (*SCNN1B* and *SCNN1G*). This finding might suggest that the relationship between bacteria and genes in the caecum of the chicken may occur at the species or strain level which could not be obtained from this study. Further analysis at the lower level of bacterial taxonomy may explain more details the relationship between *Oscillospira spp* and the immune system of the chicken.

In this study, *SCNN1B* and *SCNN1G* which relate to the intestinal absorption of sodium were up-regulated in non-vaccinated chickens. As *SCNN1B* and *SCNN1G* are related to sodium absorption, decreasing absorption of sodium has been found to result in impaired water balance that relates to electrolyte imbalance and diarrhoea in human ulcerative colitis and Crohn's disease (Sandle, 1998). Miranda et al. (2018) found that mice fed with high-salt diets had a positive correlation with bacteria in the genus *Oscillospira* and showed symptoms of colitis with the increased expression of *CCL4* gene. In this study, several OTUs classified as *Oscillospira* had either positive or negative correlations with the *SCNN1B, SCNN1G* and *CCL4* genes. Therefore, the direction of the correlation between the bacteria genus *Oscillospira* and these genes could not be concluded at the genus level.

In conclusion, the correlations found between intestinal bacteria and gene expression shown in this study provided more information and a possible explanation of how the bacteria and host communicate. Although the relationship between them does not prove a cause-effect relationship, hypotheses on how to manipulate the gut microbiota, host gene expression and host phenotype could be generated and tested based on these results. Further studies still need to be conducted to test these correlations. Large numbers of samples are required to increase confidence in the differences determined here. Gene expression analysis of the germ-free chickens or cell culture after the inoculation of these correlated bacteria could be performed to test some of the results. Once the correlation is confirmed, improvement of gut

health through the manipulation of host gene expression or bacteria could help the poultry industry in improving animal welfare and improve feed efficiency. Direct environmental impacts from poor gut health such as chicken mortality and nutrient waste, including indirect environmental impact such as less use of anti-microbial could benefit from improved gut health chicken. With improved gut health, the demand for the poultry meat by the rising world population could be met with more sustainable production.

Chapter 7

General discussion

7.1 Introduction

As introduced in Chapter 1, the definition of gut health is the ability of the gut to perform normal physiological functions such as digestion, absorption and maintenance of intestinal barrier function (Kogut *et al.*, 2017). Major interdependent components that could influence gut health are the immune system, gut microbiota and nutrition (Kogut and Klasing, 2009; Kogut *et al.*, 2017). Therefore, a wide range of analyses is required to comprehensively study gut health in chickens. In this thesis, anti-coccidial vaccination and enzyme supplementation were used as factors to alter the immune system and nutrition respectively. The effects of these factors on the gut microbiome, host gene expression and their correlation were analysed. However, other parameters could also be studied to provide further in-depth knowledge of chicken gut health. In **Figure 7.1**, analyses performed in this thesis are shown in orange boxes and additional analysis which could be performed in further investigations are shown in blue boxes.

As the 16S metabarcoding analysis only shows changes in bacterial composition, additional analyses are required in order to answer gut-health-related questions such as “what is the impact of those changes?”, “what are the bacteria doing?” or “how does the host respond to the compositional changes?” (Jovel *et al.*, 2016). These questions lead to the correlation analysis which aims to understand how the host responds or relates to the change of bacteria rather than just describing the changes in microbial composition. With the use of multiple bioinformatics analyses within a single experiment, it is

considered that the correlation results could be able to answer these questions or generate new hypotheses to study gut health. Significant findings from multi-omics analyses in this thesis, along with the weaknesses and strengths of the performed analyses will be discussed in this chapter.

7.2 Exogenous enzyme supplementation effects on chicken gut health and growth performance

Bedford and Cowieson (2012), reviewed the direct effects of enzyme supplementation on the animal gut microbiome, which were the removal of rapidly fermentable nutrients from the distal small intestine and the provision of fermentable oligosaccharides The consequences of supplemented enzyme activity in the animal intestine are changes in bacterial fermentation products such as SCFAs (Choct, Hughes and Bedford, 1999) and lactic acid bacteria numbers (Hübener, Vahjen and Simon, 2002; Jia *et al.*, 2009). In addition, indirect effects from enzyme supplementation could also affect host immunity or intestinal integrity as a result of the altered structure of the microbiome (Fernandez *et al.*, 2000; Bedford and Cowieson, 2012; Kiarie, Romero and Nyachoti, 2013). Therefore, it was anticipated that the supplementation of exogenous enzymes in this study should affect the chicken gut microbiome and intestinal gene expression.

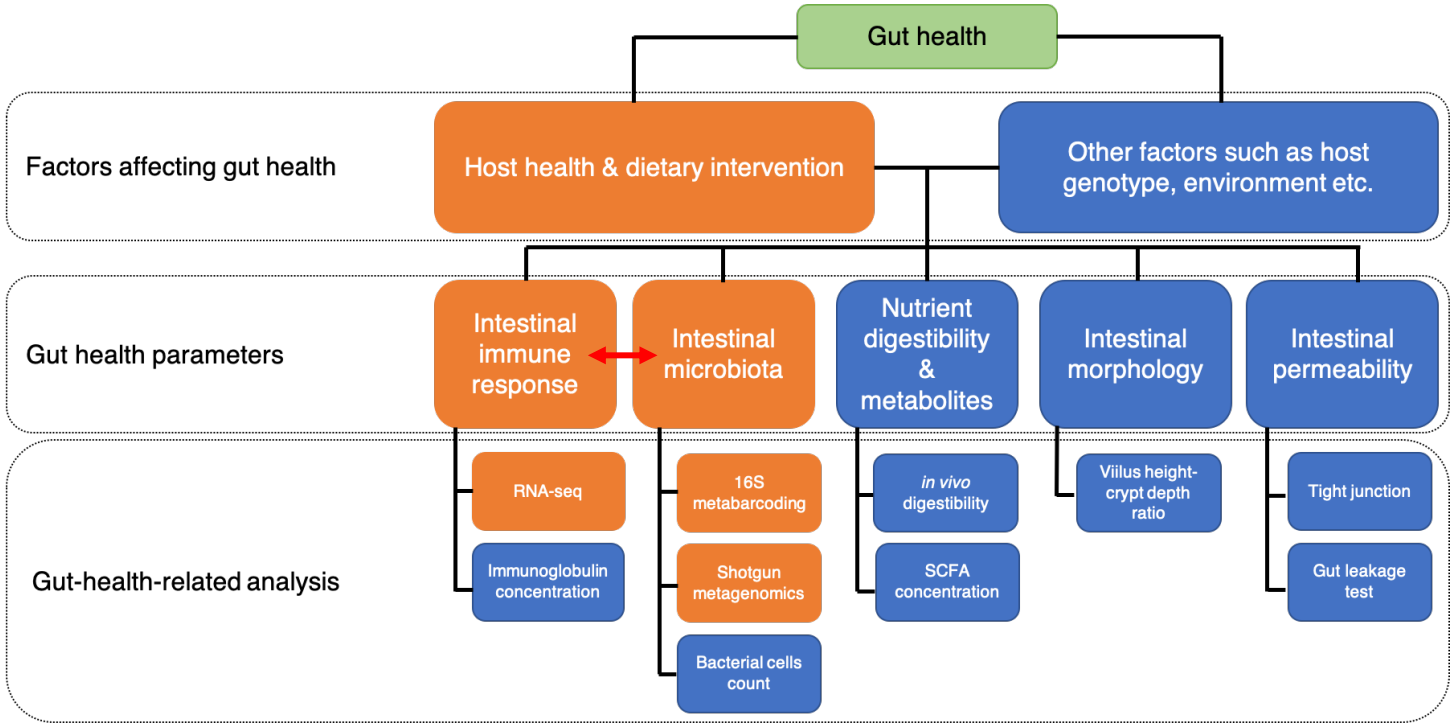


Figure 7.1: Schematic diagram of a gut health study in chickens. Analyses and paramaeters studied in this thesis are shown in orange boxes with the correlation study between intestinal immune response and microbiota (red double arrow). Example additional analyses and parameters which could be studied to improve gut health related studies are shown in blue boxes.

However, in this study, exogenous enzymes had only small effects on the gut microbiome and intestinal gene expression at both the ileum and caecum. The minimal effects on the gut microbiome found in this study are similar to a previous study by Engberg et al. (2004), who counted multiple types of bacteria (coliform bacteria, anaerobic bacteria, lactose-negative enterobacteria, *Clostridium perfringens*, lactic acid bacteria, *Lactobacillus salivaris*, other lactobacilli and Enterococci) at several GI regions (gizzard, duodenum/jejunum, ileum, caecum and rectum) of chickens and found significant effects of xylanase supplementation only on lactic acid bacteria and anaerobic bacteria plate counts at the ileum and duodenum/jejunum respectively. Although non-culture dependent methods for gut microbiota analysis (16S metabarcoding and shotgun metagenomics) were used in this study, similarly small effects of exogenous enzymes on the gut microbiota were still observed. In contrast, other studies showed large effects from exogenous enzyme supplementation on the gut microbiome when using non-culture dependent methods (Munyaka *et al.*, 2015; Ptak *et al.*, 2015). The different microbiome outcomes between studies could be caused by diet formulation, different ingredients used, chicken breed, environment, bioinformatics technique and many more factors (Kers *et al.*, 2018; Pollock *et al.*, 2018).

Indirect effects of enzyme supplementation on chicken gut health could be examined by studying intestinal barrier function, intestinal morphology, nutrient transporter genes and fermentation products, as performed in other studies

(Choct, Hughes and Bedford, 1999; Wu *et al.*, 2004; Kuzmuk *et al.*, 2005; Józefiak *et al.*, 2006; Kuttappan *et al.*, 2015). Unfortunately, these analyses were not performed in this study as previous studies could identify changes the expression of intestinal barrier genes (e.g. claudin-1 and occludin) and nutrient transport genes (e.g. peptide transporter-2) as the effects of enzymes supplementation (Liu, Guo and Guo, 2012; Cowieson *et al.*, 2019). Therefore, the expression of these genes were expected to be seen from the RNA-seq analysis. However, from the RNA-seq analysis, they were not differentially expressed.Future studies should include more of these additional analyses to provide more evidence of the effects of enzyme supplementation on gut health.

The positive effect of enzyme supplementation on growth performance without changes in the gut microbiome and gene expression could be due to the availability of nutrients in the gut. Several studies showed the benefit of enzyme supplementation on chicken growth but no significant difference in ileal digestible energy or metabolisable energy was observed (Wu *et al.*, 2004; Olukosi, Cowieson and Adeola, 2007). These energy values can be measured from the complete combustion of the sample in a bomb calorimeter. As the energy measured in the samples originates from carbohydrates, proteins and fats, non-significant difference in energy from these nutrients found in the digesta could lead to subtle changes in the gut microbiota and host gene expression. It is unfortunate that *in vivo* digestibility analysis was not able to perform in this study as the chicken digesta could not be shipped to the UK

due to biosecurity reasons and no laboratory could analyse the non-digestible marker in the digesta in Thailand.

7.3 Effects of anti-coccidial vaccination on chicken gut health and growth performance

Anti-coccidial vaccination significantly affected chicken gut health, as shown in the microbiome analysis, intestinal gene expression analysis and WGCNA analysis. In **Chapter 4**, vaccination significantly affected the gut microbiota at the ileum and caecum of the chicken. In the ileum, the abundance of 11 bacterial genera showed a significant difference between vaccinated and non-vaccinated chickens, while only 2 significantly differential abundant bacterial genera were observed in the caecum. This was an unexpected result as the bacterial diversity of the caecum was higher than the ileum. A possible explanation for the high number of differentially abundant genera between vaccinated and non-vaccinated chickens at the ileum is the nutrient digestion and absorption function at this site. Changes in the digestion at proximal intestinal regions (duodenum) and absorption in the ileum were expected where intestinal epithelium damage occurred, as shown by the coccidiosis score. This poor digestion and absorption might result in non-digested, non-absorbed particles leading to a difference in the bacterial composition between vaccinated and non-vaccinated chickens at the ileum, and these particles could also pass through the caecal opening (Rinttilä and Apajalahti, 2013). Unfortunately, details of the gross appearance or nutrient composition of the ileal digesta were not collected or analysed in this study. A further study on the

ileal microbiome should collect more data on the appearance of the ileal digesta, location within the ileum including the nutrient composition of the digesta. This information might show a relationship with the high number of significantly differently abundant members of the ileal microbiome in the16S metabarcoding analysis.

A protective immune response was observed in the 24-day-old vaccinated chickens as shown in the upregulation of Th1-related cytokines such as *CCL1*, *CCL4* and especially IFN-gamma (Yun, Lillehoj and Choi, 2000). Moreover, vaccinated chickens showed evidence of protozoa clearance from the duodenum. Although the coccidiosis lesion score was still observed in the ileum and caecum, vaccinated chicken showed compensatory growth when compared to the non-vaccinated chickens during the finisher phase. This evidence indicates that the anti-coccidial vaccination protected the vaccinated chickens from *Eimeria* re-infection. Therefore, *CCL1*, *CCL4* and *IFNG* genes could be used as a target for chicken genetic or expression manipulation for improved protection against *Eimeria* protozoa.

