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Executive Summary

Improving growth performance in chickens has long been one of the most important goals in poultry research. Previous Poultry CRC projects demonstrated that there was considerable bird-to-bird variation in performance and that particular gut microbes and intestinal gene expression patterns were linked to birds with high or low apparent metabolisable energy (AME). Further CRC projects identified a number of specific gut microbes that were indicative of high or low-performing birds, and demonstrated the important role of gene expression in the small intestine in affecting performance of meat chickens. Despite all the improvements in genetics, nutrition, disease control and husbandry over many decades, performance within and between flocks varies considerably and this variation causes significant losses to the industry both during production and processing.

This sub-project brought together the collective knowledge and skills of many researchers in six institutions (five in Australia and one in the USA) to undertake excellent quality research to deliver significant benefits to the Australian chicken meat industry. The research team utilised recent technological advances in this area of science to comprehensively characterise the gut environment which is a critical factor in determining productivity of meat chickens, and reducing reliance on in-feed antibiotics.

The main aims of this sub-projects were (1) customise an existing gut bacterial microarray chip for usage in Australia, (2) advance our understanding of gut microbes and host gene expression patterns underpinning bird performance, (3) identify feeding regimes (including probiotics) that can consistently facilitate the establishment of a healthy gut environment, (4) identify organisms with the potential to become new probiotics, and (5) advance our understanding of microbial changes during necrotic enteritis infection and identify dietary additives to reduce the severity of necrotic enteritis.

The first generation gut microarray, referred to as Poultry Intestinal Tract Chip 1 or PITChip 1 originating from The Ohio State University was customised for usage in Australia by addition of DNA probes for caecal bacteria taken from low and high performing chickens in a series of experiments conducted at the University of Adelaide. PITChip2 was then used to evaluate bacterial DNA from further experiments conducted in Australia. PITChip3 was an advancement on earlier versions by inclusion of a wider region of highly conserved DNA to enable more accurate classification of gut bacteria. Differences in gut microbiota revealed by PITChip3 point to interactions between the gut microbiota and diet, and subsequent effects on growth performance and NE resistance seen in chickens fed with either corn- and wheat-based diets.

The most technologically advanced methods available for microbiota analysis have been implemented and applied within this project, putting this poultry capability at the cutting edge within this area of research. During the life of this sub-project, DNA sequencing technologies advanced at such a rapid rate that it now possible to analyse hundreds of gut samples by 16S mRNA sequencing for less than AUD$8 per sample. Furthermore, the current technologies identify all the significant bacterial populations present in the samples, which make these technologies the preferred choice of most if not all researchers conducting the latest studies on gut microbial ecology.

A key starting point for this project was to determine the reproducibility of previous results that had indicated correlations between microbiota composition and growth performance. Therefore, a series of three experiments were conducted on different batches of chickens all fed the same commercial diet and grown in the same facility under identical physical conditions. Growth rate and feed conversion of the chickens were comparable to the breeder's expectations, however, within each trial there were differences in the performance parameters of individual birds and so relatively high and low performance birds could be identified. Analyses of the same bacterial DNA samples by PITChip2 and Roche pyrosequencing demonstrated (1) that there was wide variation in background gut microbiota between different batches of birds, (2) there were no specific gut microbes that were consistently associated with good or poor performance in three separate experiments, and (3) numerous consortia of gut microbes can support high performance in meat chickens. A further series of three experiments utilising different antibiotics and cereal grains further demonstrated that numerous consortia of gut microbes can support high performance in meat chickens. This finding of great variability in the microbiota of different batches of high performance birds was new and somewhat unexpected. It has very important implications for the direction of future research to understand the basis of gut health and is of fundamental importance in helping to understand the way in which products aimed at manipulating gut health (e.g., phytogenics, prebiotics, and probiotics) can be identified, developed and implemented. It is clear that they must be effective on and within a wide variety of different gut environments or must be effectively tailored for use under defined circumstances.

Gene expression differences observed in the duodenum and other intestinal sections were not directly linked to differences in performance and hence did not provide useful tools to monitor the effects of dietary manipulation for productivity gain or identify host genes for marker assisted breeding for improved performance. We concluded that differences in gene expression seen in and across batches of birds may be due to influences other than performance, for example, slight variations in environmental conditions, differences in colonising gut microflora, breeder flock age and health status, and cleanliness of breeder farms and hatchery. Relatively early in the life of the

project the decision was made to discontinue work on gene expression in the gut as it was not clear that there would be any downstream implementation of the knowledge that would be of direct benefit to the industry.

The *in vitro* methods to identify potentially probiotic strains of bacteria that have been used by others and reported in the scientific literature were recognised as being largely unproven and having limitations. Therefore we initiated work to develop new *in vivo* based methods to identify probiotic strains. The resulting gut bacterial isolates from healthy chickens have potential for development into commercial probiotic products, not only for application in Australia, but also as exportable commodities. *In ovo* application of these pure cultures should be explored, along with administration of probiotics via other routes and at other times during the life of the flock. For example, encapsulated probiotics could be provided via drinking water to offset gut dysbiosis and performance losses arising from clinical and subclinical disease challenges, and other stresses incurred on flocks such as through mechanical breakdowns or by temporary feed withdrawal during partial harvesting of the flock.

The necrotic enteritis challenge model to test alternatives to in-feed antibiotics was simplified to usage of *Eimeria* as a predisposing factor without need to feed a high level of fishmeal prior to infection with *C. perfringens* in chickens fed an industry standard diet. Some feed additives based on commercial enzyme products and organic acids had ameliorating effects on gut damage in birds challenged with *C. perfringens*. Understanding of the mechanisms underlying the improvement will be beneficial to the application of these additives as alternatives to in-feed antibiotics in the poultry industry. Results from other studies in this sub-project suggest how the utilisation of the NE challenge model would be enhanced if gut samples were to be taken for microbiome analysis to establish the pre-existing gut ecology prior to administration of *Eimeria* and *C. perfringens*.

Finally, the impact of this sub-project on our understanding of importance of the role of a healthy gut environment is demonstrated by publication of results in 14 papers in high impact scientific journals, 7 presentations at national and international conferences, and one PhD dissertation to date, with more publications to follow. Two currently enrolled Poultry CRC-supported PhD students at the University of Adelaide have utilised tissue samples and data from this sub-project. The wider scientific community has recognised the value of this work through over 200 citations in scientific journals, with this number expected to increase substantially.

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Introduction

Background

Improving growth performance in chickens has long been one of the most important goals in poultry research. Despite all the improvements, performance within and between flocks varies considerably and this variation causes significant losses to the industry.

Birds use large amounts of energy compared to other animals (Clench and Mathias 1995) and their digestive system is adapted to extract energy from difficult to digest food sources. Intensive selection over the last six or seven decades has resulted in chickens that very efficiently convert food into body mass, making them the most efficient agricultural animal in terms of the input required to produce high quality meat protein. Energy and nutrient extraction from feed requires interplay between the biochemical functions provided by the chicken and the microbiota present within the gastrointestinal tract. Highly productive chickens have been developed by selection for elite genetic traits; it is possible that in the future gains in productivity and health outcomes will be dependent on consistent establishment of elite microbiota in the gastrointestinal tract (GIT). Hence developing a clear understanding of the role of the GIT microbiota in both productivity and health is paramount.

Bird-to-bird variability in performance and apparent metabolisable energy (AME) has been reported previously in birds fed an identical diet (Hughes and Choct 1997). Recently, CRC-funded projects (including 05-2, 06-25 and 09-20) have attempted to understand key host and microbial factors underlying variable bird performance and associated with either high or low-performing birds. Preliminary investigations have identified a series of genes and microbes potentially associated with bird performance (see summaries of previous CRC Projects); however, the variable nature of animal physiology dictates that to establish a solid link between host and microbial factors and bird performance, more comprehensive and repeatable studies must be performed and validated. Identification and subsequent manipulation and harnessing of these factors driving high performance will lead to improvements in flock efficiency and uniformity; thus providing significant economic and environmental benefits to the Australian poultry industry.

It is clear that in order to develop a greater understanding of the factors linked to bird performance, a more comprehensive picture of the intestinal environment and host-microbial interactions is required.

The intestinal environment

The gut microbiota

The intestinal microbiota of the broiler chicken contains over 900 different bacterial species (Apajalahti *et al*. 2004; Wei *et a*l. 2013), and benefits the host by facilitating gut and immune development (Umesaki *et al*. 1999; Lu *et al*. 2003), preventing the colonisation of harmful bacteria by competitive exclusion (Nurmi *et al*. 1992) and the production of antimicrobial compounds (Brisbin *et al.* 2008), increasing energy availability via the fermentation of indigestible carbohydrates (Jozefiak *et al*. 2004), and improving the availability and uptake of vitamins and minerals (Patterson and Burkholder 2003). The microbiota utilise a significant proportion of the nutrients derived from the feed (Apajalahti *et al*. 2004); therefore a healthy microbial balance must be maintained in order to ensure that host benefits outweigh this loss of nutrients. The maintenance of an optimal microbiota is important for sustained bird health; however, unfavourable microbial shifts can occur, which lead to infectious diseases and impaired performance.

In recent years, the capacity for researchers to study the microbiota has increased enormously. Initially, microbial analysis was restricted to organisms that could be grown on artificial media; however molecular techniques targeted at 16S rRNA genes including terminal-restriction fragment length polymorphism and denaturing gradient gel electrophoresis facilitated characterisation of the overall microbiota and the identification of unculturable organisms. More recently, the use of next-generation sequencing technologies, in particular the Roche/454 FLX Genome Sequencer, has allowed the rapid and quantitative analysis of hundreds of thousands of individual bacteria; providing a comprehensive picture of the microbiota at a depth not previously manageable.

Development and temporal fluctuations in microbiota of the gastrointestinal tract of chickens

Colonisation of the gastrointestinal tract is thought to start immediately after hatching and therefore the hatching environment has a major influence on a chicken's microbial profile. Large differences in microbiota profiles have been noted between groups of birds from replicate trials and high individual bird to bird variation within groups has also been reported in chickens (Stanley *et al.* 2013b) and turkeys (Scupham 2009). This kind of variation has also been found in the GIT microbiota of humans and other animals (Zoetendal *et al.* 1998; Simpson *et al.* 2000) and has been attributed to both host and environmental factors.

Amongst the animal production systems, poultry are somewhat unusual in that the young are generally separated from the parents and hence there is a markedly reduced parental influence on the development of microbiota. Within commercial hatcheries, hygiene measures reduce the bacterial load in the hatching environment to limit the spread of bacterial pathogens. A consequence of this is that newly hatched chicks are exposed to a diverse range of bacteria from

environmental sources such as human handlers, bedding material, feed and transport boxes, rather than from parental sources. Widely varying colonisation of the chicken GIT may be a consequence of the high diversity in non-avian bacterial sources and lack of parental bacteria during the first hours and days of life (Fuller 1989; Stanley *et al.* 2013b). This situation is somewhat analogous to the finding that human infants delivered in hospitals can harbour abhorrent microbiota rather than human specialised microbiota (Fryklund *et al.* 1992) and infants delivered by caesarean section have been shown to have different microbiota compared to naturally delivered infants (Dominguez-Bello *et al.* 2010). However, a key difference with chickens is that the eggs are physically separated from hens on breeder farms and hence once eggs have been washed or fumigated prior to hatching, there is no contact with adults during incubation.

Pathogens within the microbiota

The presence of zoonotic bacterial pathogens within chicken microbiota is an important issue for both chicken and human health. Pathogens such as *C. jejuni* and *S. enterica* are leading causes of food poisoning in humans and a significant proportion of human infections can be traced back to the consumption of improperly prepared chicken meat or contaminated eggs. The incidence of these pathogens and others such as *E. coli* and *C. perfringens* is an important aspect of microbiota studies. For decades many poultry producers have added low level antibiotics to feed to control pathogens such as *C. perfringens* and promote increased growth efficiency in birds and so the response of the GIT microbiota to antibiotics has been well studied. The antibiotic effect on microbiota appears to be dose and age dependant (Zhou et al. 2007), however, individual communities respond differently. They appear to reduce *Lactobacillus* and promote *Clostridia* while reducing overall diversity (Lu et al. 2008). The community usually recovers rapidly after removal of antibiotics; however some taxa did not recover within 6 months (Dethlefsen et al. 2008). The increasing occurrence of antibiotic resistance in human pathogens has raised concerns about the wisdom of routinely using antibiotics in the feed of intensively reared animals and has led to the total ban of antibiotic growth promoters in the European Union (Dibner and Richards 2005). This has prompted a number of research projects looking into ways of developing beneficial microbiota in chickens that will provide protection from pathogens. Microbiota communities resist change in composition (Dethlefsen et al. 2008); its members can actively compete by the production of antibacterial molecules or passively compete by occupying a niche and excluding other bacteria.

Necrotic enteritis

Necrotic enteritis, caused by *Clostridium perfringens*, is one of the world's most prominent and severe poultry diseases (Van Immerseel *et al.* 2004; Cooper and Songer 2009). The economic impact of NE on the world-wide poultry industry is estimated at over US$2 billion per annum (Choct and Kocher 2008). In many countries, NE is controlled by the in-feed supplementation of antibiotics. The European Union has enforced a ban on the use of in-feed antibiotics (Timbermont

et al. 2009), and consumer pressure in other regions may force similar restrictions on antibiotic use (Stringfellow *et al.* 2009). Therefore, alternative strategies for the control of NE are needed which can limit the health and economic impact of the disease.

It has been observed that *Clostridium perfringens* types A and C can cause NE with type A predominating (Wages and Opengart 2003). Whilst other factors such as coccidiosis (caused by Eimeria infection), temperature and diet also contribute to the outbreak of this disease (Dahiya *et al*. 2006). α-toxin was considered as the key virulence factor produced by *C. perfringens* (van Immerseel *et al*. 2009), but recent studies suggested that a novel toxin, NetB, plays a major role in NE pathogenesis (Cooper *et al*. 2010, Keyburn *et al*. 2008).

The role of gut microbial balance in poultry health and production has been studied extensively (Apajalahti and Kettunen 2006, Choct 2009). Reports on the dynamics of microbial responses to NE outbreak are sporadic, and focussed predominantly on *Clostridium perfringens* or a few selected bacterial groups (Feng *et al.* 2010, McReynolds *et al.* 2007, Mikkelsen *et al.* 2009). For example, Feng *et al.* (2010) indicated that *C. perfringens* infection suppressed *Lactobacillus aviarius* but not *Lactobacillus salivarius*. To the best of our knowledge, a thorough survey has not been conducted on whole microbial community changes in the gut of the birds subjected to NE challenge.

As a result of the removal of in-feed antibiotics in Europe, and consumer pressure worldwide, potential alternatives to antibiotics have been investigated (Huyghebaert *et al.* 2010, Yang *et al.* 2009, Yegani and Korver 2010). These include prebiotics, probiotics, enzymes, organic acids, antibodies and phytobiotics. Exogenous enzymes (Choct 2006, Jia *et al.* 2009, Liu *et al.* 2010, McReynolds *et al.* 2007), mannan-oligosaccharide (Bio-Mos) (Nollet *et al.* 2007), and probiotics (McReynolds *et al.* 2009) can improve bird growth performance and mitigate the negative effects of *C. perfringens* or coccidial challenge to a certain extent. Nevertheless, none of the tested feed additives can completely or consistently substitute for in-feed antibiotics (Huyghebaert *et al.* 2010), and their beneficial effects vary in different experiments (Yang *et al.* 2009).