As shown in **Chapter 3**, coccidiosis lesion scores found in 10-day-old vaccinated chickens led to a significant decrease in growth performance at the end of the starter and grower phase. Previous studies have shown similar negative impacts of vaccination on chicken growth performance (J. T. Lee *et al.*, 2011; Walk *et al.*, 2011); however, the 12% decrease in average body weight of 24-day-old vaccinated chickens is considered to be high when compared to previous studies (8.3% at 21 days old and 2.2% at 18 days old)

(J. T. Lee *et al.*, 2011; Walk *et al.*, 2011). This highly negative effect on body weight might be the results of either the updated anti-coccidial vaccine producing a more rapid immune response, rearing environment, diet, chicken breed, chicken age at study, or the accelerated growth by the updated chicken breed. The chicken breed used in this experiment was Ross, and Fortegra® was used for vaccination. Lee et al., (2011) and Walk et al., (2011) used Cobb and Coccivac-B® as the chicken breed and vaccination type. In experimental settings, researchers have found that Cobb was more susceptible to *Eimeria* protozoa than Ross, while in field research no significant difference in coccidiosis prevalence has been observed (Jang *et al.*, 2013; Gharekhani, Sadeghi-Dehkordi and Bahrami, 2014). Advanced growth rates in updated chicken breeds may cause great decreases in the bodyweight of vaccinated chicken. The average chicken bodyweight in previous studies were 705 grammes at 21-day-old (J. T. Lee *et al.*, 2011) and 628.7 grammes at 18-day-old (Walk *et al.*, 2011) while in this study, average bodyweight of the chickens was 1.38 kilogramme at 24-day-old. The results in this study showed that the anti-coccidial vaccination can be used as a model for gut-damage studies but the degree of the negative impact depends on several factors such as host, vaccine, diet and rearing environment.

7.4 Correlation between chicken gut microbiota and intestinal gene expression

From **Chapter 6**, surprisingly, from the correlation analysis using differentially expressed genes, the bacteria that correlated with the most differentially

expressed genes were *Coprococcus spp* and *Oscillospira spp*, in the ileum and caecum of the chickens, respectively. These bacteria were not the major population of the intestinal bacteria as the relative abundance of *Coprococcus spp* in the ileum was 0.27% while *Oscillospira spp* was 4.09% in the caecum of the chickens. These findings might show that the cross-link between gut bacteria and host may occur through low abundance populations, similar to previous findings on the role of segmented filamentous bacteria, *Akkermansia municiphila* and *Faecalibacterium prausnitzii* to the host immune response (Sokol *et al.*, 2008; Everard *et al.*, 2013; Ericsson *et al.*, 2014).

From the correlation findings, correlated genes and microbes could be used as biomarkers for improved immune responses and to further investigate host-microbe interactions. However, limitations of correlation studies are that they do not imply causation and could occur co-incidentally (Altman and Krzywinski, 2015; Rohrer, 2018). Correlated bacteria and genes need to be investigated further to identify their relationships and roles in the chicken immune system. Several advanced technologies such as gene knockout in chickens, germ-free chickens, cell/tissue culture and CRISPR/Cas9 systems could be used to investigate the role of correlated genes and bacteria (Zaffuto, Estevez and Afonso, 2008; B. W. Kong *et al.*, 2011; Park *et al.*, 2014; Sid and Schusser, 2018; Guitton *et al.*, 2020). More in-depth knowledge of their mechanisms may help improve gut health and result in overall improvements in health and growth of chickens in the future (Carrasco, Casanova and Miyakawa, 2019; Kogut, 2019). For example, a recent study showed that manipulation of the gut

bacteria using faecal transplantation could inhibit the colonisation of pathogens (Gilroy *et al.*, 2018). Therefore, using similar techniques, it may be possible to change chicken phenotypes, such as better feed efficiency or improved immune status. However, the use of faecal transplantation techniques in chicken still needs to be improved as other studies found negative results from transplantation (Siegerstetter *et al.*, 2018; Metzler-Zebeli *et al.*, 2019; Chintoan-Uta *et al.*, 2020).

7.5 Anti-coccidial vaccination as chicken gut health study model

The main reason for the use of anti-coccidial vaccination as a gut-damage or mild infection model in chickens in this study was to cause inflammation and epithelial damage that resembled what would be commonly found in the industry. Anti-coccidial vaccination resulted in a mild intestinal infection resembling subclinical infection found in industry (Gussem, 2006; Blake and Tomley, 2014). Subclinical infection of coccidiosis occurs when the chicken has not developed clinical signs but the growth performance is decreased (Gussem, 2006). This leads to significant loss to the industry as no obvious signs are observed and no treatment or intervention has been made. Therefore, if the results from this study showed large benefits from the exogenous enzyme supplementation on vaccinated chickens, the application could be a benefit to industry and easily adopted into industrial practice.

Other infection models have used pathogens such as *Salmonella* spp or *Campylobacter jejuni* to damage or cause inflammation to the chicken gut (Han *et al.*, 2016; Dar *et al.*, 2019). However, these pathogens have different pathogenesis when compared with the *Eimeria* protozoa (**Figure 1.3**). *Campylobacter jejuni* colonises in the chicken intestine by rapid replication in the mucous and invasion of the intestinal epithelium or other organs without developing clinical signs (Awad, Hess and Hess, 2018). *Salmonella* bacterium attaches to the host epithelium and transports effector proteins into the cells (Foley *et al.*, 2013). These *Salmonella* proteins cause intestinal inflammation which results in damage and death of the host cells, however, most *Salmonella* infections in chicken are subclinical (Foley *et al.*, 2013; Kauber *et al.*, 2017). In addition, if these pathogens are detected in industry, significant actions will be taken by the veterinarian or animal husbandry, such as antibiotic treatment or culling all the chickens in the flock, as these pathogens are zoonotic. Therefore, the use of anti-coccidial vaccination to induce gut-damage is more preferable to *Campylobacter jejuni* and *Salmonella* spp models due to direct gut damage by the life cycle developed by the *Eimeria* protozoa, less systemic infection of the chicken without potential zoonotic risks and more resemblance to industrial practice.

However, in this study, the unintended infection of *Eimeria* protozoa in non-vaccinated chickens significantly affected the results of this study. The *Eimeria* infection to non-vaccinated chickens could have occurred because of cross-contamination or infection. The infection could have been caused by wild-type

Eimeria. Although molecular techniques such as PCR could be able to distinguish between wild-type infection and vaccination, this step was not performed in this study as the aim of this study is to find the correlation where the cause of infection in non-vaccinated chicken could be ignored. The solution to this cross-contamination or infection could be an increased level of biosecurity in the rearing house. Husbandry and management should be carefully performed with the supervision of the veterinarian. Rearing the chickens in separate houses with similar environments could also be performed but the confounding factors from different houses could affect the microbiome results (Ludvigsen, Svihus and Rudi, 2016).

Another factor that could have a significant impact on the cross-contamination is the anti-coccidial vaccine selection. In my previous experience, using Coccivac-B® which is discontinued in Thailand, coccidiosis lesion scores were observed in the chicken GI tract at 18 days post-vaccination while using Fortegra®, in this study, coccidiosis lesion scores were observed at 7 days post-vaccination. From the vaccine representatives, Fortegra® could induce the immune response quicker than Coccivac-B® which is important in fast-growing commercial chickens. In the experiment in fast-growing commercial chickens, using the vaccine which quickly develops the lesion score may result in a higher chance of cross-contamination. Therefore, the use of anti-coccidial vaccine as mild infection model in chickens could still be a useful model but the character of the vaccine, such as lesion score developing time or pathogenicity, should be determined before the study.

7.6 Concluding remarks

Poultry producers aim to improve production performance, the welfare of the chickens and to reduce environmental impact from poultry production. Maintenance of gut health is believed to be the key to achieve this goal. However, gut health is an interdisciplinary system and it requires multiple parameters to quantitatively measure gut health. In this study, growth performance, gut microbiome and intestinal gene expression were used as parameters to study gut health. Anti-coccidial vaccination was used as mild intestinal infection model while exogenous enzyme supplementation was used as dietary intervention. In this study, the cross-contamination from vaccinated to non-vaccinated chickens showed a major drawback of using anti-coccidial vaccination as mild intestinal infection model. Improved biosecurity of the rearing house is required if anti-coccidial vaccination model is repeated.

Growth performance was improved by exogenous enzyme supplementation but the gut health parameters such as gut microbiota and intestinal gene expression were only slightly affected. Further analysis such as metabolite and intestinal permeability measurements to explore the additional role of nutrition on gut health is required. On the contrary, pathogen infection significantly affected the growth performance and was related to the gut health parameters studied. Anti-coccidial vaccination reduced chicken growth performance and significantly affected the gut microbiota and host gene expression especially immune-related gene. Microbiome analyses performed in this study showed the weaknesses and strengths of both 16S metabarcoding and shotgun

metagenomic analysis. The correlations between gut microbiome and host intestinal gene expression provide possible direct or indirect relationships between them, based on the immune system and dietary intervention. However, correlations found in this study do not necessarily imply causation and could have occurred co-incidentally.

From this study, although comprehensive study of gut health using several parameters could provide more information such as correlation between gut microbiome and gene expression, additional analysis such as intestinal metabolites or nutrient measurements could be performed as they may provide cause of the changes of gut microbiome or intestinal gene expression. Moreover, further investigation on these genes and bacteria may improve chicken gut health knowledge and lead to improvements in chicken health and feed efficiency that results in increased sustainability of production in the future.

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Appendices

Appendix 1: Supplemental material for Chapter 2

Table S1: Datasets from Mockrobiota selected for bioinformatic pipelines comparison.

Dataset	Number of strains	Note	Reference
mock13	21	Even distribution (4.7% of each bacterial strain), mock 13, 14 and 15 have similar strains with different quality and read counts	(Kozich *et al.*, 2013)
mock14	21		
mock15	21		
mock18	15	Even distribution (6.7% of each bacterial strain)	(Tourlousse *et al.*, 2017)
mock19	27	Similar to mock 18 with 12 more strains included with even distribution (3.7% of each bacterial strain)	(Tourlousse *et al.*, 2017)
mock22	20	Even distribution (5% of each bacterial strain)	(Gohl *et al.*, 2016)
mock23	20	Similar dataset as mock 22 but the strains are unevenly distributed	(Gohl *et al.*, 2016)

Table S2: Bacterial strains and their percentage abundance in the mock13, mock14 and mock15 datasets from Mockrobiota*.

Bacterial strains	Percentage
Acinetobacter baumannii ATCC_17978	4.76
Actinomyces odontolyticus ATCC_17982	4.76
Bacillus cereus ATCC_10987	4.76
Bacteroides vulgatus ATCC_8482	4.76
Clostridium beijerinckii ATCC_51743	4.76
Deinococcus radiodurans DSM_20539	4.76
Enterococcus faecalis ATCC_47077	4.76
Escherichia coli ATCC_700926	4.76
Helicobacter pylori ATCC_700392	4.76
Lactobacillus gasseri DSM_20243	4.76
Listeria monocytogenes ATCC_BAA-679	4.76
Neisseria meningitidis ATCC_BAA-335	4.76
Porphyromonas gingivalis ATCC_33277	4.76
Propionibacterium acnes DSM16379_	4.76
Pseudomonas aeruginosa ATCC_47085	4.76
Rhodobacter sphaeroides ATCC_17023	4.76
Staphylococcus aureus ATCC_BAA-1718	4.76
Staphylococcus epidermidis ATCC_12228	4.76
Streptococcus agalactiae ATCC_BAA-611	4.76
Streptococcus mutans ATCC_700610	4.76
Streptococcus pneumoniae ATCC_BAA-334	4.76

* Data taken from Kozich et al., 2013 and Bokulich et al., 2016.

Table S3: Bacterial strains and their percentage abundance in the mock18 and mock19 datasets from Mockrobiota*.

Bacterial strains	Percentage in mock18	Percentage in mock19
Anaerolinea thermophila UNI-1	6.67	3.70
Bacillus subtilis ATCC-6051	6.67	3.70
Bacteroides vulgatus ATCC-8482	6.67	3.70
Chloroflexus aurantiacus J-10-fl	6.67	3.70
Clostridium acetobutylicum ATCC-824	6.67	3.70
Deinococcus grandis DSM-3963	6.67	3.70
Desulfitobacterium hafniense DCB-2	6.67	3.70
Desulfovibrio vulgaris Hildenborough	6.67	3.70
Escherichia coli DH5a	6.67	3.70
Gemmatimonas aurantiaca T-27	6.67	3.70
Microlunatus phosphovorus NM-1	6.67	3.70
Nitrobacter winogradskyi ATCC-14123	6.67	3.70
Nitrosomonas europaea ATCC-19178	6.67	3.70
Pseudomonas putida KT2440	6.67	3.70
Treponema bryantii ATCC-33254	6.67	3.70
Bacteroides vulgatus JCM-5826 5501	0	3.70
Clostridium acetobutylicum ATCC-8245501	0	3.70
Escherichia coli ATCC-11775 5001	0	3.70
Escherichia coli ATCC-11775 5002	0	3.70
Escherichia coli ATCC-11775 5003	0	3.70
Escherichia coli ATCC-11775 5004	0	3.70
Escherichia coli ATCC-11775 5005	0	3.70
Escherichia coli ATCC-11775 5501	0	3.70
Escherichia coli ATCC-11775 5502	0	3.70
Escherichia coli ATCC-11775 6001	0	3.70
Gemmatimonas aurantiaca T-27 5501	0	3.70
Treponema bryantii DSM-1788 5501	0	3.70

* Data taken from Bokulich et al., 2016 and Tourlousse et al., 2017.

Table S4: Bacterial strains and their percentage abundance in the mock22 and mock23 datasets from Mockrobiota*.