Beneficial microbes and probiotics

Certain microbes have been identified to be more beneficial to the host due to properties which include pathogen exclusion and immune modulation (Patterson and Burkholder 2003). Some species, typically of the *Lactobacillus* genera, have been identified as being particularly beneficial to the host (including avian and human). These bacteria have often been isolated and probiotic products developed in order to increase the relative concentrations of these strains within the microbiota. The majority of probiotics developed for poultry have been targeted at the reduction of

organisms related to poultry diseases (Hofacre *et al.* 1998; Lee *et al.* 2007), or food-related pathogens (Noujaim *et al.* 2008; Willis and Reid 2008). Recently the use of probiotics in poultry has gained increased attention as a natural growth promoter (Khan *et al.* 2007; Apata 2008; Mountzouris *et al.* 2010). Probiotic-mediated improvements in bird performance (Khan *et al*. 2007) and immunity (Haghighi *et al.* 2006) have been documented previously; however, the reported effects of probiotics are inconsistent, with some strains producing beneficial results (Mountzouris *et al.* 2010), and others having no observable effect on performance (Li *et al.* 2008; Lee *et al.* 2010).

Major factors likely to be driving these variable effects include the strains of probiotics used, the composition and structure of the microbiota in these birds, and a lack of understanding of the complex interactions among microbes within the gut environment; in addition to non-microbial factors including management and diet. It is clear that the optimal probiotic strains or combinations thereof, have yet to be identified. It has also yet to be proven that specific microbial products can consistently facilitate the establishment of a healthy microbiota and support a high level of performance across multiple flocks and in different environments. A more rational approach to the development of probiotics that can consistently improve bird performance is to first understand the indigenous gut microbiota and the interactions of probiotics with the indigenous gut microbiota and host. High-performing birds may serve as a source of probiotics. Recent advances in microbial profiling techniques allow comprehensive microbial analysis at a depth not previously possible.

Microbes linked to energy metabolism

Previously, specific microbial profiles have been associated with body weight gain in mice (Turnbaugh *et al.* 2006; Turnbaugh *et al.* 2008); a finding believed to be due to variable efficiency of energy harvest among microbes, specifically *Bacteroidetes* and *Firmicutes*. In terms of poultry, shifts in microbial profiles in the intestine of broiler chickens have been linked to bird AME (Torok *et al.* 2008). Furthermore, a suite of core bacteria consistently linked with broiler performance (FCR) across numerous Australian feeding trials have been identified (Torok; CRC 06-25). This demonstrates that the presence/absence of specific bacterial groups may be important to performance despite the inevitable overall shifts occurring in gut microbiota, which are usually associated with various dietary compositions, environmental conditions and trials. More recently, Moore and colleagues (2010; unpublished results from CRC 09-20) have used next-generation sequencing technology to profile the microbiota and again identified microbial shifts associated with high and low AME birds.

Host intestinal gene expression

The structure and function of the gut is directed by the genes that are expressed. During embryogenesis the cellular differentiation and formation of the various gut tissues is directed by changing patterns of gene expression. In the mature gut, a wide variety of genes must be

expressed to provide the metabolic and catabolic functions of the gut as well as the constant renewal of gut tissue. Analysis of differences in gene expression may indicate aspects of gut biology which vary between different groups of birds and may provide some explanation for the differences observed. Gene expression can be characterised in chickens using microarray technology. The availability of the whole chicken genome (ICGSC 2004) has facilitated the construction of chicken microarrays containing probes for virtually all genes, thus allowing in-depth characterisation of host gene expression.

In preliminary trials from previous CRC-funded projects (05-2 and 09-20) we have identified a number of genes associated with high and low AME birds. Some of the genes identified were involved in pathways such as metabolism, nutrient and ion transport, growth, gut health and gut hormone action. More comprehensive studies are now required to identify which genes are consistently up- and down-regulated in high and low AME birds, and to investigate links between gene expression and other key performance parameters such as FCR and body weight gain.

Summary

It is clear that a healthy gut environment is required for optimal bird performance, and that host-microbial interactions are key determinants of gut health and feed efficiency. A better understanding of host intestinal gene expression and microbial communities will allow simultaneous assessment of the key gut factors driving bird performance. A healthy gut microbiota is a critical component of a healthy, high-performing bird. A better understanding of which specific microbes are linked to high-performing birds is a crucial step towards identification of important host-microbial interactions in the chicken; this may lead to the development of a probiotic product that could improve flock performance and health status. In order to obtain a comprehensive understanding of gut microbial communities, the most up-to-date technologies are required in order to study the microbiota at a depth not previously possible. Critically, an evaluation of the capacity for targeted microbial manipulations, by using probiotics and feed additives to consistently produce a healthy microbiota, is needed in order to confidently assess the potential for the commercialisation of new probiotics.

Objectives

In accordance with the three selection criteria set by the Commonwealth for establishment of a new CRC, this sub-project brought together the collective knowledge and skills of many researchers in six institutions (five in Australia and one in the USA) to undertake excellent quality research to deliver significant benefits to Australia.

The aims of this ambitious sub-project were:-

- To develop an accurate, rapid and inexpensive technology, in the form of a gut bacterial microarray chip, to assess broiler gut microbial communities in Australian conditions.
- To identify key gut factors driving variable bird performance.
- To assess the flock variability in microbial community composition and structure and host gene expression patterns.
- To evaluate the reproducibility and reliability of the impact of feed additives on the gut environment, encompassing microbial communities and intestinal gene expression.
- To improve bird performance and uniformity of performance via targeted microbial manipulations.
- To identify microbes with potential to be developed as new probiotics, targeted at improving performance.
- To study the microbial changes that occur during NE challenge and understand how different feed additives influence the microbiota of NE challenged birds.

The main outcomes achieved by this sub-project were:-

- Development and validation of a gut bacterial microarray, designed to detect microbes present in Australian conditions
- An understanding of key microbes and host genes that are consistently linked to high and low-performing birds.
- Knowledge of how reliably and reproducibly specific feeding regimes and feed additives can influence the gut microbiota and gene expression patterns.
- Identification of microbes with potential to be developed and commercialised as new probiotics, identified under Australian conditions, targeted at improving performance.
- Understanding of the microbial changes that occur during NE infection.
- Significant support for several Poultry CRC-funded higher degree students.

Methodology

This sub-project brought together the successful research team that has worked previously on CRC project 05-2: *An integrated approach to understanding gut function and gut health in chickens*. Members of the current collaborative team have also worked together successfully on CRC Projects 06-18, 09-20, 09-21 and 09-22. The additional expertise of Prof Zhongtang Yu from The Ohio State University, and Dr Shubiao Wu from the University of New England further strengthened the credentials of this collaborative research team.

Whilst it is clearly evident that there is substantial variation in broiler performance within a flock, few studies have attempted to understand the key microbial and gut factors underlying such variation. Recent technological advances in the study of the gut microbiota and host gene expression provide us with an ideal opportunity to identify the main factors responsible for driving bird performance and will allow us to develop feeding regimens and robust probiotics to promote and maintain optimal bird performance in the absence of in-feed antibiotics.

This proposal adopted the novel approach of studying individual birds to identify microbial and host gene expression factors linked to high and low performing birds in order to identify targets for microbial manipulations to facilitate the establishment of a gut environment to ensure consistent high levels of performance.

Furthermore, we undertook a series of in-depth studies to understand the nature of the microbial shifts that occur during the development of necrotic enteritis in meat chickens and the impact that classes of feed additives may on the microbiota of birds infected with *Clostridium perfringens*, the causative agent of necrotic enteritis.

During the course of this sub-project we utilised the latest, most advanced technologies required for high throughput microbial DNA profiling of chicken gut contents, two separate platforms to assess host gene expression in gut tissue, and super computer based bioinformatics programs to analyse many terabytes of data obtained during these studies.

Finally, the effectiveness of this strong collaboration is demonstrated by 15 high quality scientific manuscripts (Appendix 1) published in high impact journals. These publications have been cited in several hundreds of publications by other researchers. Further publications will continue to result long after this sub-project is finalised.

Chapter 1. Identification of microbes and gene expression patterns associated with high-performing birds

Extracted from an original article by Stanley et al. (2016)

Introduction

Previous Poultry CRC projects 03-3, 05-2, 09-19 and 09-20 identified that there is considerable bird-to-bird variation in performance and that particular gut microbes and gene expression patterns are linked to birds with high or low apparent metabolisable energy, and feed conversion ratio.

Whilst it is clearly evident that there is substantial variation in broiler performance within a flock, few studies have attempted to understand the key microbial and gut factors underlying such variation. Recent technological advances in the study of the gut microbiota and host gene expression provided us with an ideal opportunity to identify the main factors responsible for driving bird performance with a view to development of feeding regimens and robust probiotics to promote and maintain optimal bird performance in the absence of in-feed antibiotics.

The series of three experiments reported here adopted the novel approach of studying individual birds to identify microbial and host gene expression factors linked to high and low performing birds. The specific aims of these experiments were:-

- To identify key gut factors driving variable bird performance.
- To assess the flock variability in microbial community composition and structure and host gene expression patterns.
- To identify microbes with potential to be developed as new probiotics, targeted at improving performance.

It was considered essential that this experiment be repeated three times in order for accurate identification of microbes consistently associated with high or low-performing birds.

Materials and methods

Animal ethics

The Animal Ethics Committees of the University of Adelaide (Approval No.S-2010-080) and the Department of Primary Industries and Resources, South Australia (Approval No. 08/10) approved this study. All animal work was conducted in accordance with the national and international guidelines for animal welfare.

Animal trials

Three animal trials (each with n=96 birds) were performed following the same procedures over a five month period in 2010/11. Briefly, one-day old male Cobb 500 broiler chickens from the Baiada Hatchery, Willaston, SA, Australia, were transferred to a chick rearing pen in a fully environmentally controlled experimental animal facility. Feed was supplied *ad libitum* throughout the experiment and was comprised of 44.4% wheat, 17% soybean meal, 15% barley, 10% canola meal, 5% peas, 3.2% meat meal, 3% tallow, 1% limestone, 0.5% vitamin mix, and traces of salt, lysine HCl, DL-methionine and threonine. The same batch of commercially prepared starter crumbles (Ridley Agriproducts, Murray Bridge, S.A.) was used in all three trials and was stored under controlled cool and dry conditions for the duration of the trials. All birds within each trial were housed together for the first 13 days of life to ensure microbiota exchange through typical bird behaviour, including copraphagy.

On day 13 chicks were transferred in pairs to 48 metabolism cages in a temperature controlled room (23-25°C). Initial placing in metabolic cages in pairs was done to minimise stress and allow the birds to adjust to cages. At day 15, birds were moved into individual cages. Individual caging allowed the precise assessment of individual feed intake, energy in feed, and unused energy remaining in faeces. The experimental design eliminated competition for feed and reduced behavioural issues affecting feed intake. Single bird caging and individual measurements and sampling were implemented in order to allow direct correlation of microbiota structure and productivity measurements on a bird by bird basis. Birds were euthanased and necropsied on day 25 and caecal contents and cloacal swabs were collected from each bird. Samples from all birds from all three trials were sequenced.

Feed conversion ratio was calculated as a ratio of feed eaten and weight gained. Thus birds with low FCR, that needed less feed per kg gained, were the most efficient in converting feed to mass. Gross energy (GE) was measured in feed and in faeces of each individual bird using a Parr isoperibol bomb calorimeter (Parr Instrument Company, Moline, IL). Apparent metabolisable energy (AME) in MJ/kg dry matter, was calculated as (AME_{diet} = [(GE_{diet} x feed eaten) x ($GE_{excreta}$ x dry excreta)]/feed eaten/dry diet content). Gain rate (GR) was calculated as [weight gain (g) / start weight (g)] and feed eaten (FE) was total amount of feed eaten during the 10 day measurement time period. All of the above measurements were taken from day 15 to day 25, during the time when single birds were housed in metabolic cages.

Microarray sample preparation

Tissue samples from the duodenum, caecum, liver, spleen and pancreas were processed for microarray analysis. Total RNA was isolated from each sample using the Meridian total RNA isolation kit (Cartagen Molecular Systems Inc., San Carlos, CA, USA). The quantity of RNA and

the 260/280 and 260/230 ratios were determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Purified RNA samples were stored at -80°C. RNA that had a 260/280 ratio and 260/230 ratio above 1.8 were used for this study. Total RNA (5 µg) was reverse transcribed into cDNA using oligo dT_{20} primer as per the Invitrogen Superscript III First Strand Synthesis System protocol (Invitrogen Corporation, Carlsbad, CA, USA). Microarray experiments, comprising cDNA labeling, hybridisation and washing, were carried out according to the NimbleGen Arrays User's Guide protocol, without modification. Briefly, cDNA was labeled with Cyanine-3 and individual samples were hybridised on our custom designed chicken NimbleGen12x135K arrays, using the NimbleGen Hybridisation system, for 16 h at 42ºC. The custom designed chicken high-density multiplex microarrays carried a set of 65,849 long oligonucleotides (60-75nt) based on chicken UniGene sequence families. Probes were present on the array in duplicate and each gene was represented by at least two independently designed probes. A NimbleGen MS200 2µm resolution laser scanner was used to capture the fluorescent hybridisation signals. All arrays were scanned individually with parameter adjustment of the scanner gain. Data extraction was performed using NimbleScan Software Version 2.5.26 (Roche NimbleGen, Inc).

Microarray analysis

Microarray analysis was performed in Bioconductor using the oligo package for data import, background correction, and quantile normalisation with the rma algorithm. This package is specially written to accommodate Nimblegen array analysis. Quality control of arrays was done using the Bioconductor arrayQualityMetrics package (Kauffmann 2009) and arrays reported as outliers were removed from further analysis. The normalised matrix of results was imported into Genowiz™ software (Ocimum Biosolutions India Ltd, Hyderabad, India) for graphical visualisation and GO analysis. Genes with t-test p-value<0.05 and were considered to be differentially expressed. Table 1.1 shows the number of arrays performed for each experiment. Microarrays were performed with samples of each of the five studied tissues from birds with the 12 highest and the 12 lowest FCR values. To maximise the difference in FCR between the compared groups for the final analysis only the results from the top and bottom 6 birds were subsequently used. The microarray analysis presented in this report is a preliminary analysis, since the the data from the arrays has only been available for a short period of time; more detailed gene by gene searches and network analysis is currently under way. AME ranked microarray experiments were also done for each tissue with 6 highest vs. 6 lowest ranked birds.

Table 1.1. Number of arrays completed for FCR ranked analysis

	Duodenum	Cecum	Liver	Spleen	Pancreas
Trial 1	12H vs 12L	12H vs 12L	12H vs 12L	12H vs 12L	12H vs 12L
Trial 2	12H vs 12L	6H vs 6L	6H vs 6L	6H vs 6L	6H vs 6L
Trial 3	6H vs 6L				

Total=204 arrays; H and L refer to individual birds with high and low FCR values.