Bacterial strains	Percentage in mock22	Percentage in mock23
Acinetobacter baumannii ATCC_17978	5.00	0.22
Actinomyces odontolyticus ATCC_17982	5.00	0.02
Bacillus cereus ATCC_10987	5.00	2.19
Bacteroides vulgatus ATCC_8482	5.00	0.02
Clostridium beijerinckii ATCC_51743	5.00	2.19
Deinococcus radiodurans DSM_20539	5.00	0.02
Enterococcus faecalis ATCC_47077	5.00	0.02
Escherichia coli ATCC_700926	5.00	21.91
Helicobacter pylori ATCC_700392	5.00	0.22
Lactobacillus gasseri DSM_20243	5.00	0.22
Listeria monocytogenes ATCC_BAA-679	5.00	0.22
Neisseria meningitidis ATCC_BAA-335	5.00	0.22
Propionibacterium acnes DSM16379	5.00	0.22
Pseudomonas aeruginosa ATCC_47085	5.00	2.19
Rhodobacter sphaeroides ATCC_17023	5.00	21.91
Staphylococcus aureus ATCC_BAA-1718	5.00	2.19
Staphylococcus epidermidis ATCC_12228	5.00	21.91
Streptococcus agalactiae ATCC_BAA-611	5.00	2.19
Streptococcus mutans ATCC_700610	5.00	21.91
Streptococcus pneumoniae ATCC_BAA-334	5.00	0.02

* Data taken from Bokulich et al., 2016 and Gohl et al., 2016.

Table S5: Number of rarefied reads in the dataset based on the reference database in each pipeline.

Datasets	Microbiome helper	mothur	QIIME 1.9.1
mock13			
mock14			
mock15	227,398	158,077	52,951
mock18			
mock19	130,086	118,126	137,844
mock22			
mock23	308,592	258,994	292,928

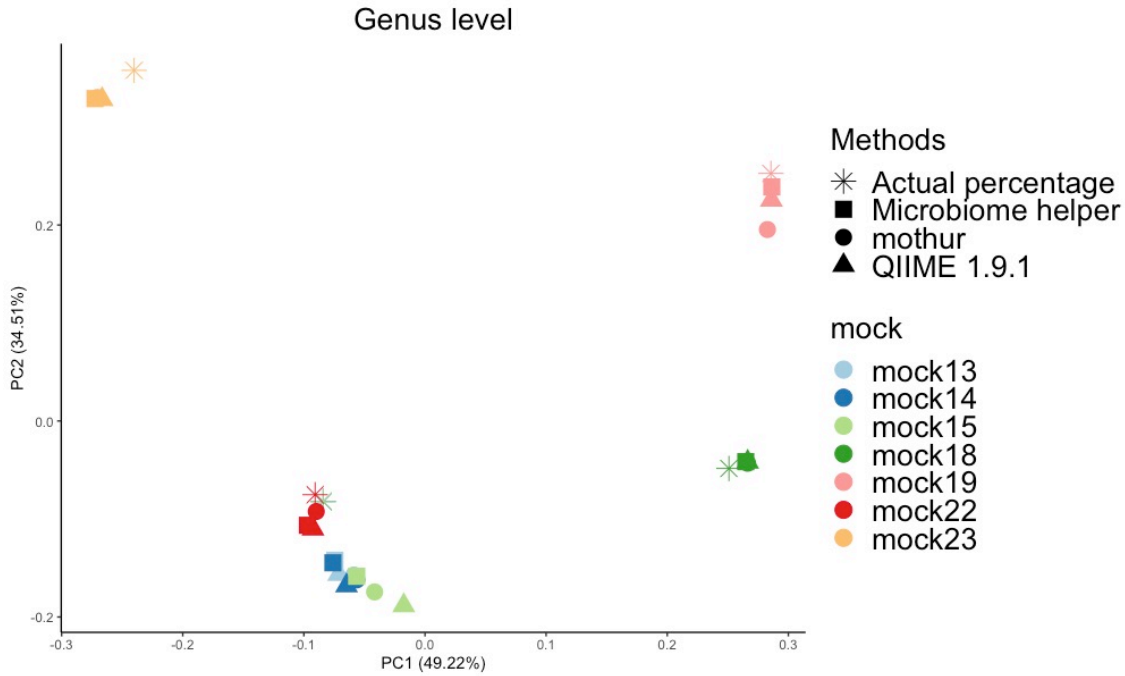

Figure S1: PCA plot showing the distance between the expected percentage abundance and the actual percentage abundance of OTUs classified by 3 pipelines at genus level using a custom database. Note that the actual percentages of mock13, mock14 and mock15 were similar and were plotted in a similar position.

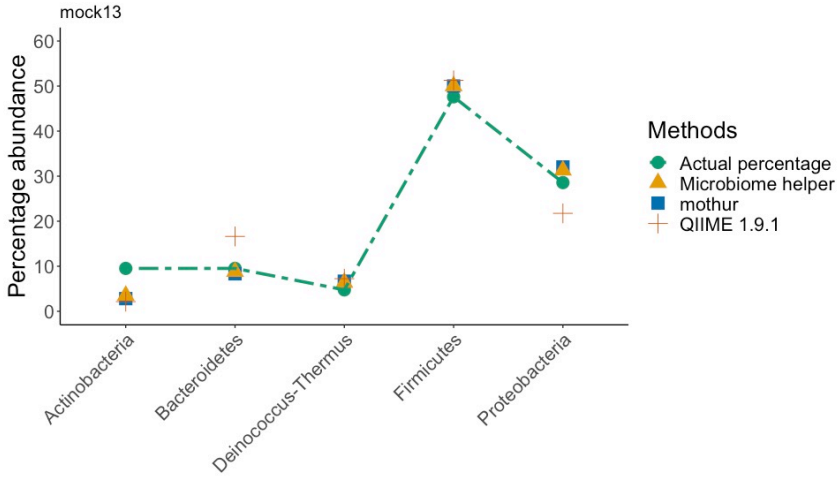

Figure S2: Percentage abundance of OTUs classified by 3 pipelines compare to the actual percentage at phylum level of the mock13 dataset

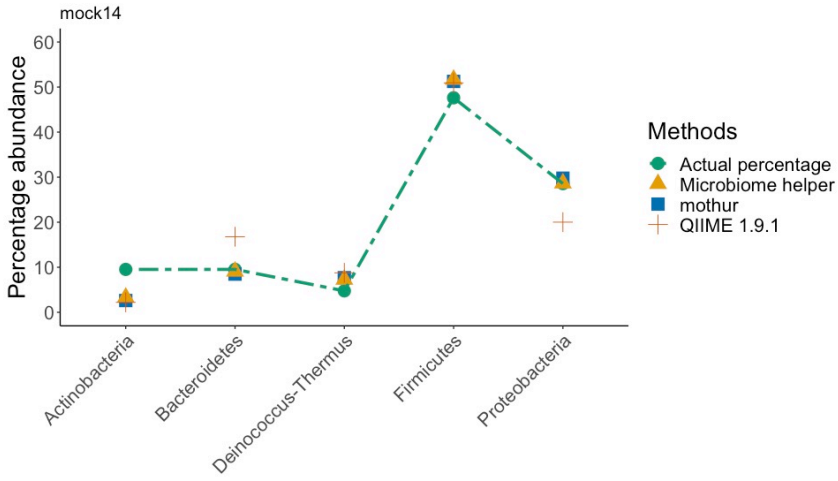

Figure S3: Percentage abundance of OTUs classified by 3 pipelines compare to the actual percentage at phylum level of the mock14 dataset

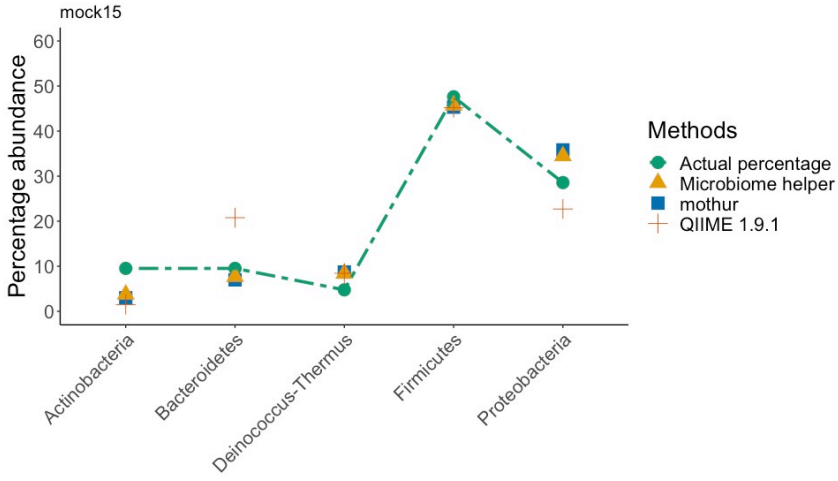

Figure S4: Percentage abundance of OTUs classified by 3 pipelines compare to the actual percentage at phylum level of the mock15 dataset

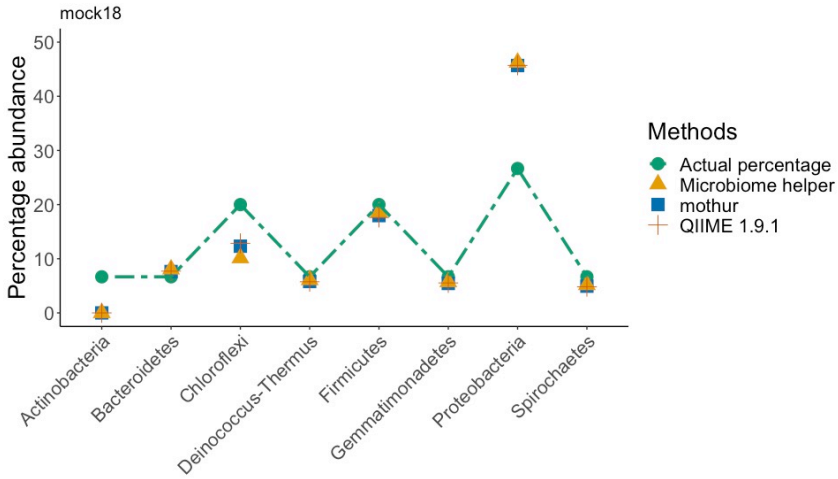

Figure S5: Percentage abundance of OTUs classified by 3 pipelines compare to the actual percentage at phylum level of the mock18 dataset

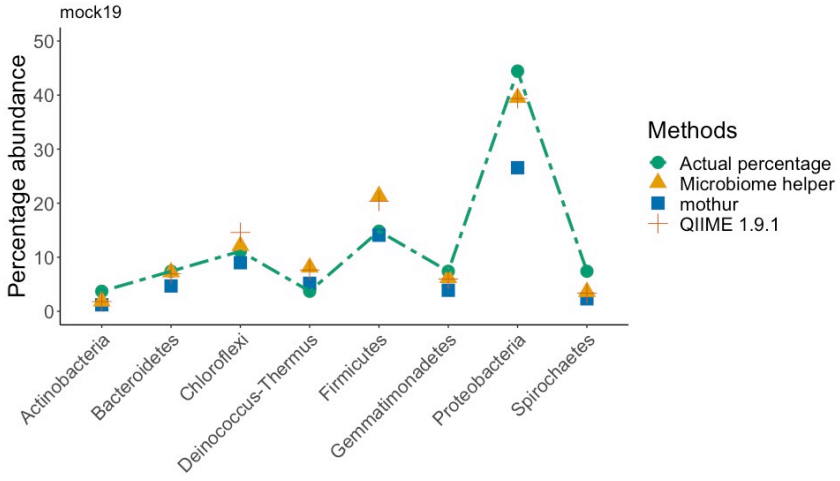

Figure S6: Percentage abundance of OTUs classified by 3 pipelines compare to the actual percentage at phylum level of the mock19 dataset

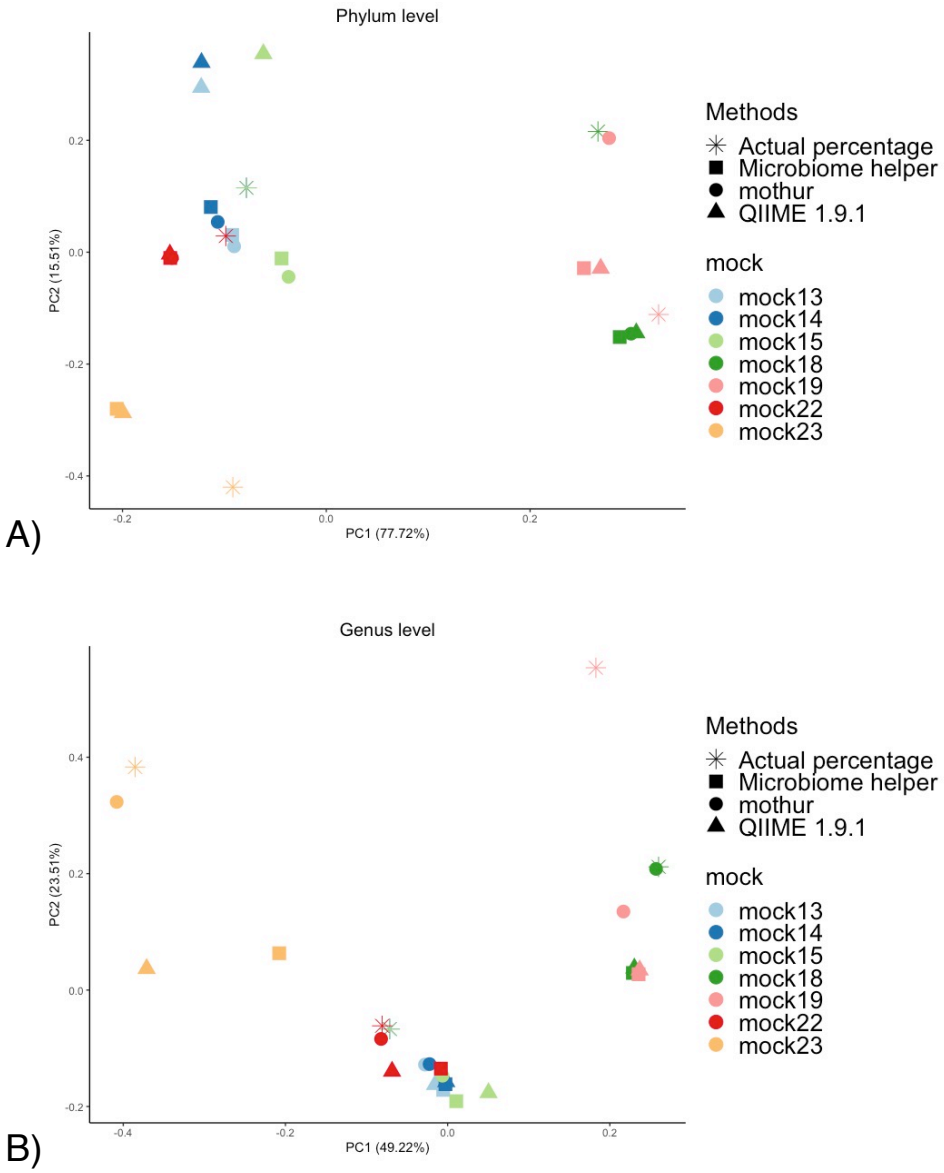

Figure S7: PCA plot showing the distance between the expected percentage and the actual percentage abundances classified by 3 pipelines at A) phylum and B) genus level of 7 datasets analysed in this study. Note that the actual percentage of mock13, mock14 and mock15 were similar and were plotted at a similar position.