DNA preparation, PCR amplification of 16S rRNA gene sequences, and bioinformatic analysis

Briefly, DNA was isolated using the method of Yu and Morrison (2004) and the V1-V3 region of the 16S rRNA gene was amplified (forward primer (Lane 1991), 5' AGAGTTTGATCCTGG 3'; reverse primer W31 (Snell-Castro *et al*. 2005), 5' TTACCGCGGCTGCT 3'). Pyrosequencing was performed using a Roche/454 FLX+ instrument and Titanium chemistry according to the manufacturer's instructions. Sff file processing was done using PyroBayes (Quinlan *et al*. 2008) and inspected for chimeric sequences with Pintail (Ashelford *et al*. 2005) and errors using Acacia (Bragg *et al*. 2012). Further trimming was done in Qiime v1.8 (Caporaso *et al*. 2010) with sequence length 300-600 bases, no ambiguous sequences, minimum average quality score of 30 and maximum of 6 bases in homopolymer runs. OTU picking was done using Uclust (Edgar 2010). Taxonomy was assigned using Blast against the GreenGenes database (DeSantis *et al*. 2006) and Qiime v1.8 defaults. All samples represented by less than 1000 sequences were removed from the analysis. Rare OTUs with relative abundance less than 0.01% were removed from further consideration. Remaining analysis was done in Qiime and some data visualised in Megan (Huson *et al*. 2007) and Calypso (http://bioinfo.qimr.edu.au/calypso/).

Faecal samples (via cloacal swab) were also collected for all birds and analysed identically to the caecal data. Statistical comparisons between the 12 highest and 12 lowest performing birds were assessed using a Qiime-based t-test, while all birds in each trial are used in Person correlation analysis for each family, genus and OTU in the dataset against all 4 variables. Alpha diversity comparisons were calculated using a two-sample nonparametric t-test and 10^6 Monte Carlo permutations. Beta diversity was based on Adonis statistics and 10^6 permutations. To identify phylotypes associated with high performance over a combination of performance variables we used a Random Forest machine-learning algorithm and RapidMiner software.

Complete sequencing data, including both faecal and caecal samples, are available from MG-RAST under project ID 4667472.3.

Results

Performance of the three flocks

All three flocks showed very good performance as indicated by FCR, AME, GR and FE as performance measures (Figure 1.1). The four variables showed significant differences in all three trials between the 12 best and 12 poorest performing birds used in microbiota comparisons. Comparison of performance measures across trials showed that there were no significant differences between trials in GR, however, there were significant differences in FE ($P < 0.0001$) with birds from Trial 3 consuming more feed than those from Trial 1 and Trial 2. Birds from Trial 3 had the poorest FCR, lowest gain rate, and highest feed eaten relative to the other two trials. However, in all three trials, including Trial 3, performance was comparable with the breeder's standard (Cobb-Vantress 2013); FCR = 1.37 and live weight = 1278 for males at 25 days of age. Inspection of the correlations between the performance parameters identified AME as significantly negatively correlated with FCR and FE (Figure 1.2). Birds with higher AME values, corresponding to better efficiency in energy extraction, tended to have lower FCR due to lower feed consumption, while having no change in GR. This trend was observed in all three trials.

Microarray analysis

A total of 204 microarrays were completed to investigate FCR related differences in gene expression. Previous investigations (Konsak *et al*., unpublished data) had found a number of differentially expressed genes in the duodenum. The current study also found differences in expression with 2294, 2920 and 2815 genes differentially expressed in the duodenum samples from experiments 1-3 respectively, using a t-test *P*-value of <0.05 (Table 1.2). However, out of this number of genes there were only six genes found differential in all three experiments and most genes showed less than a 2-fold difference in expression. Considering that the *P*-value was relaxed, these subtle differences, that cannot be reproduced, are not presenting an opportunity for further investigation. Similar results, including the lack of gene overlap, have been found in other tissues as presented in Table 1.3. The accumulated data will also be analysed to determine whether there are any consistent gene expression changes correlated with differences in AME, but it is not anticipated that the conclusions will be different from the FCR analysis.

Table 1.2. Number of differentially expressed genes (*P* < 0.05) found in each tissue type.

Tissue	Trial 1	Trial 2	Trial 3
Duodenum	2294	2920	2815
Caecum	3399	1689	
Pancreas	5497	1534	
Liver	4562	526	
Spleen	3961	971	

Table 1.3. Number of differentially expressed genes (*P* < 0.05) that also showed a greater than 2-fold change in expression level in the between group comparisons for each of five tissues in the trials 1 and 2.

Tissue	Trial 1 – FCR comparison		Trial 2 – FCR comparison	
	Higher expression in low FCR birds	Higher expression in high FCR birds	Higher expression in low FCR birds	Higher expression in high FCR birds
Duodenum**	7	5	265	64
Caecum	101	60	131	58
Liver	25	60	0	0
Pancreas	34	22	37	37
Spleen	12	50	37	70

** For experiment 3 duodenal samples there were nine genes expressed more than 2-fold higher in the low FCR birds and no genes expressed more than 2-fold higher in the high FCR birds.

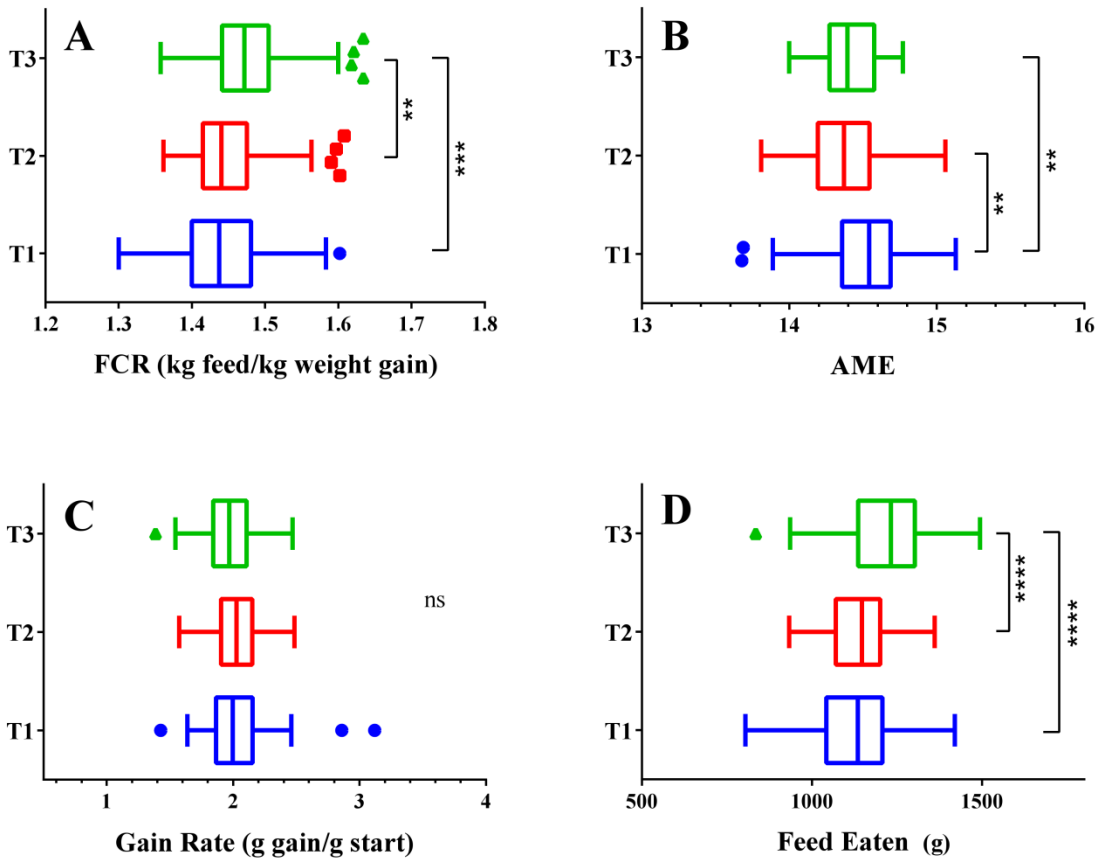


Figure 1.1. Performance of the three flocks shown as feed conversion ratio (FCR in kg feed/kg weight gain), apparent metabolisable energy (AME in MJ/kg dry matter), gain rate (GR in g gain/g start) and feed eaten (FE in g/bird). Trial 1 is shown in blue, Trial 2 in red and Trial 3 in green. * *P* < 0.05, ** *P* < 0.01) and * *P* < 0.001.**

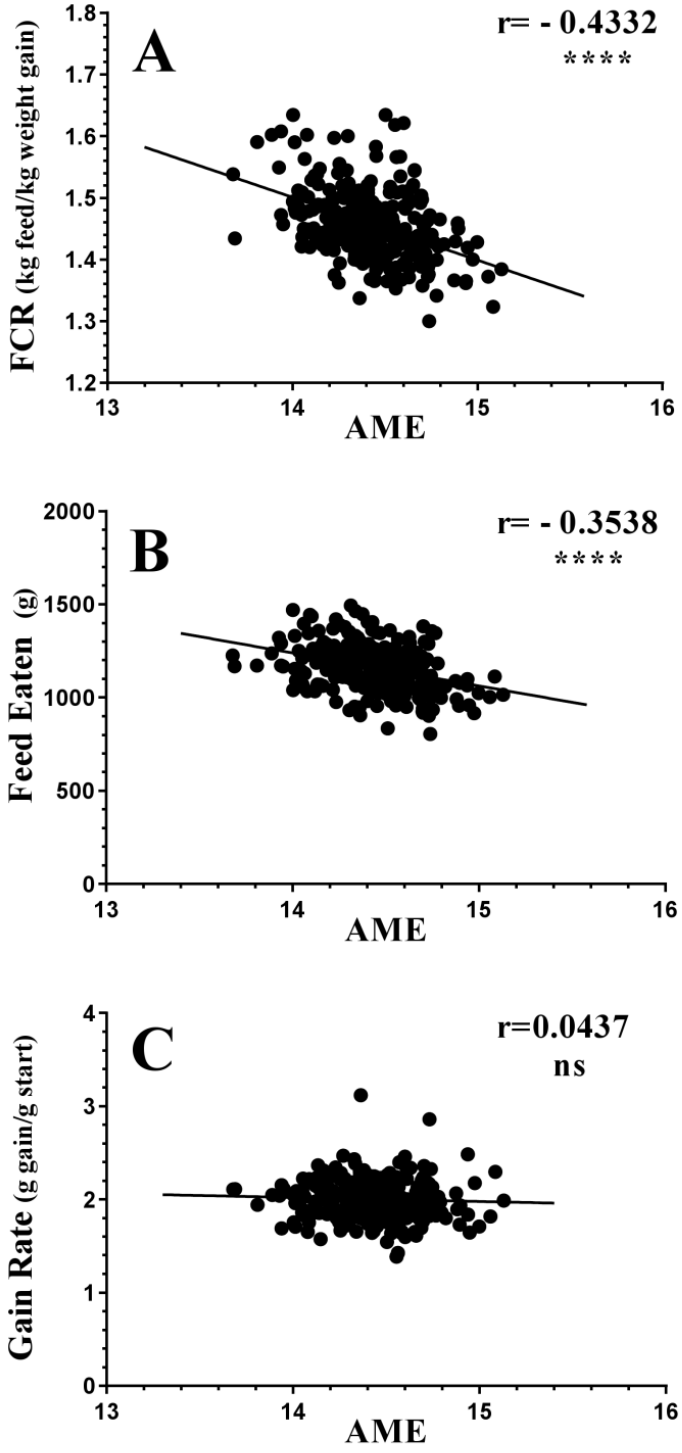


Figure 1.2. Correlations of AME against FCR, FE and GR. All birds from all three trials are represented as circles. The birds with higher AME values corresponding to better efficiency in energy extraction are likely to have lower (better) FCR, mostly due to eating less feed while having no change in gain rate. These trends were reproducible when plotted separately for each trial (data not shown).

Overall microbial structure of the three flocks

Comparison of the microbiota composition across the three replicate trials showed that there were highly significant differences between the trials; as high as at a phylum level. There were substantial differences in the abundance of *Lactobacillus*, *Bacteroides*, *Ruminococcus* and *Faecalibacterium* between trials. It was noted that Trial 3 had very different alpha diversity from the other 2 trials, having significantly more OTUs, especially rare microbiota. The differences between the trials warranted individual analysis of each trial to identify OTUs associated with productivity and then comparison of these differential OTUs across the trials. The caecal microbiota of the 12 best and 12 poorest performing birds from each trial for each of the four variables, were analysed to identify differential phylotypes. However, we used all samples from the trials to detect Pearson correlations between phylotypes at a family, genus and OTU level for FCR, AME, GR and FE.

Microbiota correlated with conversion of feed to body weight

Although differences between the birds with highest and lowest variables (FCR, AME, GR and FE) were significant (Nonparametric Mann-Whitney U test, ($P < 0.0001$) in all variables and all three trials, FCR was the only performance variable that showed convincing difference in microbiota between good and poor performing birds. There were fewer microbiota differences between birds with good and poor AME, FE and GR. The three trials showed very different microbiota correlations to FCR with Trials 1 and 2 responding strongly but differently, even at higher taxonomic levels, while the microbiota in Trial 3 birds showed comparatively mild responses to FCR extremes.

Qiime-based alpha diversity statistical analysis was performed using nonparametric, two-sample t-test and 1000 Monte Carlo permutations to inspect statistical significance of Chao1, observed species, Shannon and Simpson alpha metrics. In Trial 1, the birds with good FCR showed higher diversity with significantly higher chao1 ($P = 0.0021$, Figure 1.3A) and observed species alpha metrics ($P = 3E^{-4}$) than the birds with poor FCR. They also displayed significantly higher richness and evenness index than poor FCR birds at a family and a genus level. There was strong separation between poor (high) and good (low) FCR birds in the phylotypes present, measured as Unweighted UniFrac (Adonis $P = 3E^{-4}$, Figure 1.3B) while there were no differences in phylotype abundance in Weighted Unifrac distance ($P = 0.2526$). The differences in community structure were evident at both genus ($P = 7E^{-5}$) and family level ($P = 3.9E^{-4}$) using Canberra distance (Figure 1.3 C-D). Significance analysis was performed (t-test, Qiime), comparing the 12 best and poorest FCR birds.

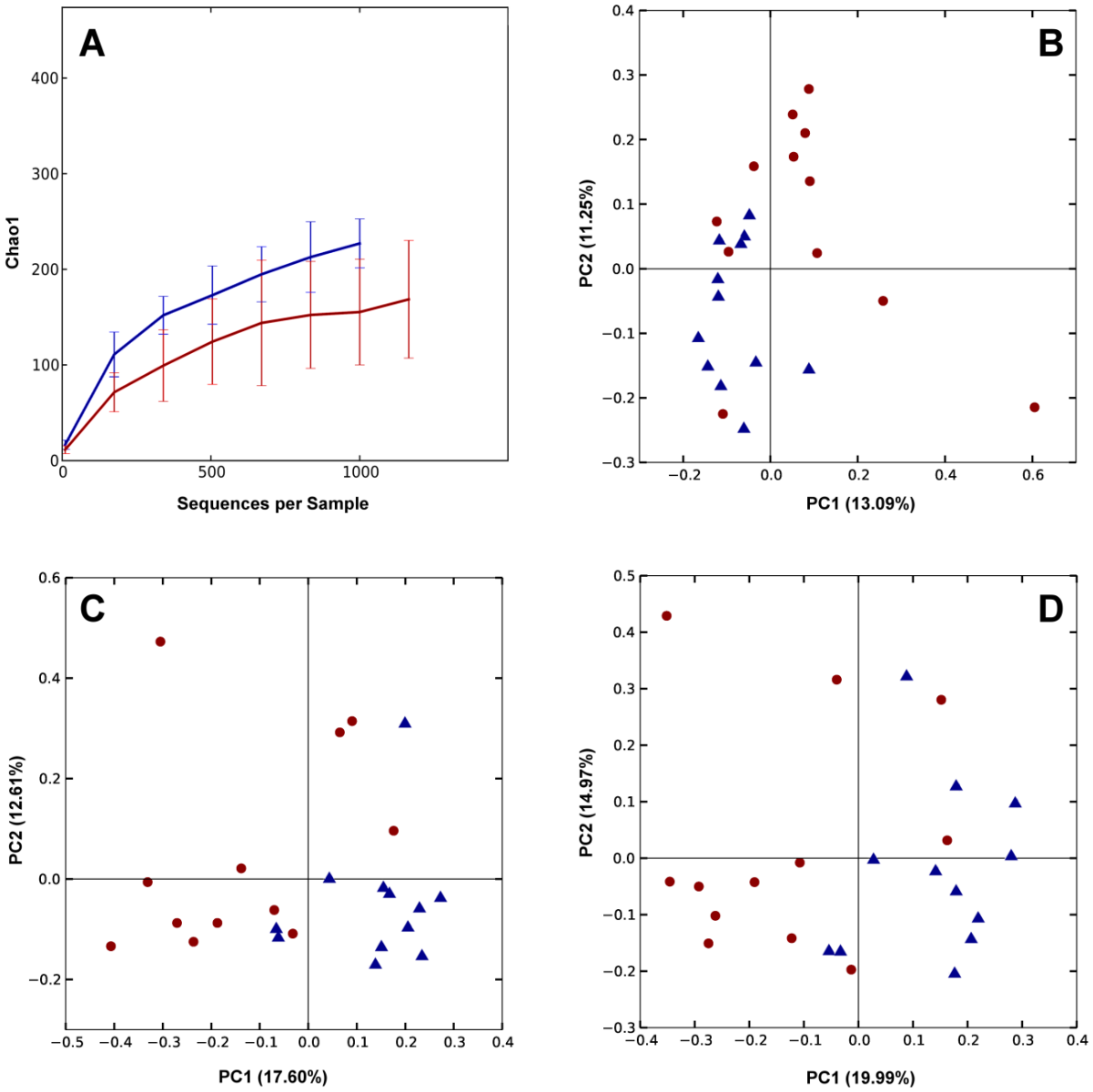


Figure 1.3. Trial 1: Differences between high (red) and low (blue) FCR microbial communities. (A) Alpha diversity metric Chao1 was significantly (*P* = 0.0021) higher in low FCR birds. (B) Unweighted UniFrac (*P* = $3E^{-4}$) PCoA plot; (C and D) PCoA plot of beta diversity using Canberra distance at a genus (C) and family level (D). Communities of high and low FCR birds were significantly different based on Canberra distance at a genus (*P* = $7E^{-5}$) or family level (*P* = $3.9E^{-4}$).

The differences were prominent at a family and genus level (Figure 1.4). Three families were responsible for high community differences at a family level: *Lachnospiraceae* (P = 1.1E^{-4}), *Ruminococcaceae* (5.09E^{-4}) and *Erysipelotrichaceae* (P = 0.026) (Figure 1.4). At the genus level, genera significantly more abundant in good FCR included *Ruminococcus*, *Faecalibacterium*, *Clostridium* and two unknown genera from the *Lachnospiraceae* family, showing differential abundance with P-value range from 0.02 to 8E^{-5} and 2.5 to 20.1 fold higher in better performing birds. Genus *Faecalibacterium* was 20.1 times more abundant in good FCR birds, while OTU18285, 16.8 times more abundant in good FCR birds (P = 0.0016), aligned with *Faecalibacterium prausnitzii* strain ATCC 27768(T) with 94.74% identity (EzTaxon database). Trial 1 had 21 OTUs differentially abundant (Qiime t-test, $P < 0.05$) between high and low FCR birds.

Correlations between phylotypes on each taxonomic level vs. FCR values were then inspected. This analysis investigated correlation in all birds between all of the phylotypes (at family, genus and species level) vs. FCR. At the family level, *Lachnospiraceae* showed the most significant (P = 3.6E^{-4}) negative correlation with FCR values followed by two other significantly negatively correlated families *Erysipelotrichaceae* (P = 0.0053) and *Ruminococcaceae* (P = 0.0259). Negative correlation with FCR values indicates a positive effect on performance as lower FCR indicates better performance.

The Trial 2 community was very different to that observed in Trial 1 and very little overlap was found between phylotypes associated with FCR, even at a family level. There were no significant differences in alpha diversity measures between good and poor FCR bird microbial communities, unlike in Trial 1. However strong differences were observed in Unweighted (P = 0.0019) and Weighted Unifrac (P = 0.0072) at an OTU as well as at higher taxonomic ranks, using Canberra distance, at a genus (P = 0.0055) and family (P = 1.8E^{-4}) levels (Figure 1.5 A:D). The order responsible for most of the differences was the same as in Trial 1 - *Clostridiales*, however, instead of families *Lachnospiraceae* and *Ruminococcaceae*, in Trial 2 significant families were *Catabacteriaceae* and an unknown family of order *Clostridiales* (Figure 1.6). Surprisingly, the genus *Lactobacillus* was enriched (P = 0.0078) in poor FCR birds (Figure 1.6). Correlation analysis included all birds from the trials and matched the results from significance analysis.

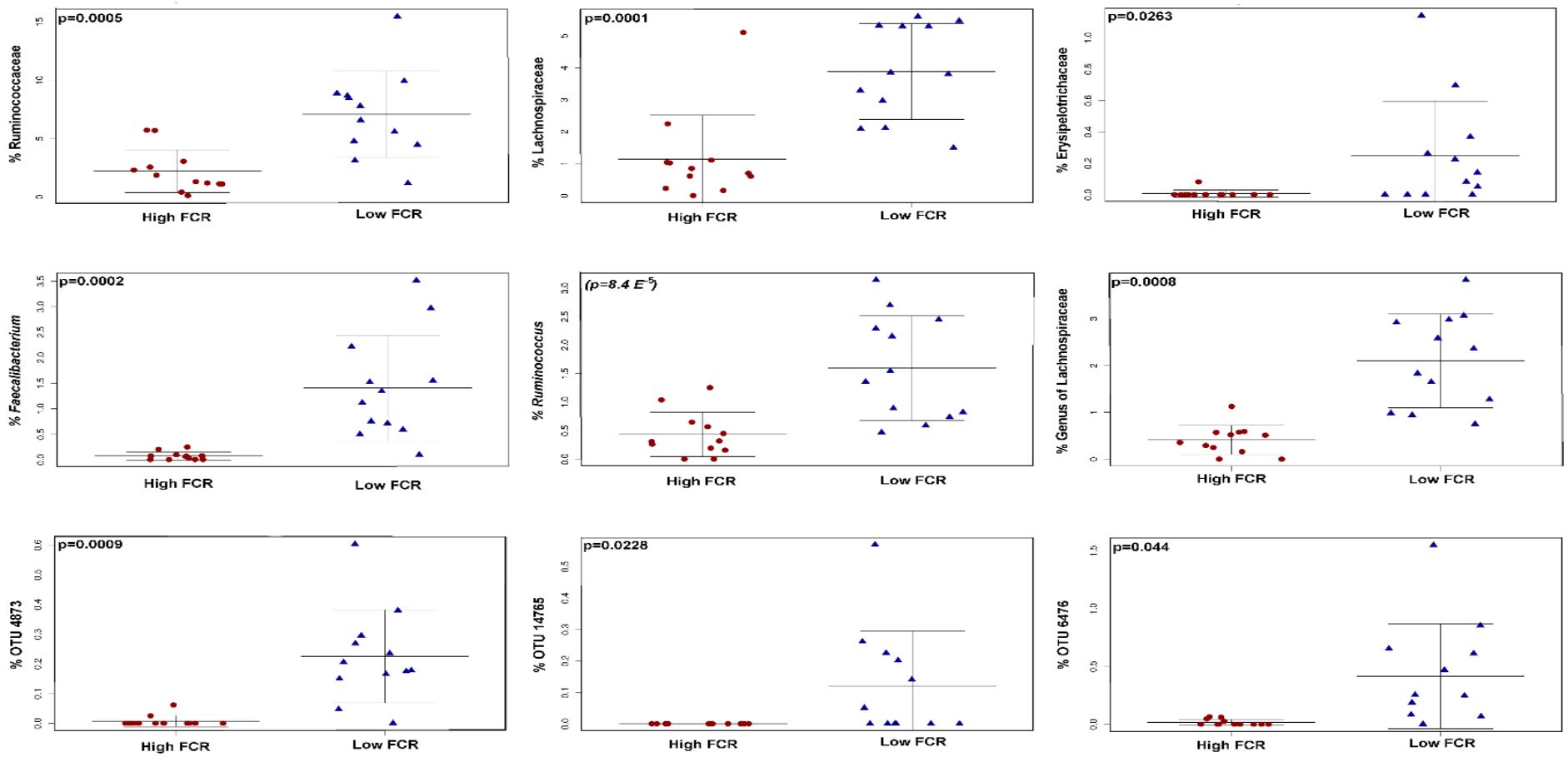


Figure 1.4. The taxa responsible for differences in high and low FCR caecal communities in Trial 1. The figure represents a family (top row), genus (middle) and OTU level (bottom row). A table with all differentially abundant phylotypes is given in Supplementary Table 1.1. The closest culturable strain (EzTaxon database) to OTU 4873 was *F. praustnizii* (ATCC 27768(T)) with 93.7% pairwise similarity, OTU 14765 was closest to *Clostridium spiroforme* (DSM 1552(T)) with 99.6% pairwise similarity to type strain and an OTU was closest to *Clostridium lactatifermentans* (89.76%).

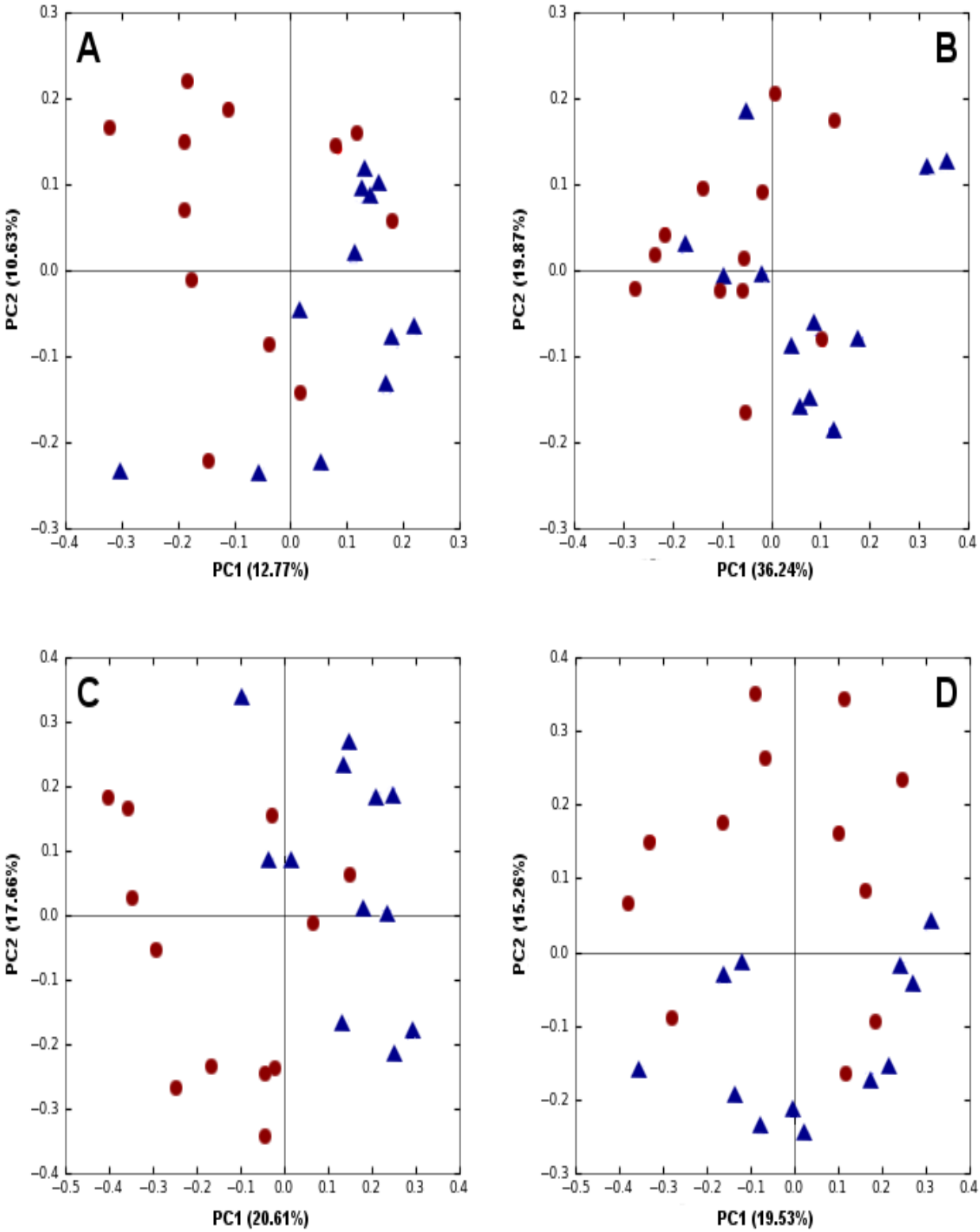


Figure 1.5. Beta diversity was significantly different between high (red) and low (blue) FCR birds microbial communities. (A) Unweighted (*P* = 0.0019) and (B) Weighted Unifrac (*P* = 0.0072) at an OTU level as well as Canberra beta diversity at a (C) genus (*P* = 0.0055) and (D) family (*P* = 1.8E^{-4}) levels were separating high and low FCR birds.