Appendix 2: Supplemental material for Chapter 3

Table S6:Ingredient composition of experimental diets given to chicken at each phase of the experiment.

Ingredients	Unit	Starter diet		Grower diet		Finisher diet	
		- Enzymes	+ Enzymes	- Enzymes	+ Enzymes	- Enzymes	+ Enzymes
Maize	%	25.00	25.00	25.00	25.00	25.00	25.00
Wheat	%	31.83	31.80	34.85	34.82	39.11	39.08
Soya bean meal	%	36.17	36.17	32.60	32.60	27.60	27.60
Soya bean oil	%	3.20	3.20	4.30	4.30	5.30	5.30
Limestone	%	1.40	1.40	1.27	1.27	1.15	1.15
Monocalcium Phosphate	%	0.73	0.73	0.55	0.55	0.40	0.40
Ronozyme Hi-phos GT	%	0.01	0.01	0.01	0.01	0.01	0.01
Ronozyme MultiGrain	%	-	0.01	-	0.01	-	0.01
Ronozyme ProAct	%	-	0.02	-	0.02	-	0.02
Others*	%	1.66	1.66	1.43	1.43	1.43	1.43

*Others are salt, sodium bicarbonate, L-lysine, DL-methionine, L-threonine, choline chloride, mold inhibitor, Vitamin premix, Mineral premix and antioxidant.

Table S7: Calculated nutritional values of the experimental diets given to chicken at each phase of the experiment.

Calculated composition	Unit	Starter diet		Grower diet		Finisher diet	
		- Enzymes	+ Enzymes	- Enzymes	+ Enzymes	- Enzymes	+ Enzymes
Metabolisable Energy	(kcal/kg)	3000.88	2999.94	3102.56	3101.62	3199.63	3198.69
Crude protein	%	23.01	23.01	21.50	21.49	19.50	19.50
Crude fat	%	5.15	5.15	6.24	6.24	7.22	7.22
Crude fibre	%	2.60	2.60	2.53	2.53	2.43	2.43
Calcium	%	0.81	0.81	0.72	0.72	0.64	0.64
Total Phosphorus	%	0.58	0.58	0.52	0.52	0.47	0.47
Available phosphorus	%	0.33	0.33	0.29	0.28	0.24	0.24
Lysine	%	1.41	1.40	1.27	1.27	1.13	1.13
Methionine	%	0.68	0.68	0.61	0.61	0.56	0.56
Cysteine	%	0.35	0.35	0.34	0.34	0.31	0.31
Methionine + Cysteine	%	1.04	1.04	0.95	0.95	0.87	0.87
Phytase (x 1000 Unit)	unit/kg	1.00	1.00	1.00	1.00	1.00	1.00
Xylanase (x 1000 Unit)	unit/kg	-	0.27	-	0.27	-	0.27
Protease (x 1000 Unit)	unit/kg	-	15.00	-	15.00	-	15.00
Glucanase (x 1000 Unit)	unit/kg	-	0.15	-	0.15	-	0.15

Table S8: Summary of chicken growth performance (mean ± SD) during the grower phase (day 11 - 24 of chicken age); EPEF = European Production Efficiency Factor.

Factor	Average body weight gain (g)	Feed consumption (g/bird)	FCR	Mortality rate (%)	EPEF
Enzyme supplement					
Without	1086 ± 75	1483 ± 81	1.369 ± 0.080 †	1.08 ± 1.7	558 ± 63
Added	1097 ± 69	1456 ± 58	1.330 ± 0.052 †	0.72 ± 1.1	584 ± 57
Vaccination					
Non-vaccinated	1157 ± 21 ‡	1506 ± 50 ‡	1.302 ± 0.041 ‡	0.96 ± 1.5	624 ± 26 ‡
Vaccinated	1026 ± 29 ‡	1433 ± 71 ‡	1.397 ± 0.059 ‡	0.84 ± 1.4	518 ± 31 ‡
P-value					
Enzyme supplement	0.161	0.169	0.011	0.431	0.003
Vaccination	< 0.001	<0.001	< 0.001	0.782	< 0.001
Interaction	0.455	0.949	0.542	0.773	0.901

†, ‡ Represent statistical significant difference between groups of the same symbol within column (P-value of <0.05)

Table S9: Summary of chicken growth performance (mean ± SD) during finisher phase (day 25 - 35 of chicken age); EPEF = European Production Efficiency Factor.

Factor	Average body weight gain(g)	Feed consumption (g/bird)	FCR	Mortality rate (%)	EPEF
Enzyme supplement					
Without	1332 ± 40	2456 ± 55	1.845± 0.048 †	1.50± 1.9	630± 34 †
Added	1342 ± 50	2429± 60	1.811± 0.046 †	1.00± 1.5	656± 40 †
Vaccination					
Non-vaccinated	1316 ± 37 ‡	2430± 55	1.847± 0.054 ‡	1.27± 2.0	628± 37 ‡
Vaccinated	1357 ± 44 ‡	2454± 61	1.810± 0.038 ‡	1.23± 1.5	658± 35 ‡
P-value					
Enzyme supplement	0.445	0.142	0.020	0.373	0.022
Vaccination	0.004	0.199	0.011	0.935	0.008
Interaction	0.859	0.353	0.506	0.392	0.526

†, ‡ Represent statistical significant difference between groups of the same symbol within column (P-value of <0.05)

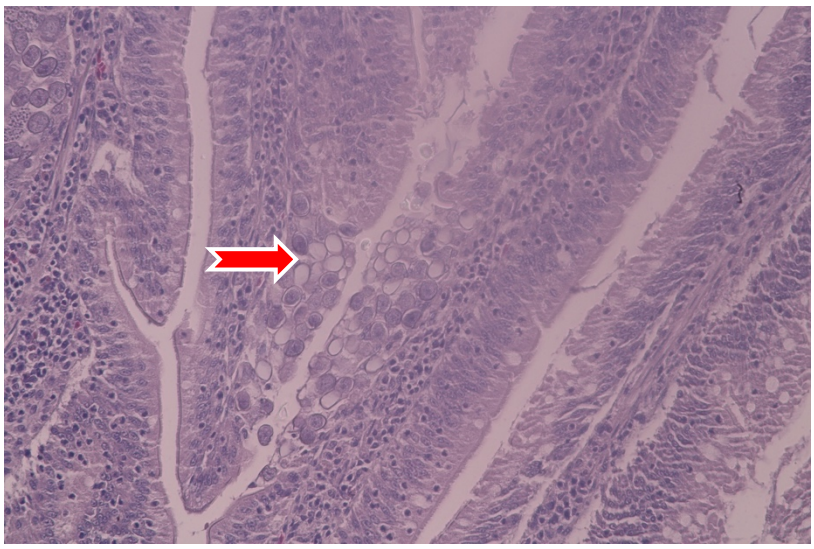

Figure S8: The duodenal tissue of a non-vaccinated chicken showed the presentation of gametocytes (red arrow) from Hematoxylin and Eosin staining.

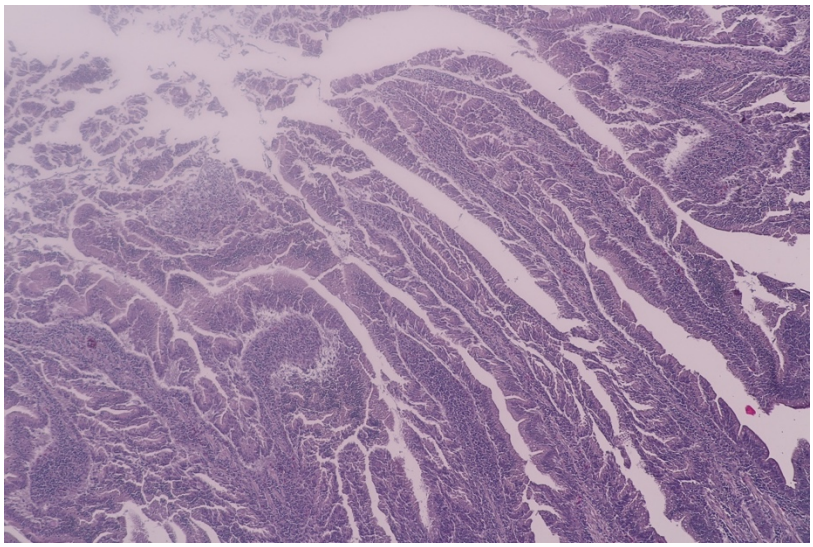

Figure S9: The duodenal tissue of a vaccinated chicken showed no evidence of gametocyte from Hematoxylin and Eosin staining.

Appendix 3: Supplemental material for Chapter 4

Table S10: Forward primer sequences including Illumina adapters and barcodes for PCR amplification of 16SrRNA gene at V4 region.

Primer	Sequence(5' – 3')
v4.SA501	AATGATACGGCGACCACCGAGATCTACACATCGTACGTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA502	AATGATACGGCGACCACCGAGATCTACACACTATCTGTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA503	AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA504	AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA505	AATGATACGGCGACCACCGAGATCTACACTCATCGAGTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA506	AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA507	AATGATACGGCGACCACCGAGATCTACACGGATATCTTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA508	AATGATACGGCGACCACCGAGATCTACACGACACCGTTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB501	AATGATACGGCGACCACCGAGATCTACACCTACTATATAT GGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB502	AATGATACGGCGACCACCGAGATCTACACCGTTACTATA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB503	AATGATACGGCGACCACCGAGATCTACACAGAGTCACTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB504	AATGATACGGCGACCACCGAGATCTACACTACGAGACTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB505	AATGATACGGCGACCACCGAGATCTACACACGTCTCGTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB506	AATGATACGGCGACCACCGAGATCTACACTCGACGAGTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB507	AATGATACGGCGACCACCGAGATCTACACGATCGTGTTA TGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB508	AATGATACGGCGACCACCGAGATCTACACGTCAGATATA TGGTAATTGTGTGCCAGCMGCCGCGGTAA

Table S11: Reverse primer sequences including Illumina adapters and barcodes for PCR amplification of 16SrRNA gene at V4 region.

Primer	Sequence(5' – 3')
v4.SA701	CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SA702	CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SA703	CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SA704	CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SA705	CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SA706	CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SA707	CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SA708	CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAG TCAGCCGGACTACHVGGGTWTCTAAT
v4.SB701	CAAGCAGAAGACGGCATACGAGATAAGTCGAGAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SB702	CAAGCAGAAGACGGCATACGAGATATACTTCGAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SB703	CAAGCAGAAGACGGCATACGAGATAGCTGCTAAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SB704	CAAGCAGAAGACGGCATACGAGATCATAGAGAAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SB705	CAAGCAGAAGACGGCATACGAGATCGTAGATCAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SB706	CAAGCAGAAGACGGCATACGAGATCTCGTTACAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SB707	CAAGCAGAAGACGGCATACGAGATGCGCACGTAGTCAG TCAGCCGGACTACHVGGGTWTCTAAT
v4.SB708	CAAGCAGAAGACGGCATACGAGATGGTACTATAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SB709	CAAGCAGAAGACGGCATACGAGATGTATACGCAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SB710	CAAGCAGAAGACGGCATACGAGATTACGAGCAAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT
v4.SB711	CAAGCAGAAGACGGCATACGAGATTCAGCGTTAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT

Table S12: Percent relative abundance results of mock community control samples compared to the actual percentage of 16S rRNA gene at the family level provided by the manufacturer (Zymo Research, Irvine, CA, USA).

Mock community member (family)	Actual percentage	Mock community DNA standard (n=1)	Mock community micro-organisms standard (n=2)
Pseudomonadaceae	4.2	6.79	13.42
Enterobacteriaceae	20.5	22.08	33.42
Lactobacillaceae	18.4	14.76	15.18
Enterococcaceae	9.9	8.26	12.08
Staphylococcaceae	15.5	17.74	11.32
Listeriaceae	14.1	12.16	3.28
Bacillaceae	17.4	18.03	11.09

Table S13: Mean percent relative abundance results of reagents-only and negative PCR control samples at the family level. OTU classification of negative control samples was performed without rarefaction.