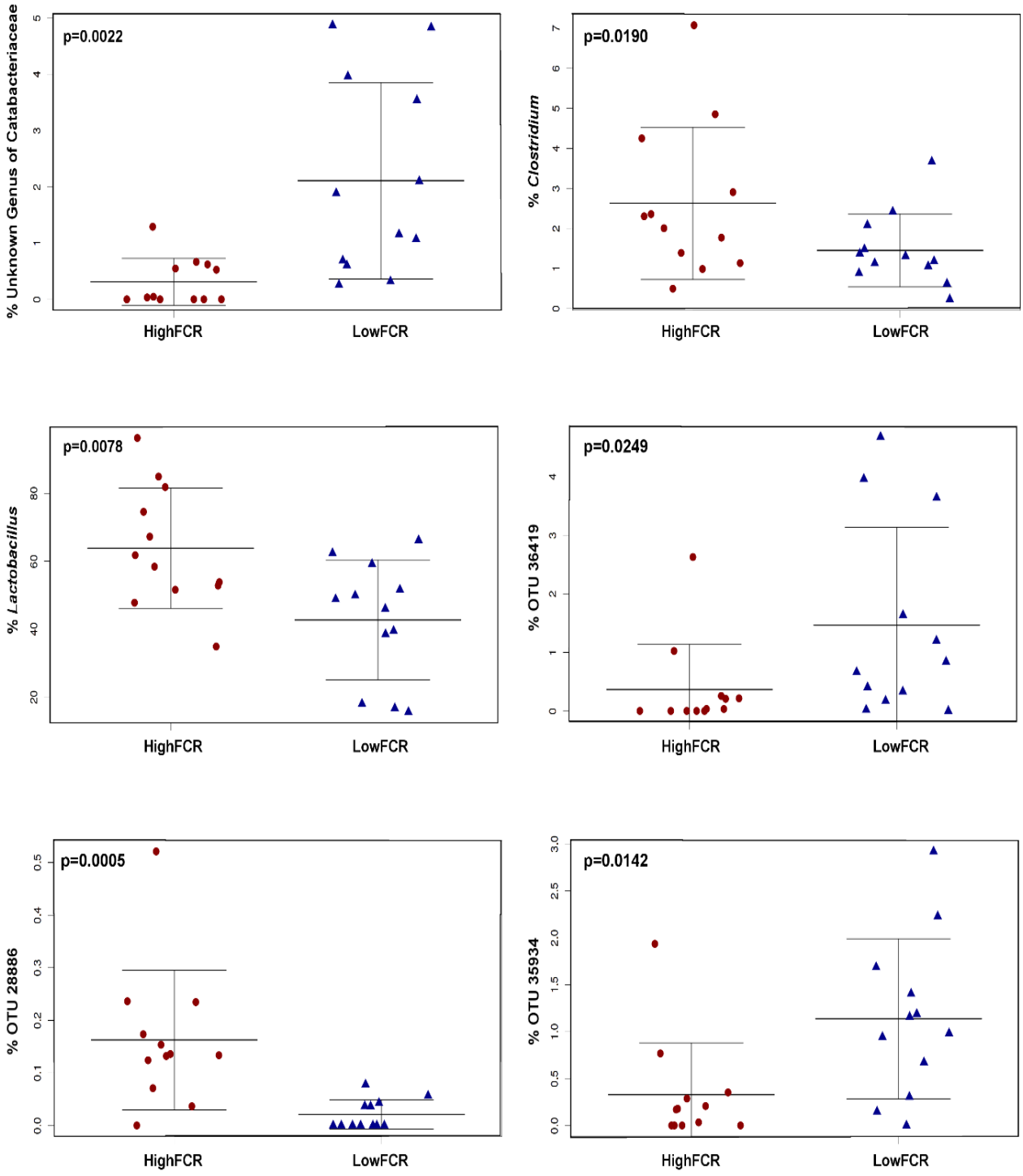


Figure 1.6. Some of the significantly (FCR) differential genera and OTUs in Trial 2. Genus *Clostridium* significantly correlated with poor performing (high FCR) birds is of lineage *Clostridiaceae/Clostridium*. Closest culturable strain to OTU36419 was *Ruminococcus albus* (similarity 90.06%), to OTU28886 *Lactobacillus reuteri* (98.98%) and OTU35934 (*Clostridium cellobioparum*, 83.13%).

Few differences were noted in the microbiota of good and poor FCR birds in Trial 3 compared to the other 2 trials. There was no significant difference in alpha or beta diversity measures at any of the taxonomic levels. There were no families or genera significantly different in abundance between good and poor birds nor were there any significant correlations at these levels. At an OTU level, four OTUs were significantly ($P < 0.01$) different in abundance between good and poor FCR birds. The OTUs enriched in poor FCR birds belonged to *Lactobacillus* and *Faecalibacterium* genera.

Families *Lachnospiraceae*, *Erysipelotrichaceae* and *Ruminococcaceae* were found to be significantly negatively correlated with FCR values. Since reduction of FCR (feed needed per kg of weight) is the aim of the animal production industries, these families are identified as a source of candidate probiotic isolates with potential to be used for performance enhancement. All three families owed their significance to the ambiguously classified genus *Clostridium*, which appeared in four different lineages (Figure 1.7). Significant *Lachnospiraceae*/*Clostridium* OTUs were comprised of sequences most similar to *C. lactatifermentans* (95.35%), *Erysipelotrichaceae*/*Clostridium* to *Clostridium spiroforme* (93%) and *Ruminococcaceae/Clostridium* to *C. leptum* (91.6%).

Trial 3 lacked significance at higher levels and showed equivocal results at an OTU level with a number of OTUs with similar phylogenetic classification differentially abundant in both good and bad FCR birds. There were 17 *Lactobacillus* OTUs among the differentially abundant ($P < 0.05$), however the genus *Lactobacillus*, as a whole, was unchanged suggesting community shifts within the Lactobacillus genus.

Phylotypes associated with efficiency of energy extraction from feed

The differences between high and low AME birds were significant ($P < 0.0001$) in each of the three trials based on a nonparametric Mann-Whitney U test. The data analysis and comparisons of the three trials pointed to Trial 2 as the trial displaying the highest, however still moderate, microbiota differences between high and low AME birds, followed by Trial 3, and with little response recorded in Trial 1, compared at all phylogenetic levels.

Differences in alpha diversity were only observed in Trial 2 where the better energy assimilating, high AME, birds had microbiota with lower dominance ($P = 0.007$) and higher equitability, Shannon and Simpson indices ($P = 0.0236$, $P = 0.0448$ and $P = 0.0064$, respectively). However, there were no differences observed in alpha diversity between high and low AME birds in Trials 1 and 3 in caecal microbiota.

No differences in overall community structure were detected between high and low AME birds in any of the three trials using Weighted and Unweighted UniFrac and Anosim statistics indicating

that differences in AME are not driven by overall total community shifts in either alpha or beta diversity. A few OTUs were found to be differentially abundant between high and low AME birds with a *Ruminococcaceae* related OTU 16-fold more abundant in the high AME birds in Trial 2. There were no family or genus level phylotypes significantly ($P < 0.05$, $r > 0.3$) correlated with AME but at the OTU level. *L. reuteri* OTU28886, ($P = 8.28E^{-6}$, $r = -0.49$) and six other OTUs identified as *L. reuteri* were all significantly negatively correlated ($P < 0.05$, $r = -0.24$ to -0.34) with energy extraction from feed. *L. crispatus* OTU 15229 abundance was positively correlated ($P = 0.0076$, $r = 0.33$) with AME values).

Correlations between bacterial abundance and gain rate and feed eaten

There were no significant differences in overall community structure, shown as either alpha or beta diversity differences between extreme GR or FE in any of the three trials. Both GR and FE extreme birds showed few individual phylotypes that were significantly different in each trial. We noted that in trials 1 and 2 the same families and genera enhanced in good FCR birds were also significantly associated with good GR (Figure 1.8). This was not reproduced with feed eaten; phylotypes involved did not overlap with phylotypes associated with FCR extremes. This observation indicates that different phylotypes were correlated with GR and performance compared to those that correlated with the amount of feed eaten (Figure 1.8).

Random Forest data modelling

Phylotypes associated with high performance over a combination of performance measures were investigated by constructing a ranked list of all birds, in each trial separately, ranking low to high FCR and high to low AME birds. The combined AME and FCR ranks with low values were used to identify the birds with a combination of desirable high AME and low FCR values. These birds were the most efficient in extracting energy from food (high AME) and converting feed to body weight (low FCR). They were identified as AME_FCR_Good. The birds with high rank values showed the opposite trend of low AME and high FCR; they were classified as AME_FCR_Bad. Upon inspecting 50 Random Forest prediction trees, no OTUs were identified as clearly implicated in differentiating the AME_FCR_Good birds from the other birds that were also identified in the correction analysis carried out independently for the AME or FCR single performance variable analysis above. However, the Random Forest data analysis approach did identify *Faecalibacterium prausnitzii*, *Bacteroides fragilis*, and members of *Ruminococcus* genus repeatedly associated with good performance in a range of prediction trees, while members of *Gammaproteobacteria* and members of the genus *Clostridium* were associated with the undesirable AME_FCR_Bad birds. *F. prausnitzii* was identified in the significance analysis of Trial 1 as being more highly abundant in the good FCR birds so the Random Forest analysis has extended this to a more general finding over all the birds and in combined AME_FCR good birds

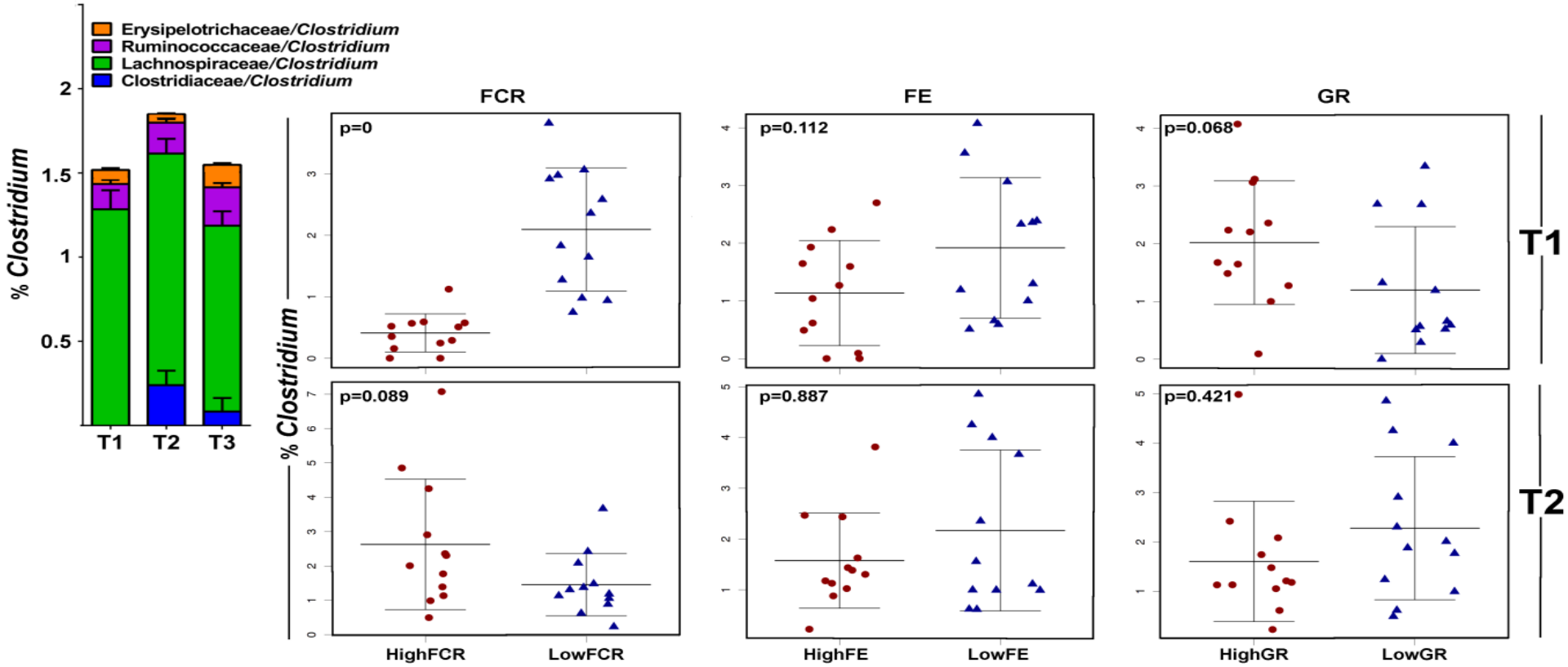


Figure 1.7. Different mode of influence of genus *Clostridium* on growth performance. In this analysis all genera listed as *Clostridium* were merged and the influence of total *Clostridium* abundance is shown in stripcharts for Trial 1 (top row) and Trial 2 (bottom row); there were no differences in Trial 3. *Clostridium* species come with somewhat confusing lineages split between families *Clostridiaceae*, *Ruminococcaceae*, *Lachnospiraceae* and *Eryspelotrichaceae*. Trial 1 *Clostridium* community with no members of *Clostridiaceae*/*Clostridium* lineage (blue on the barchart above), improved FCR while slightly reducing feed eaten and increasing growth rate. In Trial 2 (and more so in Trial 3), *Clostridiaceae* lineage of *Clostridium* gave non-significant differences in the opposite FCR direction.

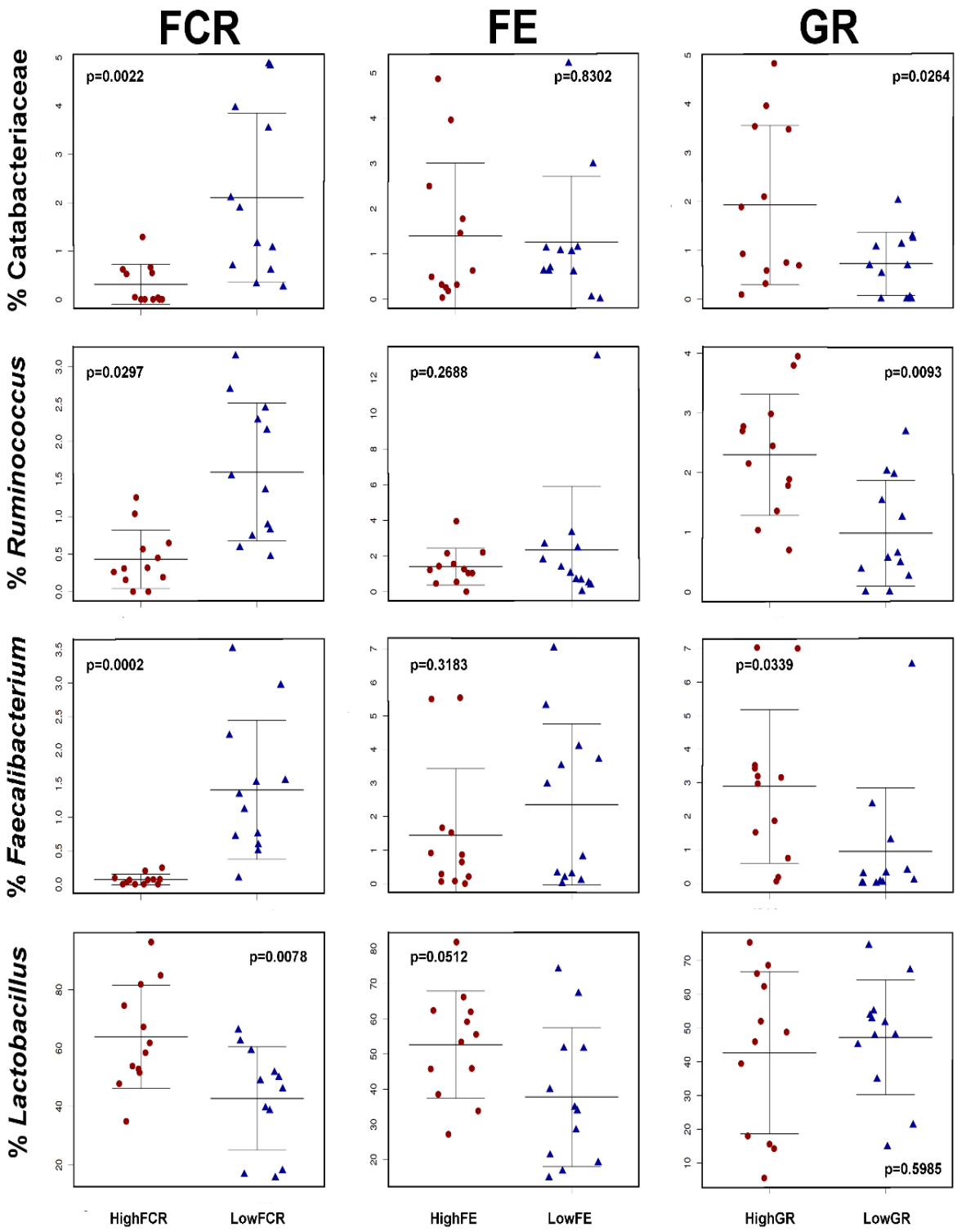


Figure 1.8. Different ways of influencing performance were observed between different genera and in different trials. All members of family *Catabacteriaceae* belonged to one unknown genus (and 1 OTU) at 3% divergence OTU picking. In Trial 2 they improved FCR by improving growth rate in a very similar way to *Ruminococcus* and *Faecalibacterium* in Trial 1 (second row). Members of *Lactobacillus* contributed to poor performance of high FCR birds and increased feed intake.

Discussion

Gene expression

The gene expression results obtained from these trials, with a single experiment design repeated in triplicate, were not as expected. Previous work on single trials had shown that there were gene expression differences between high and low performing birds – the aim of the present work was to define how reproducible these changes were. The results we have obtained confirm the earlier work, in that within a trial statistically significant gene expression differences are seen. However, we now clearly show that the pattern of differential gene expression differs from trial to trial. That is, a different group of genes is seen as differentially expressed in each trial. There is very little overlap in the list of differential genes produced for each trial. We investigated whether the overlap in differential gene lists could be more at the functional level rather than at the gene level. The thought here was that similar cellular functions or biochemical pathways could be influenced by at different points in the pathways, but result in similar outcomes, i.e. improved energy efficiency. We addressed this possibility by investigating the gene ontology (GO) classification of genes in the differentially expressed gene lists. Gene ontology gives a functional assignment for genes, indicating what cellular processes the gene is involved in. This analysis (Figure 1.9) gave a similar result to the straight gene expression analysis, indicating that there was very little overlap between trials.

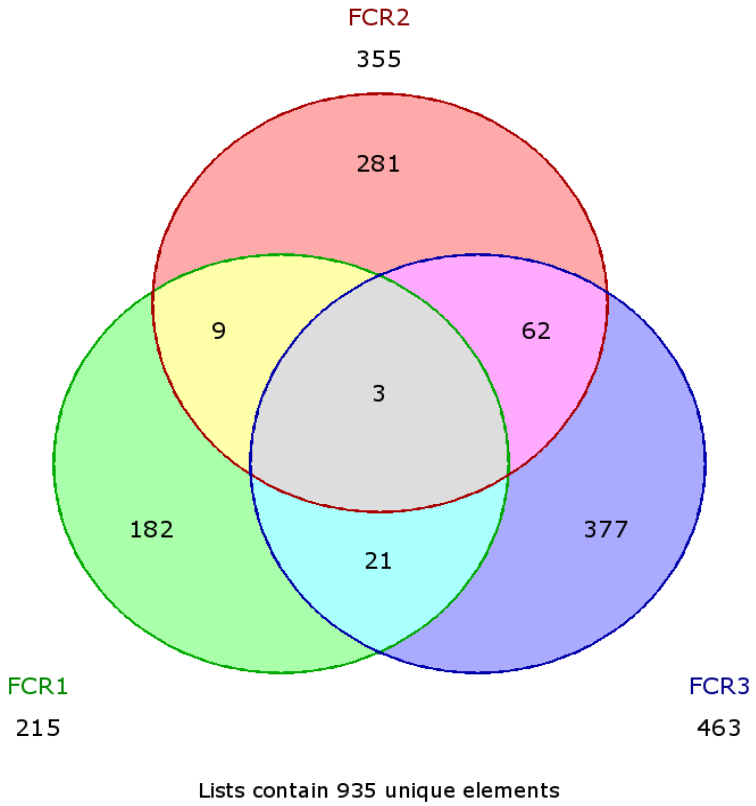


Figure 1.9. Analysis of the overlaps in gene ontology assignments for the differentially expressed genes from three trials.

Microbiota

In all three trials growth rate and feed conversion of the chickens met or exceeded the breeder's expectations (Cobb-Vantress 2013), however, within each trial there were differences in the performance parameters of birds and so relatively high and low performance birds could be identified. The current analysis performed here was aimed at determining whether in the face of this sort of microbiota variation there were common changes in the phylotype profiles that characterised the enhanced performance birds in the different trials. In each of the three trials there where bacterial phylotypes that were differentially abundant between the high and low performance birds. However, it was found that some phylotypes associated with high performance in one trial were completely absent in another trial. With the variation in background microbiota between trials there were no OTUs that were consistently associated with good or bad performance across all three trials.

In the present study, several probiotic candidates and taxonomic groups with potential benefits to performance were identified. In the first trial, there was a strong dominance of *Clostridia* influence. The *Clostridia* are a highly polyphyletic class of *Firmicutes* converged from multiple ancestors thus believed to be in need of reclassification. There are over 140 annotated and described *Clostridium* species, however, 16S rRNA gene sequencing resulted in a major revision of the genus (Stackebrandt *et al*. 1999), describing many *Clostridium* species as new genera and associating many others to different known genera. Despite major revisions the *Clostridium* species occupy different positions within the 16S rDNA phylogenetic tree (Stackebrandt *et al*. 1999; Biddle *et al*. 2013). Many of the *Clostridium* species moved to different families kept the genus name *Clostridium* adding to the taxonomic confusion. Moreover, different databases take different views on the issue and display different lineages for the same species. *Clostridium* species are now referred to as a number of gram positive and negative bacteria, with some not even being anaerobes (Fak and Backhed 2012). In 2013 renaming of ambiguously named *Clostridium* species was suggested (Yutin and Galperin 2013). In our Trial 1, *Clostridium* species were classified using GreenGenes database, to four different families (*Clostridiaceae, Ruminococcaceae, Lachnospiraceae* and *Erysipelotrichaceae*). Species of *Clostridium,* from family *Clostridiaceae*, were implicated in poor performance, however, the species of *Clostridium* classified as *Lachnospiraceae, Ruminococcaceae* and *Erysipelotrichaceae,* based on 16S rRNA gene sequence, showed significant positive correlation between relative abundance and good FCR performance at a family, genus and species level. *Lachnospiraceae* and *Ruminococcaceae* are associated with gut health through short chain fatty acid (SCFA) production and degradation of plant materials (Biddle *et al*. 2013) while *Erysipelotrichaceae* family members were implicated in weight gain in a human study where they were present only in obese individuals (Zhang *et al*. 2009).

The relative abundance of the *Lachnospiraceae* family as a whole was positively correlated with good FCR performance. A *Lachnospiraceae* family classified genus *Clostridium*, with members closest (95.35%) to *C. lactatifermentans,* was significantly increased in low FCR birds (4.7 fold). It was recently suggested that the genus should be renamed to *Lachnoclostridium* (Yutin and Galperin 2013). *C. lactatifermentans* was isolated from the ceca of chicken and described by van der Wielen *et al*. (2002b). The same group has investigated the use this lactate-fermenting strain as a chicken probiotic. They demonstrated that *C. lactatifermentans* inhibited the growth of *Salmonella enterica* in batch culture simulating caecal conditions (van der Wielen *et al*. 2002a). The authors argued that the ability of *C. lactatifermentans* to ferment lactate to short chain fatty acids, namely acetate, propionate and traces of butyrate and isovalerate, ought to be beneficial to the host (van der Wielen *et al*. 2002a; van der Wielen *et al*. 2002b; van der Wielen *et al*. 2002c). SCFAs are known to be important in colonic health and reduce the risk of inflammatory bowel disease, irritable bowel syndrome, cancer and cardiovascular disease in humans (Hijova and Chmelarova 2007). SCFAs can increase the growth of beneficial bacteria, *Lactobacillus* and *Bifidobacteria* (Roy *et al*. 2006), and are a major energy source in the intestine. Lactic acid produced by *Lactobacillus* strains can be converted to the most beneficial SCFAs such as acetate, propionate and butyrate by *C. lactatifermentans*. The vast majority of produced acetate is readily transported to the liver and metabolised. The liver also uses residual butyrate and propionate for gluconeogenesis. Butyrate is the main energy source for caecal and colonic epithelial cells. Acetate is an energy source for muscle (reviewed by Hijova and Chmelarova 2007) and propionate and butyrate regulate the expression of the *FFAR3* gene, which leads to fat deposition in adipocytes (Vangaveti *et al*. 2010). Thus, there are good theoretical reasons why the increased abundance of *C. lactatifermentans* could potentially improve colonic and liver heath of the chicken, stimulate muscle and control fat deposits via the production of SCFAs. It seems likely that *C. lactatifermentans* within the microbial community, could increase performance via conversion of *Lactobacillus* metabolites to beneficial SCFAs. Similarly, the whole butyrate-producing *Lachnospiraceae* family potentially have beneficial effects.

The *Clostridium spiroforme* related OTU with the strongest correlation with FCR performance was 99.6% identical to the type strain, while the most abundant *C. spiroforme* OTU, comprising most of the microbiota contribution from the *Erysipelotrichaceae* family, was only 93% similar to the type strain. *C. spiroforme* was recently transferred (reviewed in Yutin and Galperin 2013) to the family *Erysipelotrichaceae* in the class *Erysipelotrichi*, within the new genus, *Erysipelatoclostridium*. *C. spiroforme* is often cited in the literature for toxin production and is implicated in gastrointestinal issues (Perelle *et a*l. 1997)). However, the most abundant strain driving this genus significance in FCR, in our data is only 93% similar to the type strain thus allowing for the possibility of non-toxic

relative being involved in broiler performance especially with the knowledge of the role of *Erysipelotrichaceae* in the weight gain (Zhang *et al*. 2009).

Among other candidates as performance enhancing probiotics, one of the most exciting is *Faecalibacterium prausnitzii*. Newly emerging evidence indicates that *F. prausnitzii* has an important role in establishing and maintaining healthy metabolism, SCFA production and appropriate development of the immune system in humans. Reduction in the abundance of *F. prausnitzii* is linked to numerous diseases including diabetes, colitis, IBD, dysbiosis and immunocompromised states (reviewed in Miquel *et al*. 2013). This bacterium was identified as correlated with good performance in Trial 1 and the extended analysis with the Random Forest approach indicated that it was more broadly indicative of high performance birds rated on joint FCR and AME rankings across the three trials. Although clearly of interest there are difficulties in developing this bacterium as a probiotic as it is a strict anaerobe and it is not clear whether it can be economically grown and delivered on a large scale.

We identified some members of the *Lactobacillus* genus as undesirable for overall performance, mostly due to an increase in feed eaten. The link between *Lactobacillus* and increased appetite and feed consumption has been shown in humans. Although there are strains of *Lactobacillus* known to improve performance, there are numerous strains retailed as weight loss probiotics and others with reported ability to reduce obesity (Fak and Backhed 2012). The same finding of a negative influence of some *Lactobacillus* strains on performance has been previously reported (Torok *et al*. 2011). Moreover, different strains of the same species may act in the opposite manner: *L. reuteri* L6798 was associated with weight gain, whereas *L. reuteri* ATCC PTA 4659 was associated with weight loss in mice (Fak and Backhed 2012). In Trial 3, most of the differential OTUs were *Lactobacillus* species correlated with both good and poor performance, while the genus *Lactobacillus*, as a whole, remained unchanged in relative abundance, suggesting a number of strains have conflicting effects on performance.

Our data suggest that the use of *Lactobacillus* isolates as probiotics must be approached with caution. Although some *Lactobacillus* OTUs are correlated with superior performance other OTUs, even of the same predicted species, are correlated with poor performance. There are other valid probiotic candidates emerging from this microbiota analysis. From the present dataset, beneficial, Clostridia members, involved in plant material degradation, cellulose utilisation and SCFA production, are associated with growth performance. This is in agreement with previously published 16S based studies in broiler performance (Torok *et al*. 2008; Torok *et al*. 2011; Stanley *et al*. 2012; Stanley *et al*. 2013a).

Significant variability in microbiota requires development of new strategies in culturing, isolate selection and i*n vivo* testing in order to identify isolates that could have utility as probiotics. Strains will need to be chosen on the basis that when introduced to newly hatched chickens, they colonise in high abundance in the face of different background microbiota and thus improve performance regardless of other organisms in the gut population. The ability to colonise in diverse microbiota backgrounds is likely to be a key attribute of probiotics that are able to reliably deliver desired benefits to the host.

Conclusions

Gene expression

We concluded that the statistically significant gene expression differences that are observed in the duodenum and other tissues are unlikely to be directly linked to differences in performance and hence will not provide useful tools to monitor the effects of dietary manipulation for productivity gain or to provide potential gene markers for marker assisted breeding for improved performance. We speculate that the differences in gene expression seen in each trial may be correlated to factors other than FCR performance. For example, the expression differences might be established by slight variations in environmental conditions, differences in gut microflora, or other minor perturbations in the experimental setup or source flock of chickens.

Candidate probiotics

Lactobacillus may not be the best option for performance improving probiotics. There are other valid candidates emerging from this microbiota analysis. From the present dataset, beneficial *Clostridia* members, involved in plant material degradation, cellulose utilisation and SCFA production were associated with growth performance. This is in agreement with previously published 16S based studies in broiler performance (Torok *et al*. 2008; Torok *et al*. 2011; Stanley *et al*. 2012; Stanley *et al*. 2013a). Significant variability in microbiota requires development of new strategies in culturing, isolate selection and *in vivo* testing. Strains will need to be chosen on the basis that when introduced to newly hatched chickens, they colonise in high abundance in the face of different background microbiota and thus improve performance regardless of other organisms in the gut population.

Chapter 2. Influence of in-feed antimicrobials on the populations of microbes associated with high and low-performing birds

Introduction

Microbes linked to improved bird performance were discussed in Chapter 1. Some of these organisms were observed to be linked to performance across three separate trials, whilst some were unique to just one experiment. All three trials involved birds fed a commercial wheat-based diet without any added antibiotics. In this chapter we describe a series of three similar experiments to investigate whether the same bacterial species are associated with performance when birds are fed diets containing in-feed antibiotics (zinc bacitracin and avilamycin being the two most commonly used in Australia), and when typical commercial diets containing wheat or sorghum are used (to cover the main grain types used in Australia). This approach provided information on performance-linked gut bacteria from birds fed different diet types that are typical of Australian conditions.

Materials and methods

Animal ethics

The Animal Ethics Committees of the University of Adelaide (Approval No.S-2011-218) and the Department of Primary Industries and Resources, South Australia (Approval No. 25/11) approved this study. All animal work was conducted in accordance with the national and international guidelines for animal welfare.

Animal trials

Three animal trials (each with n=96 birds) were performed in the period February to May 2012, using the same procedures as described in Chapter 1. Briefly, one-day old male Cobb 500 broiler chickens from the Baiada Hatchery, Willaston, SA, Australia, were transferred to a chick rearing pen in a fully environmentally controlled experimental animal facility. Feed and water were supplied *ad libitum* throughout the experiment. The same batch of commercially prepared starter crumbles based on wheat as the main cereal component (Ridley Agriproducts, Murray Bridge, S.A.) was used in all three trials as the Control and was stored under controlled cool and dry conditions for the duration of the trials. All birds within each trial were housed together for the first 13 days of life to ensure microbiota exchange through typical bird behaviour, including copraphagy.

The experimental diets for Trial 1 were (1) Control and (2) Control with zinc bacitracin (50 ppm active ingredient, for Trial 2 were (1) Control and (2) Control with avilamycin (15 ppm active

ingredient), and for Trial 3 were (1) wheat-based diet and (2) sorghum-based diet with the same nutrient specifications as the wheat-based diet.

On day 13 chicks were transferred in pairs to 48 metabolism cages in a temperature controlled room (23-25°C). Initial placing in metabolic cages in pairs was done to minimise stress and allow the birds to adjust to cages. At day 15, birds were moved into individual cages. Individual caging allowed the precise assessment of individual feed intake, energy in feed, and unused energy remaining in faeces. The experimental design eliminated competition for feed and reduced behavioural issues affecting feed intake. Single bird caging and individual measurements and sampling were implemented in order to allow direct correlation of microbiota structure and productivity measurements on a bird by bird basis. Birds were euthanised and necropsied on day 25 and caecal contents and cloacal swabs were collected from each bird. Tissue samples were transported on dry-ice and arrived at CSIRO Australian Animal Health Laboratory in good condition; then were immediately transferred to a -80^{o}C freezer for storage before analysis. Samples from all birds from all three trials were analysed.