Bacterial family	Reagents-only samples (n=15)	Negative PCR control samples (n=2)
Lachnospiraceae	34.82	5.60
Ruminococcaceae	28.25	18.25
Unknown family in order Clostridiales	8.97	11.38
Lactobacillaceae	5.91	6.08
Bacteroidaceae	5.19	20.82
Other family in order Clostridiales	2.04	0.00
Rikenellaceae	1.42	0.39
Erysipelotrichaceae	1.40	0.39
Enterobacteriaceae	1.24	6.87
Unknown family in order RF32	1.03	2.05
Porphyromonadaceae	0.98	1.87
Oxalobacteraceae	0.77	0.00
Veillonellaceae	0.75	0.39
Unknown family in order RF39	0.73	2.16
Campylobacteraceae	0.68	2.65
Unknown family in order YS2	0.60	2.84
Streptococcaceae	0.57	0.68
Unknown family in order Streptophyta	0.50	0.00
Alcaligenaceae	0.42	0.00
Enterococcaceae	0.32	0.68
Bacillaceae	0.30	0.39
Clostridiaceae	0.28	1.08
Odoribacteraceae	0.25	4.23
Verrucomicrobiaceae	0.23	1.57
Desulfovibrionaceae	0.19	0.00
Staphylococcaceae	0.16	0.39
Moraxellaceae	0.15	2.05
Coriobacteriaceae	0.15	0.68
Corynebacteriaceae	0.14	0.00
Dietziaceae	0.13	0.00
Unassigned	0.12	1.57
Xanthomonadaceae	0.12	0.00

Table S13: continued

Bacterial family	Reagents-only samples (n=15)	Negative PCR control samples (n=2)
Cytophagaceae	0.11	0.00
Halomonadaceae	0.10	0.00
mitochondria	0.08	0.00
Unknown family in order MLE1-12	0.07	4.11
Prevotellaceae	0.07	0.00
Caulobacteraceae	0.07	0.00
Rhizobiaceae	0.07	0.00
Sphingomonadaceae	0.06	0.00
Anaeroplasmataceae	0.05	0.00
Planctomycetaceae	0.05	0.00
Comamonadaceae	0.05	0.00
Rhodobacteraceae	0.05	0.00
Christensenellaceae	0.05	0.00
Planococcaceae	0.04	0.00
Sphingobacteriaceae	0.03	0.00
Micrococcaceae	0.03	0.00
Unknown family in order ML615J-28	0.03	0.00
Victivallaceae	0.03	0.39
Peptostreptococcaceae	0.03	0.00
Mogibacteriaceae	0.03	0.00
Eubacteriaceae	0.02	0.00
Barnesiellaceae	0.02	0.00
Burkholderiaceae	0.01	0.00
Bifidobacteriaceae	0.01	0.00
Dehalobacteriaceae	0.01	0.00
Other family in class Clostridia	0.01	0.00
Dermabacteraceae	0.01	0.00
Aerococcaceae	0.01	0.00
Pseudomonadaceae	0.01	0.00
Leuconostocaceae	< 0.01	0.00
Gordoniaceae	< 0.01	0.00
Other family in orde Bacilliales	< 0.01	0.00
Unknown family in order CAB-I	< 0.01	0.00
Listeriaceae	< 0.01	0.00

Table S14: Taxonomical classification of 322 MAGs in the caecum of 24-day-old chicken at the species level with the number of MAGs classified into the same species.

Family	Genus	Species	MAGs count
Acidaminococcaceae	CAG-207	GCA_000436295.1	1
Akkermansiaceae	Akkermansia	Akkermansia_muciniphila	1
Anaerotignaceae	undefined	undefined	1
Anaerotignaceae	An114	undefined	1
Anaerotignaceae	An114	GCF_002161055.1	1
Anaerotignaceae	Anaerotignum	undefined	1
Anaerotignaceae	Anaerotignum	Anaerotignum_lactatifermentans	3
Anaerotignaceae	ASF356	undefined	1
Anaerovoracaceae	CAG-145	undefined	2
Bacteroidaceae	Bacteroides	undefined	1
Bacteroidaceae	Bacteroides	Bacteroides_caccae	1
Bacteroidaceae	Bacteroides	Bacteroides_clarus	1
Bacteroidaceae	Bacteroides	Bacteroides_fragilis	1
Bacteroidaceae	Bacteroides	Bacteroides_intestinalis	1
Bacteroidaceae	Bacteroides	Bacteroides_ovatus	1
Bacteroidaceae	Bacteroides	Bacteroides_thetaiotaomicron	1
Bacteroidaceae	Bacteroides	Bacteroides_uniformis	6
Bacteroidaceae	Bacteroides	Bacteroides_xylanisolvens_B	1
Bacteroidaceae	Bacteroides	GCF_001185845.1	1

Table S14: Continued

Family	Genus	Species	MAGs count
Barnesiellaceae	Barnesiella	Barnesiella_intestinihominis	1
Burkholderiaceae	Parasutterella	Parasutterella_excrementihominis	1
Burkholderiaceae	Turicimonas	Turicimonas_muris	1
Butyricicoccaceae	undefined	undefined	1
Butyricicoccaceae	Agathobaculum	undefined	7
Butyricicoccaceae	Butyricicoccus	undefined	3
Butyricicoccaceae	Butyricicoccus	Butyricicoccus_pullicaecorum	1
CAG-239	51-20	GCA_001917175.1	2
CAG-239	CAG-495	undefined	2
CAG-239	CAG-495	GCA_000432275.1	2
CAG-239	CAG-495	GCA_000436375.1	1
CAG-239	CAG-495	GCA_001917125.1	1
CAG-508	CAG-269	undefined	2
CAG-552	undefined	undefined	1
CAG-631	CAG-631	GCA_000433015.1	1
CAG-727	undefined	undefined	15
CAG-727	UBA11940	undefined	19
CAG-727	UBA7597	undefined	4
CAG-917	undefined	undefined	1
Desulfovibrionaceae	Bilophila	Bilophila_wadsworthia	1
Desulfovibrionaceae	Mailhella	undefined	2

Table S14: Continued

Family	Genus	Species	MAGs count
DTU089	undefined	undefined	5
DTU089	An172	GCF_002160515.1	2
DTU089	CAG-180	undefined	2
DTU089	Eubacterium_R	undefined	1
DTU089	Neglecta	undefined	1
DTU089	Neglecta	GCA_000435395.1	5
DTU089	UBA1417	undefined	1
DTU089	UBA1691	undefined	1
Eggerthellaceae	CHKCI002	GCF_002159935.1	1
Enterobacteriaceae	Escherichia	Escherichia_coli	3
Erysipelatoclostridiaceae	Erysipelatoclostridium	Erysipelatoclostridium_spiroforme	1
Erysipelatoclostridiaceae	Erysipelatoclostridium	GCF_002160495.1	1
Erysipelotrichaceae	undefined	undefined	1
Erysipelotrichaceae	Merdibacter	undefined	1
Gastranaerophilaceae	undefined	undefined	1
Gastranaerophilaceae	CAG-196	GCA_002102725.1	3
Gastranaerophilaceae	CAG-306	undefined	1
Gastranaerophilaceae	CAG-306	CAG-306_sp1	1
Gastranaerophilaceae	CAG-484	GCA_000431315.1	1
Lachnospiraceae	undefined	undefined	11
Lachnospiraceae	Anaerostipes	undefined	4

Table S14: Continued

Family	Genus	Species	MAGs count
Lachnospiraceae	Blautia_A	undefined	4
Lachnospiraceae	Blautia_A	GCF_002159835.1	1
Lachnospiraceae	Blautia	undefined	6
Lachnospiraceae	Blautia	GCF_002161285.1	5
Lachnospiraceae	CHKCI001	undefined	2
Lachnospiraceae	Clostridium_M	undefined	3
Lachnospiraceae	Dorea	Dorea_faecis	2
Lachnospiraceae	Dorea	GCF_002160985.1	1
Lachnospiraceae	Eisenbergiella	undefined	4
Lachnospiraceae	Eubacterium_E	GCF_002161065.1	1
Lachnospiraceae	Fusicatenibacter	undefined	1
Lachnospiraceae	GCA-900066575	GCF_002160765.1	3
Lachnospiraceae	Lachnoclostridium_A	undefined	1
Lachnospiraceae	Lachnoclostridium_A	GCF_002160755.1	1
Lachnospiraceae	Ruminococcus_B	undefined	13
Lachnospiraceae	Ruminococcus_B	GCA_002314255.1	1
Lachnospiraceae	Ruminococcus_B	GCF_002161355.1	2
Lachnospiraceae	Sellimonas	GCF_002159995.1	1
Lachnospiraceae	UBA7160	undefined	1
Lachnospiraceae	UBA7182	undefined	1
Lachnospiraceae	UBA7182	GCF_002160135.1	6

Table S14: Continued

Family	Genus	Species	MAGs count
Lachnospiraceae	UBA9502	undefined	2
Lactobacillaceae	Lactobacillus_B	Lactobacillus_B_salivarius	1
Lactobacillaceae	Lactobacillus	Lactobacillus_crispatus	1
Lactobacillaceae	Lactobacillus	Lactobacillus_johnsonii	1
Marinifilaceae	Butyricimonas	Butyricimonas_synergistica_A	1
Marinifilaceae	Butyricimonas	GCF_002161485.1	1
Marinifilaceae	Odoribacter	undefined	1
Marinifilaceae	Odoribacter	Odoribacter_massiliensis	1
Marinifilaceae	Odoribacter	Odoribacter_splanchnicus	1
Oscillospiraceae	undefined	undefined	6
Oscillospiraceae	CAG-110	undefined	2
Oscillospiraceae	Clostridium_AJ	undefined	2
Oscillospiraceae	Clostridium_AJ	GCF_002160305.1	2
Oscillospiraceae	Flavonifractor	undefined	4
Oscillospiraceae	Flavonifractor	GCF_002161215.1	2
Oscillospiraceae	Flavonifractor	GCF_002161245.1	1
Oscillospiraceae	Intestinimonas	undefined	1
Oscillospiraceae	Oscillibacter	undefined	4
Oscillospiraceae	UBA5446	undefined	1
Oscillospiraceae	UBA9475	undefined	2
Oscillospiraceae	UBA9475	GCF_002160435.1	2

Table S14: Continued

Family	Genus	Species	MAGs count
Peptostreptococcaceae	Romboutsia	undefined	1
Rikenellaceae	Alistipes_A	Alistipes_A_ihumii	1
Rikenellaceae	Alistipes_A	Alistipes_A_sp1	1
Rikenellaceae	Alistipes	undefined	2
Rikenellaceae	Alistipes	Alistipes_finegoldii	1
Rikenellaceae	Alistipes	Alistipes_onderdonkii	1
Rikenellaceae	Alistipes	Alistipes_putredinis	2
Rikenellaceae	Alistipes	Alistipes_senegalensis	1
Rikenellaceae	Alistipes	Alistipes_shahii	1
Rikenellaceae	Alistipes	GCF_900021155.1	1
Rikenellaceae	Rikenella	Rikenella_microfusus	1
Rikenellaceae	Tidjanibacter	Tidjanibacter_massiliensis	1
Ruminococcaceae	undefined	undefined	1
Ruminococcaceae	Anaerofilum	undefined	1
Ruminococcaceae	Anaerofilum	GCF_002160015.1	1
Ruminococcaceae	Anaerotruncus	undefined	1
Ruminococcaceae	Anaerotruncus	Anaerotruncus_colihominis	6
Ruminococcaceae	Angelakisella	Angelakisella_massiliensis	1
Ruminococcaceae	D5	undefined	1
Ruminococcaceae	Faecalibacterium	undefined	1
Ruminococcaceae	Fournierella	GCF_002161595.1	1

Table S14: Continued

Family	Genus	Species	MAGs count
Ruminococcaceae	Negativibacillus	undefined	1
Ruminococcaceae	Ruthenibacterium	undefined	2
Ruminococcaceae	Ruthenibacterium	GCA_002315015.1	2
Ruminococcaceae	Subdoligranulum	undefined	8
Ruminococcaceae	UBA1448	undefined	1
Ruminococcaceae	UBA7177	undefined	1
Tannerellaceae	Parabacteroides	Parabacteroides_distasonis	4
Tannerellaceae	Parabacteroides	Parabacteroides_merdae	1
UBA1381	UBA4716	undefined	2
UBA1390	UBA1390	GCA_002305315.1	1
UBA1784	UBA11493	undefined	1
UBA1784	UBA11493	UBA11493	1
UBA3700	undefined	undefined	3
UBA660	CAG-451	undefined	2
UBA660	CAG-460	undefined	2
UBA660	CAG-594	undefined	1
UBA660	CAG-776	undefined	2
UBA660	CAG-877	undefined	4

Table S14: Continued

Family	Genus	Species	MAGs count
UBA660	CAG-914	undefined	2
UBA660	CAG-988	GCA_000437335.1	5
UBA660	UBA5026	undefined	4
undefined in order Exiguobacterales	undefined	undefined	1

Table S15: Significant higher abundance MAGs in the caecum of 24-day-old vaccinated chickens compared to non-vaccinated chickens at the family to genus level (adjusted P-value < 0.05); padj = adjusted P-value.

Family	Genus	Species	log2FoldChange	padj
Bacteroidaceae	Bacteroides	Bacteroides_fragilis	-4.28	0.019
Ruminococcaceae	Fournierella	GCF_002161595.1	-4.13	0.043
Ruminococcaceae	Ruthenibacterium	undefined	-3.33	0.043
Oscillospiraceae	Flavonifractor	undefined	-3.10	0.035

Table S16: Significant higher abundance MAGs in the caecum of 24-day-old non-vaccinated chickens compared to vaccinated chickens at the family to genus level (adjusted P-value < 0.05); padj = adjusted P-value.

Family	Genus	Species	log2FoldChange	padj
CAG-727	UBA11940	undefined	6.34	<0.001
Gastranaerophilaceae	CAG-196	GCA_002102725.1	5.61	0.008
CAG-727	UBA11940	undefined	5.55	0.019
CAG-508	CAG-269	undefined	5.43	0.008
CAG-727	UBA11940	undefined	5.41	0.019
CAG-727	UBA11940	undefined	5.35	0.035
CAG-727	undefined	undefined	5.20	0.010
CAG-727	undefined	undefined	4.89	0.037
CAG-727	UBA11940	undefined	4.36	0.022
Gastranaerophilaceae	undefined	undefined	4.32	0.019
CAG-727	UBA11940	undefined	4.23	0.021
CAG-727	undefined	undefined	4.23	0.035
Ruminococcaceae	Subdoligranulum	undefined	4.11	0.008
Oscillospiraceae	Flavonifractor	GCF_002161215.1	2.87	0.035
Oscillospiraceae	Flavonifractor	GCF_002161215.1	2.72	0.035

Table S17: Significant higher abundance MAGs in the caecum of 24-day-old enzymes supplemented chickens compared to non-enzyme supplemented chickens at the family to genus level (adjusted P-value < 0.05); padj = adjusted P-value.

Family	Genus	Species	log2FoldChange	padj
Bacteroidaceae	Bacteroides	Bacteroides_clarus	-8.08	0.022

Table S18: Significant differential abundance MAGs from interaction effects between anti-coccidial vaccination and exogenous enzymes supplement in the caecum of 24-day-old chickens at the family to genus level (adjusted P-value < 0.05). Positive interaction between main factors was shown in positive log2 fold change and negative interaction effect was shown in negative log2 fold change.; padj = adjusted P-value.

Family	Genus	Species	log2FoldChange	padj
Bacteroidaceae	Bacteroides	Bacteroides_clarus	10.70	0.029

Appendix 4: Supplemental material for Chapter 5.

Table S19: Significant up-regulated genes at the ileum of enzymes supplemented chickens (adjusted P-value < 0.05); padj = adjusted P-value, NA = unannotated gene.

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000053173	NA	-21.227	0.002
ENSGALG00000006354	GAL3ST2	-9.406	0.002
ENSGALG00000006361	NEU4	-6.180	0.040

Table S20: Significant up-regulated genes at the ileum of non-enzymes supplemented chickens (adjusted P-value < 0.05); padj = adjusted P-value, NA = unannotated gene.

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000052158	NA	21.431	0.002

Table S21: Significant up-regulated genes at the ileum of vaccinated chickens (adjusted P-value < 0.05); padj = adjusted P-value, NA = unannotated gene.

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000052158	NA	-19.494	<0.001
ENSGALG00000039876	LOC107055133	-6.765	<0.001
ENSGALG00000053626	LOC107050390	-6.429	0.001
ENSGALG00000048441	NA	-6.173	0.004
ENSGALG00000050242	LOC107054464	-5.963	0.003
ENSGALG00000009929	SOST	-3.998	0.007
ENSGALG00000003554	OPTC	-3.990	0.020
ENSGALG00000052207	NA	-3.653	0.027
ENSGALG00000023395	PLIN1	-3.556	0.019
ENSGALG00000011219	GCGR	-3.390	0.014
ENSGALG00000033729	NA	-3.345	0.006
ENSGALG00000015425	LPL	-3.318	0.007
ENSGALG00000005739	SCD	-3.052	0.004
ENSGALG00000040434	RAB18L	-3.000	0.039
ENSGALG00000030030	RSPO2	-2.786	0.013
ENSGALG00000053245	VTG3	-2.692	0.004
ENSGALG00000028273	HBE1	-2.667	0.018
ENSGALG00000015053	GLDC	-2.666	0.004
ENSGALG00000011875	TRPC3	-2.619	<0.001
ENSGALG00000040573	FMO3	-2.615	0.025
ENSGALG00000014585	CCL26	-2.587	<0.001
ENSGALG00000005763	VILL	-2.574	0.004
ENSGALG00000000558	SLC1A6	-2.538	0.030
ENSGALG00000054033	NA	-2.515	0.033
ENSGALG00000046316	CFAP97D1	-2.504	0.008

Table S21: (continued)

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000042944	LOC101748650	-2.488	0.042
ENSGALG00000000168	ADORA1	-2.478	0.039
ENSGALG00000009903	IFNG	-2.466	0.003
ENSGALG00000032683	ADTRP	-2.463	0.047
ENSGALG00000000871	ART1	-2.447	0.002
ENSGALG00000023933	G0S2	-2.438	0.002
ENSGALG00000001325	CYP1A1	-2.434	0.038
ENSGALG00000048325	GBP	-2.412	<0.001
ENSGALG00000004916	DRC3	-2.297	<0.001
ENSGALG00000034478	CCL4	-2.212	<0.001
ENSGALG00000029140	CRABP2	-2.195	0.003
ENSGALG00000054102	NA	-2.129	0.049
ENSGALG00000043234	HBA1	-2.122	<0.001
ENSGALG00000041491	ACKR4	-2.121	0.008
ENSGALG00000053794	NA	-2.114	0.007
ENSGALG00000031597	HBAD	-2.100	<0.001
ENSGALG00000002329	CCL1	-2.085	<0.001
ENSGALG00000000081	IL4I1	-2.080	<0.001
ENSGALG00000046358	FBLN7	-2.078	0.012
ENSGALG00000006375	TM4SF19	-2.072	<0.001
ENSGALG00000046937	NA	-2.050	0.001
ENSGALG00000053792	NA	-2.043	0.002
ENSGALG00000005493	ITGB1BP2	-2.039	0.013
ENSGALG00000013056	TUBA8A	-2.024	0.020
ENSGALG00000047152	HBBA	-2.008	<0.001
ENSGALG00000052767	NA	-2.000	<0.001

Table S22: Significant up-regulated genes at the ileum of non-vaccinated chickens (adjusted P-value < 0.05); padj = adjusted P-value, NA = unannotated gene.

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000040021	HOXA11	8.383	0.025
ENSGALG00000040666	ATP6V0A4	6.208	0.001
ENSGALG00000004545	WDR72	5.792	0.016
ENSGALG00000007998	SLC26A4	5.765	0.001
ENSGALG00000043909	MSMB	4.594	0.014
ENSGALG00000044387	SLC4A9	4.178	0.020
ENSGALG00000053074	NA	4.067	0.003
ENSGALG00000053343	NA	3.762	0.026
ENSGALG00000039536	C2H8ORF22	3.687	<0.001
ENSGALG00000016431	PAX5	3.600	0.002
ENSGALG00000008599	LOC101747844	3.526	<0.001
ENSGALG00000014975	DRD5	3.473	0.004
ENSGALG00000048697	NA	2.991	0.045
ENSGALG00000030614	ADH1C	2.987	0.010
ENSGALG00000034294	ATP6V0D2	2.950	0.015
ENSGALG00000015970	COL9A1	2.942	<0.001
ENSGALG00000046331	NA	2.918	0.034
ENSGALG00000045607	CXCL13	2.910	0.004
ENSGALG00000054799	SPIC	2.867	0.001
ENSGALG00000010338	CXCL13L2	2.842	0.006
ENSGALG00000044668	LOC421856	2.794	0.013
ENSGALG00000053848	NA	2.779	0.033
ENSGALG00000007596	LOC416086	2.775	0.006
ENSGALG00000013304	SLC5A12	2.765	0.004

Table S22: (continued)

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000002893	STC2	2.720	<0.001
ENSGALG00000002207	ATP6V1G3	2.576	0.036
ENSGALG00000001244	TBC1D24L, CFAP52	2.566	0.021
ENSGALG00000007959	SLC26A3	2.542	0.006
ENSGALG00000042505	DSEL	2.489	<0.001
ENSGALG00000050737	NA	2.486	0.047
ENSGALG00000014766	HAO2	2.384	0.007
ENSGALG00000052283	NA	2.377	0.018
ENSGALG00000010781	GLRA3	2.294	0.014
ENSGALG00000047807	CD163	2.284	0.040
ENSGALG00000022815	AvBD1	2.264	0.020
ENSGALG00000010822	SPATA4	2.263	0.011
ENSGALG00000048815	NA	2.238	0.018
ENSGALG00000054856	LOC100857280	2.217	0.027
ENSGALG00000042679	FAM134B	2.205	<0.001
ENSGALG00000035052	BMP3	2.135	0.001
ENSGALG00000049379	IRS2	2.132	<0.001
ENSGALG00000014537	BMF	2.120	<0.001
ENSGALG00000013085	PTPRO	2.110	0.005
ENSGALG00000013535	CYP4V2	2.045	0.002
ENSGALG00000006112	SCN5A	2.036	0.012

Table S23: Significant differentiallly expressed genes from interaction effects between anti-coccidial vaccination and exogenous enzymes supplement at the ileum of 24-day-old chickens (adjusted P-value < 0.05). Positive interaction between main factors was shown in positive log2 fold change and negative interaction effect was shown in negative log2 fold change.; padj = adjusted P-value, NA = unannotated gene.

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000052158	NA	-24.092	0.046
ENSGALG00000052767	NA	-3.081	<0.001
ENSGALG00000031597	HBAD	-2.965	0.001
ENSGALG00000043234	HBA1	-2.755	0.001
ENSGALG00000047152	HBBA	-2.639	0.013
ENSGALG00000015970	COL9A1	3.760	0.020
ENSGALG00000002207	ATP6V1G3	5.709	0.039
ENSGALG00000044387	SLC4A9	8.386	0.039
ENSGALG00000007998	SLC26A4	9.850	0.002
ENSGALG00000040666	ATP6V0A4	10.414	0.001
ENSGALG00000053173	NA	26.508	0.016

Table S24: Significant up-regulated genes at the caecum of enzymes supplemented chickens (adjusted P-value < 0.05); padj = adjusted P-value, NA = unannotated gene.

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000053173	NA	-21.759	0.001
ENSGALG00000050856	NA	-20.273	0.001

Table S25: Significant up-regulated genes at the caecum of non-enzymes supplemented chickens (adjusted P-value < 0.05); padj = adjusted P-value, NA = unannotated gene.

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000048343	CES1L2	2.203	0.048

Table S26: Significant up-regulated genes at the caecum of vaccinated chickens (adjusted P-value < 0.05); padj = adjusted P-value, NA = unannotated gene.

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000053266	NA	-8.626	0.030
ENSGALG00000039876	LOC107055133	-5.970	0.013
ENSGALG00000010949	GPNMB	-5.528	<0.001
ENSGALG00000013548	GZMA	-4.447	<0.001
ENSGALG00000049973	TMEM252	-4.301	0.033
ENSGALG00000038918	NA	-4.166	0.001
ENSGALG00000016556	GVINP1	-3.298	<0.001
ENSGALG00000009903	IFNG	-3.287	0.025
ENSGALG00000013546	GZMK	-3.287	<0.001
ENSGALG00000006290	HS3ST4	-3.181	0.032
ENSGALG00000051726	NA	-2.927	<0.001
ENSGALG00000002329	CCL1	-2.826	<0.001
ENSGALG00000000871	ART1	-2.693	0.017
ENSGALG00000032614	AT2	-2.658	<0.001
ENSGALG00000041025	FOXJ1	-2.630	0.001
ENSGALG00000027247	EOMES	-2.573	<0.001
ENSGALG00000042227	GNLY	-2.572	<0.001
ENSGALG00000028016	GBE	-2.517	<0.001
ENSGALG00000048617	LOC112532337	-2.398	0.025
ENSGALG00000011859	GBE	-2.378	0.009
ENSGALG00000028376	FGF19	-2.232	0.005
ENSGALG00000003690	KRT14	-2.194	0.050
ENSGALG00000015141	GDA	-2.193	0.005
ENSGALG00000050240	TBX21	-2.191	<0.001

Table S26: (continued)

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000040557	TFEC	-2.180	0.034
ENSGALG00000032717	CCL4	-2.170	<0.001
ENSGALG00000038096	NOS2	-2.169	0.017
ENSGALG00000019719	KRT24	-2.111	0.040
ENSGALG00000000136	BLEC2	-2.105	0.006
ENSGALG00000005739	SCD	-2.048	0.006
ENSGALG00000053568	LOC107049158	-2.037	<0.001
ENSGALG00000007171	CIITA	-2.033	0.032
ENSGALG00000052425	BATF3	-2.014	0.035

Table S27: Significant up-regulated genes at the caecum of non-vaccinated chickens (adjusted P-value < 0.05); padj = adjusted P-value, NA = unannotated gene.