Feed conversion ratio was calculated as a ratio of feed eaten and weight gained. Thus birds with low FCR, that needed less feed per kg gained, were the most efficient in converting feed to mass. Gross energy (GE) was measured in feed and in faeces of each individual bird using a Parr isoperibol bomb calorimeter (Parr Instrument Company, Moline, IL). Apparent metabolisable energy (AME) in MJ/kg dry matter, was calculated as (AME_{diet} = [(GE_{diet} x feed eaten) x ($GE_{excreta}$ x dry excreta)]/feed eaten/dry diet content). Gain rate (GR) was calculated as [weight gain (g) / start weight (g)] and feed eaten (FE) was total amount of feed eaten during the 10 day measurement time period. All of the above measurements were taken from day 15 to day 25, during the time when single birds were housed in metabolic cages.

DNA preparation, PCR amplification of 16S rRNA gene sequences, and bioinformatic analysis

Briefly, DNA was isolated using the method of Yu and Morrison (2004) and the V1-V3 region of the 16S rRNA gene was amplified (forward primer (Lane 1991), 5' AGAGTTTGATCCTGG 3'; reverse primer W31 (Snell-Castro *et al*. 2005), 5' TTACCGCGGCTGCT 3'). Pyrosequencing was performed using a Roche/454 FLX+ instrument and Titanium chemistry according to the manufacturer's instructions. Sff file processing was done using PyroBayes (Quinlan *et al*. 2008) and inspected for chimeric sequences with Pintail (Ashelford *et al*. 2005) and errors using Acacia (Bragg *et al*. 2012). Further trimming was done in Qiime v1.8 (Caporaso *et al*. 2010) with sequence length 300-600 bases, no ambiguous sequences, minimum average quality score of 30 and maximum of 6 bases in homopolymer runs. OTU picking was done using Uclust (Edgar 2010). Taxonomy was assigned using Blast against the GreenGenes database (DeSantis *et al*. 2006) and

Qiime v1.8 defaults. All samples represented by less than 1000 sequences were removed from the analysis. Rare OTUs with relative abundance less than 0.01% were removed from further consideration. Remaining analysis was done in Qiime and some data visualised in Megan (Huson *et al*. 2007) and Calypso (http://bioinfo.qimr.edu.au/calypso/).

Results

Performance of the three flocks

All three flocks showed good performance as indicated by FCR, BWG and AME (Table 2.1). Within each trial and within each diet, FCR, BWG and AME were superior for 12 birds in the cohort with lowest FCR. In Trial 1, zinc bacitracin improved FCR and BWG but did not affect AME. In Trial 2, avilamycin did not affect FCR, BWG or AME. In Trial 3, birds given the wheat diet had better FCR than birds fed the sorghum diet but showed lower BWG and AME.

Microbiota in caecal samples in three experiments

Good yields of bacterial DNA were recovered from the caecal samples and faecal samples (data not shown) and the DNA was suitable for PCR amplification (Table 2.2). Typical results of 16S rRNA gene amplification are shown in Figure 2.1.

The 16S sequence data were processed to cluster into operational taxonomic units (OTUs) and an example of the summary data is shown in Figures 2.1, 2.2 and 2.3 for Trials 1 3, respectively. Figure 2.3 shows OTU colour codes used in Figures 2.1, 2.2 and 2.3. It was immediately apparent that the overall structure of the caecal microbiota was quite different in each experiment. This can be most obviously seen in Trial 2 which was dominated by a *Bacteriodes* OTU whereas Trials 1 and 3 were dominated by *Lactobacillus* and *Clostridiales* OTUs, although the proportions varied between each trial. Also notable was the great variation in microbiota composition from bird to bird within any one trial.

Statistical tools were used to compare the microbiota composition both within groups from each trial and between trials. Monte Carlo analysis (Figure 2.4) indicated that all three trials had quite distinct microbiota and further analysis (Figure 2.5) identified the OTUs that were most responsible for differentiating the different trial and treatment groups.

Individual trials were also investigated using these analysis methods (Figures 6.1, 6.2 and 6.3). For Trial 1 (Figure 2.6.1), the Monte Carlo analysis showed that there was little separation between the high and low FCR birds on the standard diet (Diet 1) but when zinc bacitracin was added to the diet the microbiota changed and the caecal microbiota from the high and low FCR birds were distinctly different. In Trial 2 (Figure 2.6.2), the trial in which the microbiota was dominated by *Bacteriodes*,

the best performing birds fed the standard diet with and without avilamycin had similar caecal microbiota whereas the poor performing birds had somewhat different microbiota. In Trial 3 (Figure 2.6.3), the high and low performance birds on the sorghum diet had similar microbiota whereas the birds on the wheat-based diet had different microbiota and the microbiota in the high and low performance birds were fairly distinct.

Within each trial OTUs were identified that were present in differential abundance between the high performance (low FCR) and poor performance (high FCR) birds (Table 2.3). The differential abundance of these OTUs was generally restricted to a single treatment group and was not reflected across all trials.

Table 2.1. Summary of performance of birds from 15 - 25 days of age in three experiments

		FCR g feed; g gain			BWG g gain/bird			AME (MJ/kg dry matter)		
Trial	Diet	Overall	High	Low	Overall	High	Low	Overall	High	Low
1	Control	1.667	1.761	1.587	852	809	886	14.22	13.37	14.69
1	Zinc bacitracin	1.607	1.683	1.533	867	842	916	14.28	13.92	14.48
2	Control	1.494	1.569	1.433	912	909	937	14.40	14.03	14.54
2	Avilamycin	1.513	1.566	1.466	900	879	912	14.42	14.15	14.57
3	Wheat-based	1.471	1.536	1.423	845	831	819	14.44	14.34	14.48
3	Sorghum-based	1.503	1.555	1.455	885	867	907	14.71	14.60	14.74

High and low refer to results from 12 individual birds in the cohort with the highest and lowest FCR values for each diet in each trial.

Table 2.2. The number of sequence reads generated and analysed for each experiment.

Trial	Raw reads	Quality trimmed reads	Number of samples	Average reads per sample
1	320,607	301,534	95	3,174
2	335,273	326,560	96	3,402
3	456,756	447,325	92	4,862

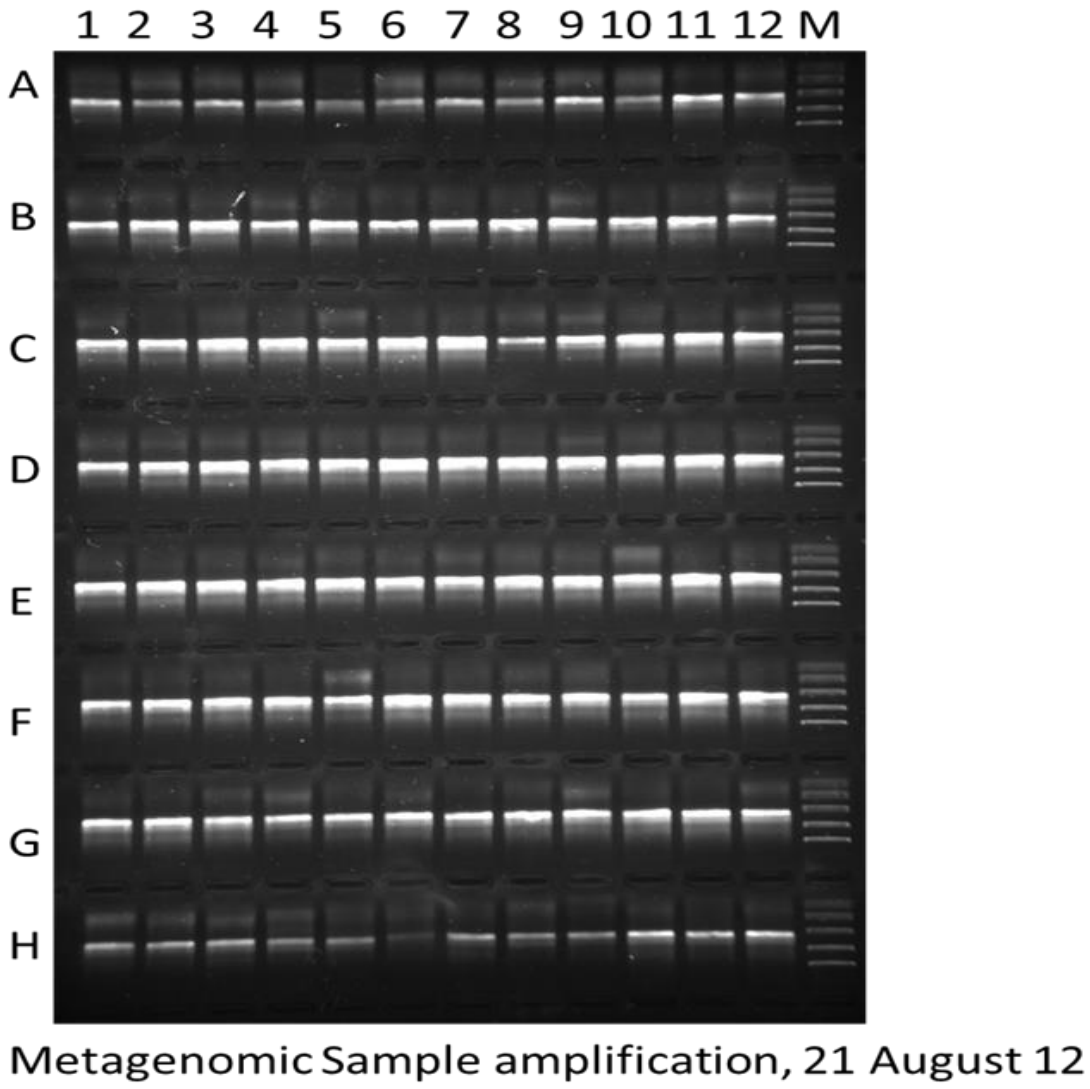


Figure 2.1. PCR amplification of V1-V3 region of 16S rRNA genes from DNA samples extracted from Trial 1 caecal samples.

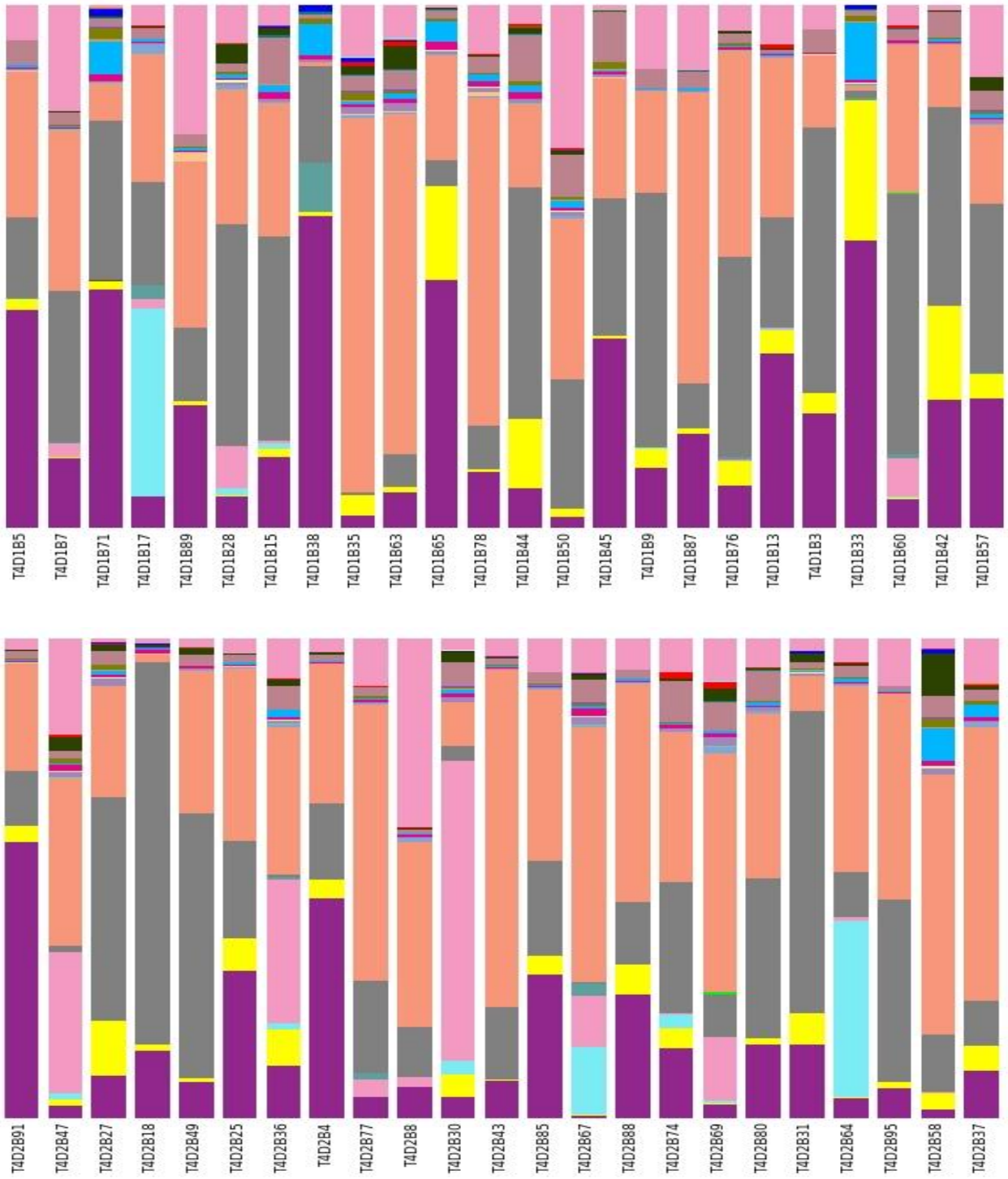


Figure 2.2.1. OTU analysis of caecal samples from Trial 1. OTUs from birds on Control diet are shown in the top panel. OTUs from birds on the diet with zinc bacitracin (50 ppm) are shown in the top panel. In each case the genus level analysis is shown for the birds with the best and worst FCR values. The data from the 12 birds with the lowest FCR within a group are shown first, followed by the data from the 12 birds with the highest FCR from that group. The colour code for the OTUs is constant across the trials (see Figure 2.3).