ENSEMBL GeneID	Gene_Name	log2FoldChange	padj
ENSGALG00000039536	C2H8ORF22	4.834	<0.001
ENSGALG00000013097	SLC15A5	3.497	0.002
ENSGALG00000049947	NA	3.162	<0.001
ENSGALG00000006202	SCNN1B	2.636	0.037
ENSGALG00000010469	CYP4B7	2.626	0.030
ENSGALG00000000820	HTR1D	2.434	0.016
ENSGALG00000005204	GSTT1L	2.306	<0.001
ENSGALG00000006270	SCNN1G	2.231	0.009
ENSGALG00000004545	WDR72	2.219	0.004
ENSGALG00000046875	NA	2.139	0.025
ENSGALG00000026727	SLC30A10	2.111	0.028
ENSGALG00000047199	RP11-400G3.5	2.106	0.028
ENSGALG00000055025	LOC112533547	2.021	0.032

Appendix 5: Supplemental material for Chapter 6

Table S28: List of significant correlations between bacterial OTUs and gene expression at the ileum of 24-day-old chickens. NA = unannotated gene

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000001325	CYP1A1	otu_134726	Lactobacillus spp.	0.740
ENSGALG00000001325	CYP1A1	otu_290235	Lactobacillus reuteri	0.779
ENSGALG00000001325	CYP1A1	otu_582616	Unknown genus in order Clostridales	-0.764
ENSGALG00000002207	ATP6V1G3	otu_561607	Unknown genus in family Ruminococcaceae	-0.814
ENSGALG00000002207	ATP6V1G3	otu_3138798	Phascolarctobacterium spp.	-0.783
ENSGALG00000002329	CCL1	otu_186881	Unknown genus in family Ruminococcaceae	-0.795
ENSGALG00000002329	CCL1	otu_4460021	Ruminococcus spp.	-0.714
ENSGALG00000002329	CCL1	otu_157840	Unknown genus in order Clostridales	-0.785
ENSGALG00000002329	CCL1	otu_325850	Unknown genus in order RF32	-0.740
ENSGALG00000002329	CCL1	otu_4381553	Bacteroides spp.	-0.844
ENSGALG00000002329	CCL1	otu_158309	Coprococcus spp.	-0.722
ENSGALG00000002329	CCL1	otu_592616	Unknown genus in family Erysipelotrichaceae	-0.753
ENSGALG00000002329	CCL1	otu_3141342	Coprococcus spp.	-0.781
ENSGALG00000002329	CCL1	otu_328905	Oscillospira spp.	-0.750

Table S28: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000002329	CCL1	otu_359809	Sutterella spp.	-0.892
ENSGALG00000002329	CCL1	otu_333348	Unknown genus in family Ruminococcaceae	-0.743
ENSGALG00000002893	STC2	otu_359809	Sutterella spp.	0.727
ENSGALG00000005739	SCD	otu_359809	Sutterella spp.	-0.747
ENSGALG00000006375	TM4SF19	otu_563086	[Ruminococcus] spp.	-0.775
ENSGALG00000006375	TM4SF19	otu_333348	Unknown genus in family Ruminococcaceae	-0.790
ENSGALG00000006375	TM4SF19	otu_592901	Unknown genus in family Ruminococcaceae	-0.799
ENSGALG00000008599	NA	otu_157840	Unknown genus in order Clostridales	0.717
ENSGALG00000008599	NA	otu_333348	Unknown genus in family Ruminococcaceae	0.792
ENSGALG00000008599	NA	otu_158309	Coprococcus spp.	0.778
ENSGALG00000008599	NA	otu_359809	Sutterella spp.	0.756
ENSGALG00000010822	SPATA4	otu_333348	Unknown genus in family Ruminococcaceae	0.794
ENSGALG00000011875	TRPC3	otu_1028036	Bacillus spp.	-0.782
ENSGALG00000011875	TRPC3	otu_158309	Coprococcus spp.	-0.794
ENSGALG00000011875	TRPC3	otu_359809	Sutterella spp.	-0.770
ENSGALG00000013056	TUBA8A	otu_358185	Ruminococcus spp.	-0.799
ENSGALG00000013085	PTPRO	otu_333348	Unknown genus in family Ruminococcaceae	0.862
ENSGALG00000014537	BMF	otu_158309	Coprococcus spp.	0.721
ENSGALG00000014537	BMF	otu_333348	Unknown genus in family Ruminococcaceae	0.710
ENSGALG00000014585	CCL26	otu_333348	Unknown genus in family Ruminococcaceae	-0.772

Table S28: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000014585	CCL26	otu_359809	Sutterella spp.	-0.714
ENSGALG00000014585	CCL26	otu_158309	Coprococcus spp.	-0.839
ENSGALG00000014975	DRD5	otu_358858	Blautia producta	0.821
ENSGALG00000014975	DRD5	otu_186881	Unknown genus in family Ruminococcaceae	0.788
ENSGALG00000014975	DRD5	otu_4381553	Bacteroides spp.	0.810
ENSGALG00000015970	COL9A1	otu_157840	Unknown genus in order Clostridales	0.735
ENSGALG00000015970	COL9A1	otu_592616	Unknown genus in family Erysipelotrichaceae	0.816
ENSGALG00000015970	COL9A1	otu_358185	Ruminococcus spp.	0.787
ENSGALG00000015970	COL9A1	otu_561607	Unknown genus in family Ruminococcaceae	0.784
ENSGALG00000015970	COL9A1	otu_3138798	Phascolarctobacterium spp.	0.741
ENSGALG00000015970	COL9A1	otu_3141342	Coprococcus spp.	0.763
ENSGALG00000015970	COL9A1	otu_186881	Unknown genus in family Ruminococcaceae	0.763
ENSGALG00000015970	COL9A1	otu_183932	Unknown genus in family Ruminococcaceae	0.813
ENSGALG00000015970	COL9A1	otu_563086	[Ruminococcus] spp.	0.730
ENSGALG00000030030	RSPO2	otu_186319	Coprococcus spp.	-0.764
ENSGALG00000030030	RSPO2	otu_132661	Enterococcus cecorum	-0.695
ENSGALG00000030030	RSPO2	otu_158971	Unknown genus in order Clostridales	-0.772
ENSGALG00000033729	LOC100858385	otu_359809	Sutterella spp.	-0.746
ENSGALG00000034478	CCL4	otu_158309	Coprococcus spp.	-0.776
ENSGALG00000035052	BMP3	otu_358185	Ruminococcus spp.	0.757

Table S28: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000039536	C2H8ORF22	otu_333348	Unknown genus in family Ruminococcaceae	0.766
ENSGALG00000039536	C2H8ORF22	otu_158309	Coprococcus spp.	0.802
ENSGALG00000039876	EDQM2	otu_325850	Unknown genus in order RF32	-0.740
ENSGALG00000039876	EDQM2	otu_158309	Coprococcus spp.	-0.725
ENSGALG00000041491	NA	otu_16195	Candidatus Arthromitus spp.	-0.774
ENSGALG00000042505	DSEL	otu_158309	Coprococcus spp.	0.763
ENSGALG00000042679	FAM134B	otu_158309	Coprococcus spp.	0.783
ENSGALG00000042679	FAM134B	otu_333348	Unknown genus in family Ruminococcaceae	0.721
ENSGALG00000044387	SLC4A9	otu_650615	Corynebacterium spp.	-0.846
ENSGALG00000044387	SLC4A9	otu_1504042	Oscillospira spp.	0.903
ENSGALG00000045607	CXCL13L3, CXCL13	otu_158047	Unknown genus in order Bacillales	-0.778
ENSGALG00000046937	NA	otu_4428313	Lactobacillus pp.	0.698
ENSGALG00000048441	NA	otu_4460021	Ruminococcus spp.	-0.761
ENSGALG00000049379	NA	otu_358185	Ruminococcus spp.	0.757
ENSGALG00000049379	NA	otu_158309	Coprococcus spp.	0.747
ENSGALG00000053245	NA	otu_193480	Unknown genus in order Clostridales	-0.724
ENSGALG00000053626	NA	otu_1028036	Bacillus spp.	-0.708
ENSGALG00000053626	NA	otu_325850	Unknown genus in order RF32	-0.702
ENSGALG00000053792	NA	otu_592616	Unknown genus in family Erysipelotrichaceae	-0.710
ENSGALG00000053792	NA	otu_359809	Sutterella spp.	-0.798

Table S28: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000053792	NA	otu_328905	Oscillospira spp.	-0.836
ENSGALG00000053792	NA	otu_368338	Unknown genus in family Lachnospiraceae	-0.727
ENSGALG00000053792	NA	otu_2415201	Unknown genus in family Lachnospiraceae	-0.793
ENSGALG00000053792	NA	otu_358858	Blautia producta	-0.791
ENSGALG00000053792	NA	otu_186881	Unknown genus in family Ruminococcaceae	-0.743
ENSGALG00000053792	NA	otu_563086	[Ruminococcus] spp.	-0.767
ENSGALG00000054799	NA	otu_333348	Unknown genus in family Ruminococcaceae	0.761
ENSGALG00000054799	NA	otu_368338	Unknown genus in family Lachnospiraceae	0.736
ENSGALG00000054799	NA	otu_158309	Coprococcus spp.	0.740
ENSGALG00000054856	NA	otu_361365	Unknown genus in family Erysipelotrichaceae	-0.826

Table S29: List of significant correlations between bacterial OTUs and gene expression at the caecum of 24-day-old chickens. NA = unannotated gene

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000000136	BLEC2	otu_157573	Unknown genus in family Rikenellaceae	0.767
ENSGALG00000000136	BLEC2	New.ReferenceOTU89	Unknown genus in order Clostridiales	0.747
ENSGALG00000000820	HTR1D	otu_316515	Lactobacillus spp.	0.703
ENSGALG00000000871	MADPRT1	otu_369097	Oscillospira spp.	0.698
ENSGALG00000000871	MADPRT1	New.ReferenceOTU100	Unknown genus in order Clostridiales	0.774
ENSGALG00000000871	MADPRT1	otu_4472195	Unknown genus in family Ruminococcaceae	0.880
ENSGALG00000000871	MADPRT1	otu_594206	Unknown genus in family Ruminococcaceae	-0.729
ENSGALG00000000871	MADPRT1	otu_582616	Unknown genus in order Clostridiales	0.749
ENSGALG00000000871	MADPRT1	otu_4402645	Unknown genus in order Clostridiales	0.736
ENSGALG00000000871	MADPRT1	New.ReferenceOTU89	Unknown genus in order Clostridiales	0.742
ENSGALG00000000871	MADPRT1	otu_130763	Unknown genus in order Clostridiales	-0.690
ENSGALG00000002329	CCL1	otu_4472195	Unknown genus in family Ruminococcaceae	0.769
ENSGALG00000002329	CCL1	New.ReferenceOTU100	Unknown genus in order Clostridiales	0.849
ENSGALG00000002329	CCL1	otu_582616	Unknown genus in order Clostridiales	0.849

Table S29: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000002329	CCL1	otu_4402645	Unknown genus in order Clostridiales	0.716
ENSGALG00000002329	CCL1	otu_585880	Unknown genus in order Clostridiales	-0.740
ENSGALG00000002329	CCL1	New.ReferenceOTU89	Unknown genus in order Clostridiales	0.770
ENSGALG00000002329	CCL1	otu_158302	Unknown genus in order Clostridiales	-0.770
ENSGALG00000002329	CCL1	otu_369097	Oscillospira spp.	0.775
ENSGALG00000002329	CCL1	otu_129401	Oscillospira spp.	-0.782
ENSGALG00000002329	CCL1	otu_158321	Unknown genus in order Clostridiales	-0.726
ENSGALG00000002329	CCL1	otu_594206	Unknown genus in family Ruminococcaceae	-0.700
ENSGALG00000003690	KRT17, KRT14	otu_4472195	Unknown genus in family Ruminococcaceae	0.750
ENSGALG00000003690	KRT17, KRT14	otu_4449054	Bacteroides spp.	-0.776
ENSGALG00000004545	WDR72	otu_4402645	Unknown genus in order Clostridiales	-0.673
ENSGALG00000004545	WDR72	otu_328905	Oscillospira spp.	-0.741
ENSGALG00000004545	WDR72	otu_369097	Oscillospira spp.	-0.705
ENSGALG00000004545	WDR72	otu_129401	Oscillospira spp.	0.760
ENSGALG00000004545	WDR72	otu_838685	Oscillospira spp.	-0.701
ENSGALG00000004545	WDR72	otu_594206	Unknown genus in family Ruminococcaceae	0.733
ENSGALG00000004545	WDR72	otu_130763	Unknown genus in order Clostridiales	0.697
ENSGALG00000004545	WDR72	otu_158321	Unknown genus in order Clostridiales	0.758
ENSGALG00000005204	GSTT1L	otu_158321	Unknown genus in order Clostridiales	0.772
ENSGALG00000005204	GSTT1L	otu_129401	Oscillospira spp.	0.756

Table S29: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000005204	GSTT1L	New.ReferenceOTU1	Unknown genus in family Ruminococcaceae	-0.669
ENSGALG00000005204	GSTT1L	otu_369097	Oscillospira spp.	-0.660
ENSGALG00000005739	SCD	otu_129401	Oscillospira spp.	-0.715
ENSGALG00000005739	SCD	otu_158321	Unknown genus in order Clostridiales	-0.718
ENSGALG00000005739	SCD	otu_328905	Oscillospira spp.	0.791
ENSGALG00000005739	SCD	otu_519763	Oscillospira spp.	0.846
ENSGALG00000006202	SCNN1B	otu_328905	Oscillospira spp.	-0.716
ENSGALG00000006202	SCNN1B	otu_129401	Oscillospira spp.	0.746
ENSGALG00000006202	SCNN1B	otu_369097	Oscillospira spp.	-0.841
ENSGALG00000006202	SCNN1B	otu_158321	Unknown genus in order Clostridiales	0.845
ENSGALG00000006202	SCNN1B	otu_359872	Bilophila spp.	0.711
ENSGALG00000006202	SCNN1B	otu_594206	Unknown genus in family Ruminococcaceae	0.767
ENSGALG00000006270	SCNN1G	otu_129692	[Ruminococcus] spp.	0.674
ENSGALG00000006270	SCNN1G	otu_158321	Unknown genus in order Clostridiales	0.860
ENSGALG00000006270	SCNN1G	otu_4402645	Unknown genus in order Clostridiales	-0.693
ENSGALG00000006270	SCNN1G	otu_369097	Oscillospira spp.	-0.880
ENSGALG00000006270	SCNN1G	otu_328905	Oscillospira spp.	-0.748
ENSGALG00000006270	SCNN1G	otu_129401	Oscillospira spp.	0.734
ENSGALG00000006270	SCNN1G	otu_838685	Oscillospira spp.	-0.735
ENSGALG00000006290	NA	New.ReferenceOTU1	Unknown genus in family Ruminococcaceae	0.705

Table S29: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000006290	NA	otu_130773	Unknown genus in order Clostridiales	0.691
ENSGALG00000007171	CIITA	otu_129401	Oscillospira spp.	-0.788
ENSGALG00000007171	CIITA	otu_561607	Unknown genus in family Ruminococcaceae	-0.696
ENSGALG00000007171	CIITA	otu_369097	Oscillospira spp.	0.780
ENSGALG00000007171	CIITA	New.ReferenceOTU89	Unknown genus in order Clostridiales	0.717
ENSGALG00000007171	CIITA	otu_4472195	Unknown genus in family Ruminococcaceae	0.721
ENSGALG00000007171	CIITA	otu_594206	Unknown genus in family Ruminococcaceae	-0.695
ENSGALG00000007171	CIITA	otu_4402645	Unknown genus in order Clostridiales	0.682
ENSGALG00000009903	IFNG	otu_582616	Unknown genus in order Clostridiales	0.769
ENSGALG00000009903	IFNG	otu_4402645	Unknown genus in order Clostridiales	0.689
ENSGALG00000009903	IFNG	otu_4472195	Unknown genus in family Ruminococcaceae	0.749
ENSGALG00000009903	IFNG	otu_369097	Oscillospira spp.	0.795
ENSGALG00000009903	IFNG	New.ReferenceOTU89	Unknown genus in order Clostridiales	0.790
ENSGALG00000010949	GPNMB	otu_4472195	Unknown genus in family Ruminococcaceae	0.710
ENSGALG00000010949	GPNMB	otu_158321	Unknown genus in order Clostridiales	-0.748
ENSGALG00000010949	GPNMB	New.ReferenceOTU100	Unknown genus in order Clostridiales	0.741
ENSGALG00000011859	GBE	otu_4472195	Unknown genus in family Ruminococcaceae	0.784
ENSGALG00000011859	GBE	otu_369097	Oscillospira spp.	0.736
ENSGALG00000011859	GBE	otu_129401	Oscillospira spp.	-0.675
ENSGALG00000011859	GBE	otu_4449054	Bacteroides spp.	-0.698

Table S29: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000013097	SLC15A5	otu_158183	Oscillospira spp.	-0.675
ENSGALG00000013097	SLC15A5	otu_129401	Oscillospira spp.	0.748
ENSGALG00000013097	SLC15A5	New.ReferenceOTU1	Unknown genus in family Ruminococcaceae	-0.701
ENSGALG00000013546	GZMK	otu_4472195	Unknown genus in family Ruminococcaceae	0.656
ENSGALG00000013546	GZMK	otu_2415201	Unknown genus in family Lachnospiraceae	0.722
ENSGALG00000013546	GZMK	otu_369097	Oscillospira spp.	0.765
ENSGALG00000013546	GZMK	otu_129401	Oscillospira spp.	-0.705
ENSGALG00000013548	GZMA	otu_2415201	Unknown genus in family Lachnospiraceae	0.705
ENSGALG00000013548	GZMA	otu_369097	Oscillospira spp.	0.758
ENSGALG00000013548	GZMA	otu_582616	Unknown genus in order Clostridiales	0.752
ENSGALG00000013548	GZMA	otu_158321	Unknown genus in order Clostridiales	-0.712
ENSGALG00000013548	GZMA	otu_129401	Oscillospira spp.	-0.725
ENSGALG00000015141	GDA	otu_191273	[Ruminococcus] spp.	-0.813
ENSGALG00000015141	GDA	otu_326936	Blautia spp.	-0.754
ENSGALG00000016556	GVINP1	otu_158321	Unknown genus in order Clostridiales	-0.727
ENSGALG00000016556	GVINP1	otu_582616	Unknown genus in order Clostridiales	0.739
ENSGALG00000016556	GVINP1	New.ReferenceOTU89	Unknown genus in order Clostridiales	0.772
ENSGALG00000016556	GVINP1	otu_129401	Oscillospira spp.	-0.749
ENSGALG00000016556	GVINP1	otu_4472195	Unknown genus in family Ruminococcaceae	0.765
ENSGALG00000016556	GVINP1	otu_594206	Unknown genus in family Ruminococcaceae	-0.744

Table S29: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000016556	GVINP1	otu_369097	Oscillospira spp.	0.798
ENSGALG00000016556	GVINP1	otu_544859	Unknown genus in family Ruminococcaceae	0.735
ENSGALG00000016556	GVINP1	otu_838685	Oscillospira spp.	0.743
ENSGALG00000019719	KRT19	otu_369097	Oscillospira spp.	0.696
ENSGALG00000019719	KRT19	otu_838685	Oscillospira spp.	0.718
ENSGALG00000019719	KRT19	otu_129401	Oscillospira spp.	-0.723
ENSGALG00000019719	KRT19	otu_328905	Oscillospira spp.	0.684
ENSGALG00000019719	KRT19	otu_4402645	Unknown genus in order Clostridiales	0.717
ENSGALG00000026727	SLC30A10	otu_158183	Oscillospira spp.	-0.731
ENSGALG00000026727	SLC30A10	otu_4402645	Unknown genus in order Clostridiales	-0.815
ENSGALG00000027247	EOMES	otu_4402645	Unknown genus in order Clostridiales	0.673
ENSGALG00000027247	EOMES	otu_129401	Oscillospira spp.	-0.752
ENSGALG00000027247	EOMES	otu_4472195	Unknown genus in family Ruminococcaceae	0.691
ENSGALG00000027247	EOMES	New.ReferenceOTU1	Unknown genus in family Ruminococcaceae	0.663
ENSGALG00000027247	EOMES	otu_369097	Oscillospira spp.	0.810
ENSGALG00000027247	EOMES	otu_158321	Unknown genus in order Clostridiales	-0.730
ENSGALG00000028016	HSPA12B	otu_129401	Oscillospira spp.	-0.686
ENSGALG00000028016	HSPA12B	otu_4472195	Unknown genus in family Ruminococcaceae	0.744
ENSGALG00000028016	HSPA12B	otu_369097	Oscillospira spp.	0.794
ENSGALG00000028016	HSPA12B	otu_158321	Unknown genus in order Clostridiales	-0.759

Table S29: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000028376	FGF19	otu_582616	Unknown genus in order Clostridiales	0.737
ENSGALG00000032614	LOC107049017	New.ReferenceOTU1	Unknown genus in family Ruminococcaceae	0.744
ENSGALG00000032614	LOC107049017	otu_157693	Unknown genus in order Clostridiales	-0.725
ENSGALG00000032614	LOC107049017	otu_164242	Unknown genus in family Ruminococcaceae	0.813
ENSGALG00000032614	LOC107049017	otu_561607	Unknown genus in family Ruminococcaceae	-0.737
ENSGALG00000032614	LOC107049017	otu_362793	Oscillospira spp.	-0.757
ENSGALG00000032614	LOC107049017	otu_4402645	Unknown genus in order Clostridiales	0.820
ENSGALG00000032614	LOC107049017	otu_183932	Unknown genus in family Ruminococcaceae	-0.817
ENSGALG00000032717	CCL4	otu_129401	Oscillospira spp.	-0.802
ENSGALG00000032717	CCL4	otu_369097	Oscillospira spp.	0.798
ENSGALG00000032717	CCL4	otu_533847	Oscillospira spp.	0.797
ENSGALG00000032717	CCL4	otu_510295	Unknown genus in family Ruminococcaceae	0.746
ENSGALG00000032717	CCL4	otu_582616	Unknown genus in order Clostridiales	0.794
ENSGALG00000032717	CCL4	New.ReferenceOTU89	Unknown genus in order Clostridiales	0.687
ENSGALG00000038096	NOS2	otu_369097	Oscillospira spp.	0.735
ENSGALG00000038096	NOS2	otu_4472195	Unknown genus in family Ruminococcaceae	0.704
ENSGALG00000038096	NOS2	otu_4447072	Bacteroides uniformis	0.742
ENSGALG00000038918	NA	otu_510295	Unknown genus in family Ruminococcaceae	0.730
ENSGALG00000038918	NA	otu_129401	Oscillospira spp.	-0.743
ENSGALG00000038918	NA	New.ReferenceOTU1	Unknown genus in family Ruminococcaceae	0.751

Table S29: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000039536	C2H8ORF22	otu_369097	Oscillospira spp.	-0.711
ENSGALG00000039536	C2H8ORF22	otu_129401	Oscillospira spp.	0.784
ENSGALG00000039536	C2H8ORF22	otu_328905	Oscillospira spp.	-0.806
ENSGALG00000039876	EDQM2	otu_368338	Unknown genus in family Lachnospiraceae	0.857
ENSGALG00000040557	TFEC	New.ReferenceOTU89	Unknown genus in order Clostridiales	0.660
ENSGALG00000040557	TFEC	otu_582616	Unknown genus in order Clostridiales	0.732
ENSGALG00000040557	TFEC	otu_369097	Oscillospira spp.	0.854
ENSGALG00000040557	TFEC	otu_158321	Unknown genus in order Clostridiales	-0.720
ENSGALG00000041025	FOXJ1	otu_328905	Oscillospira spp.	0.704
ENSGALG00000042227	GNLY	otu_594206	Unknown genus in family Ruminococcaceae	-0.673
ENSGALG00000042227	GNLY	otu_585880	Unknown genus in order Clostridiales	-0.655
ENSGALG00000042227	GNLY	otu_4402645	Unknown genus in order Clostridiales	0.766
ENSGALG00000042227	GNLY	otu_4472195	Unknown genus in family Ruminococcaceae	0.715
ENSGALG00000042227	GNLY	otu_158321	Unknown genus in order Clostridiales	-0.670
ENSGALG00000042227	GNLY	otu_158302	Unknown genus in order Clostridiales	-0.700
ENSGALG00000042227	GNLY	otu_369097	Oscillospira spp.	0.779
ENSGALG00000042227	GNLY	otu_129401	Oscillospira spp.	-0.703
ENSGALG00000046875	NA	New.ReferenceOTU7	Unknown genus in order Clostridiales	-0.824
ENSGALG00000047199	NA	New.ReferenceOTU9	Unknown genus in family Ruminococcaceae	0.712
ENSGALG00000047199	NA	otu_129401	Oscillospira spp.	0.758

Table S29: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000047199	NA	otu_4472195	Unknown genus in family Ruminococcaceae	-0.718
ENSGALG00000047199	NA	otu_328905	Oscillospira spp.	-0.798
ENSGALG00000047199	NA	otu_158321	Unknown genus in order Clostridiales	0.771
ENSGALG00000047199	NA	otu_369097	Oscillospira spp.	-0.859
ENSGALG00000048617	NA	otu_4472195	Unknown genus in family Ruminococcaceae	0.792
ENSGALG00000048617	NA	otu_369097	Oscillospira spp.	0.689
ENSGALG00000049947	NA	otu_197072	Bacteroides uniformis	-0.722
ENSGALG00000049947	NA	New.ReferenceOTU1	Unknown genus in family Ruminococcaceae	-0.729
ENSGALG00000050240	NA	otu_4472195	Unknown genus in family Ruminococcaceae	0.729
ENSGALG00000050240	NA	otu_582616	Unknown genus in order Clostridiales	0.842
ENSGALG00000050240	NA	otu_129401	Oscillospira spp.	-0.716
ENSGALG00000050240	NA	New.ReferenceOTU89	Unknown genus in order Clostridiales	0.765
ENSGALG00000050240	NA	otu_369097	Oscillospira spp.	0.807
ENSGALG00000050240	NA	otu_4402645	Unknown genus in order Clostridiales	0.765
ENSGALG00000050240	NA	New.ReferenceOTU100	Unknown genus in order Clostridiales	0.775
ENSGALG00000051726	NA	otu_129401	Oscillospira spp.	-0.815
ENSGALG00000051726	NA	otu_158321	Unknown genus in order Clostridiales	-0.694
ENSGALG00000051726	NA	otu_4472195	Unknown genus in family Ruminococcaceae	0.734
ENSGALG00000051726	NA	otu_369097	Oscillospira spp.	0.840
ENSGALG00000051726	NA	otu_328905	Oscillospira spp.	0.731

Table S29: (continued):

ENSEMBL ID	Gene Name	OTU_ID	Taxa	Correlation coefficient
ENSGALG00000052425	NA	otu_328905	Oscillospira spp.	0.681
ENSGALG00000052425	NA	otu_4476780	Unknown genus in family Rikenellaceae	0.741
ENSGALG00000052425	NA	otu_369097	Oscillospira spp.	0.792
ENSGALG00000052425	NA	otu_4472195	Unknown genus in family Ruminococcaceae	0.746
ENSGALG00000052425	NA	otu_130763	Unknown genus in order Clostridiales	-0.700
ENSGALG00000052425	NA	otu_129692	[Ruminococcus] spp.	-0.711
ENSGALG00000053266	NA	otu_306633	Unknown genus in family Ruminococcaceae	-0.733
ENSGALG00000053568	NA	otu_130763	Unknown genus in order Clostridiales	-0.720
ENSGALG00000053568	NA	otu_369097	Oscillospira spp.	0.760
ENSGALG00000053568	NA	otu_594206	Unknown genus in family Ruminococcaceae	-0.765
ENSGALG00000053568	NA	otu_4472195	Unknown genus in family Ruminococcaceae	0.735
ENSGALG00000053568	NA	otu_158321	Unknown genus in order Clostridiales	-0.764
ENSGALG00000053568	NA	New.ReferenceOTU1	Unknown genus in family Ruminococcaceae	0.655
ENSGALG00000053568	NA	otu_592913	Unknown genus in order Clostridiales	-0.721
ENSGALG00000053568	NA	otu_328905	Oscillospira spp.	0.685
ENSGALG00000055025	NA	otu_4449054	Bacteroides spp.	0.750
ENSGALG00000055025	NA	otu_4460021	Ruminococcus spp.	0.737