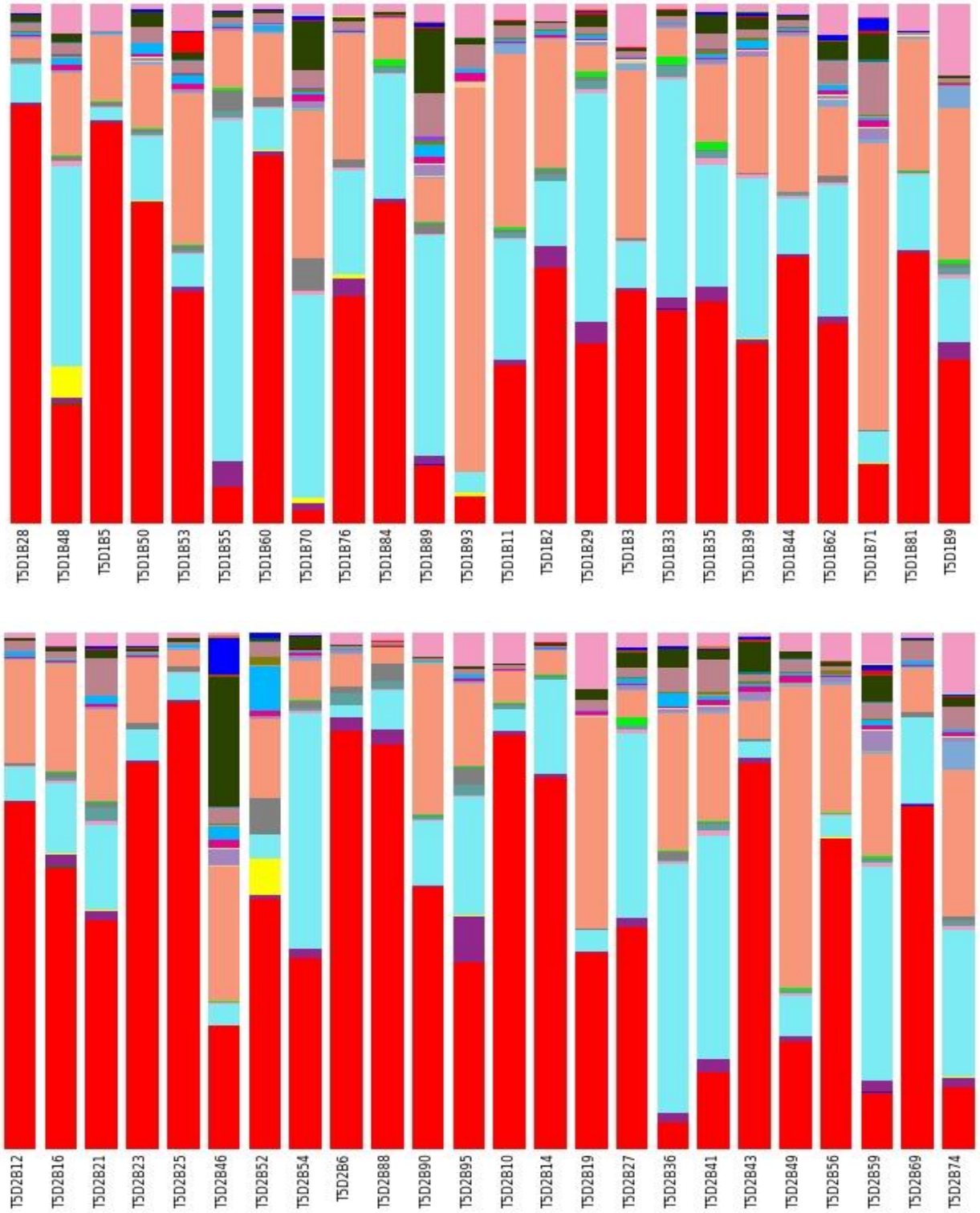


Figure 2.2.2. OTU analysis of caecal samples from Trial 2. OTUs from birds on Control diet are shown in the top panel. OTUs from birds on the diet with avilamycin (15 ppm) are shown in the top panel. In each case the genus level analysis is shown for the birds with the best and worst FCR values. The data from the 12 birds with the lowest FCR within a group are shown first, followed by the data from the 12 birds with the highest FCR from that group. The colour code for the OTUs is constant across the trials (see Figure 2.3).

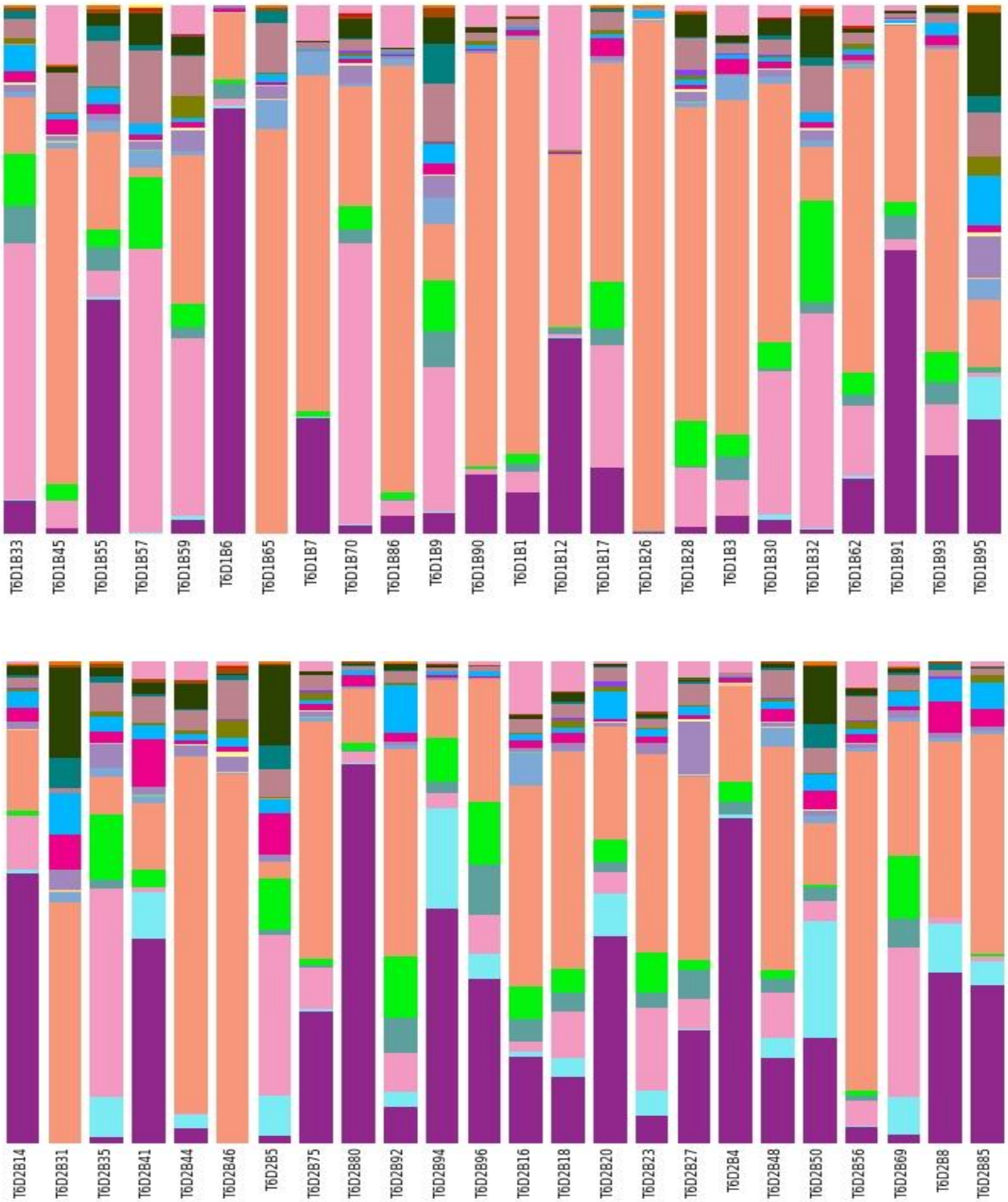


Figure 2.2.3. OTU analysis of caecal samples from Trial 3. OTUs from birds on the wheat diet are shown in the top panel. OTUs from birds on the sorghum diet are shown in the top panel. In each case the genus level analysis is shown for the birds with the best and worst FCR values. The data from the 12 birds with the lowest FCR within a group are shown first, followed by the data from the 12 birds with the highest FCR from that group. The colour code for the OTUs is constant across the trials (see Figure 2.3).

Figure 2.3: Legend showing OTU colour code for data presented in Figures 2.1, 2.2 and 2.3.

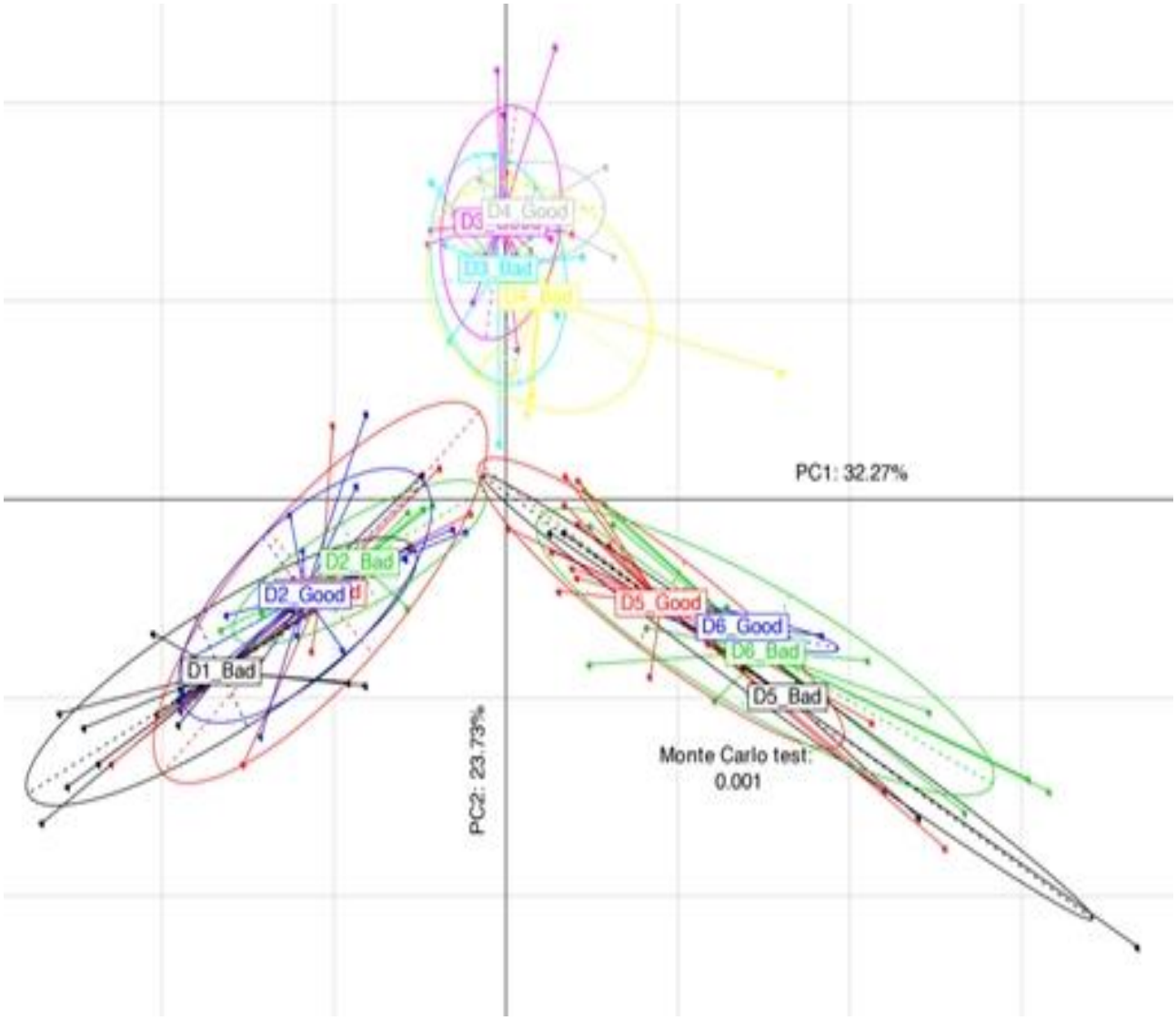


Figure 2.4. Monte Carlo analysis showing relatedness of the microbiota recovered from each bird. The groups are colour coded according to diet and performance level as indicated. Each dot represents an individual bird – distance from the other dots gives an indication of the relatedness of the microbiota – the closer the dots the more similar the microbiota. D1 is control diet from Trial 1, D2 is zinc bacitracin diet from Trial 1. D3 id control diet from Trial 2, D4 is avilamycin diet from Trial 2. D5 is wheat diet from Trial 3, D6 is sorghum diet from Trial 3.

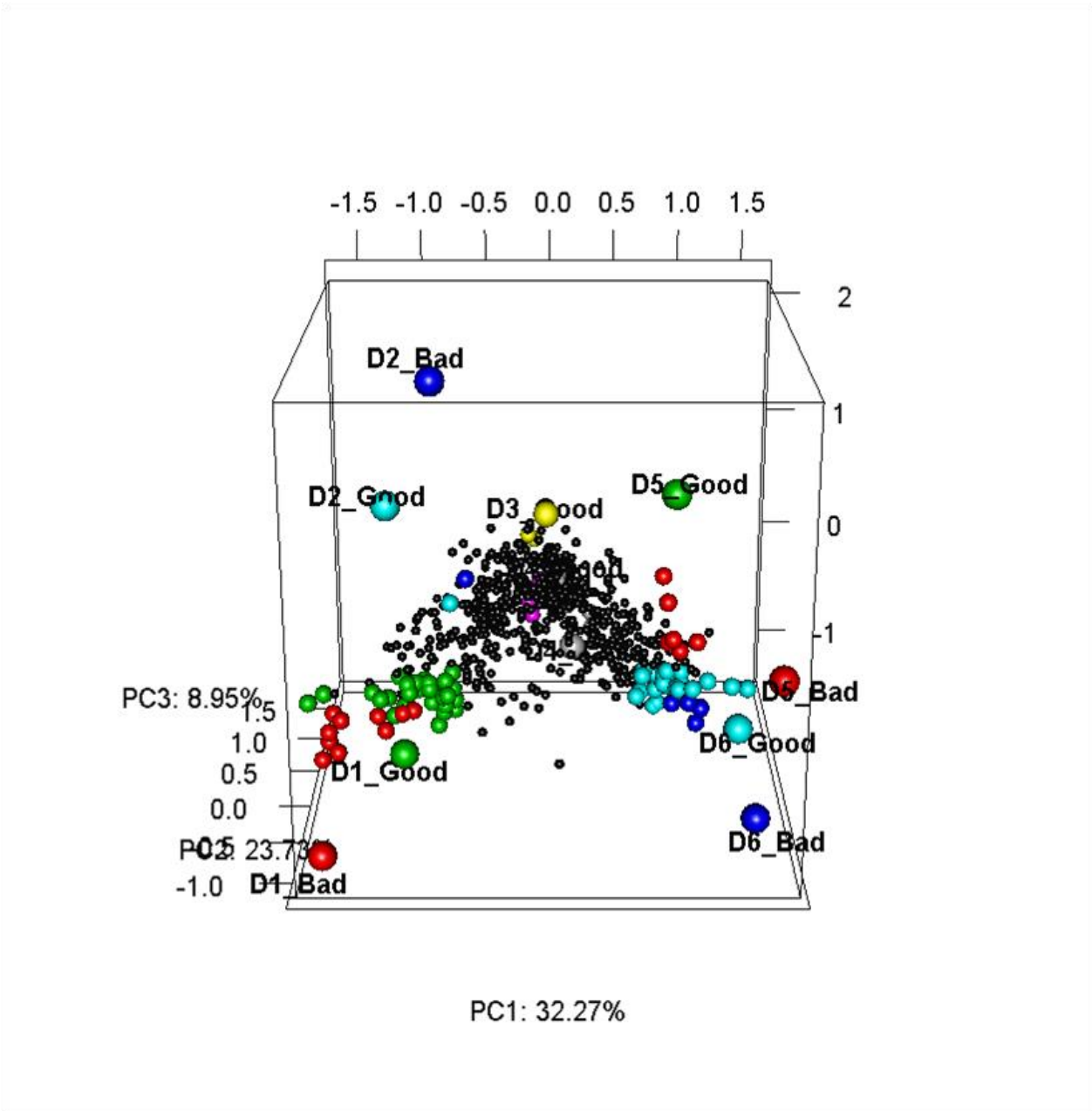
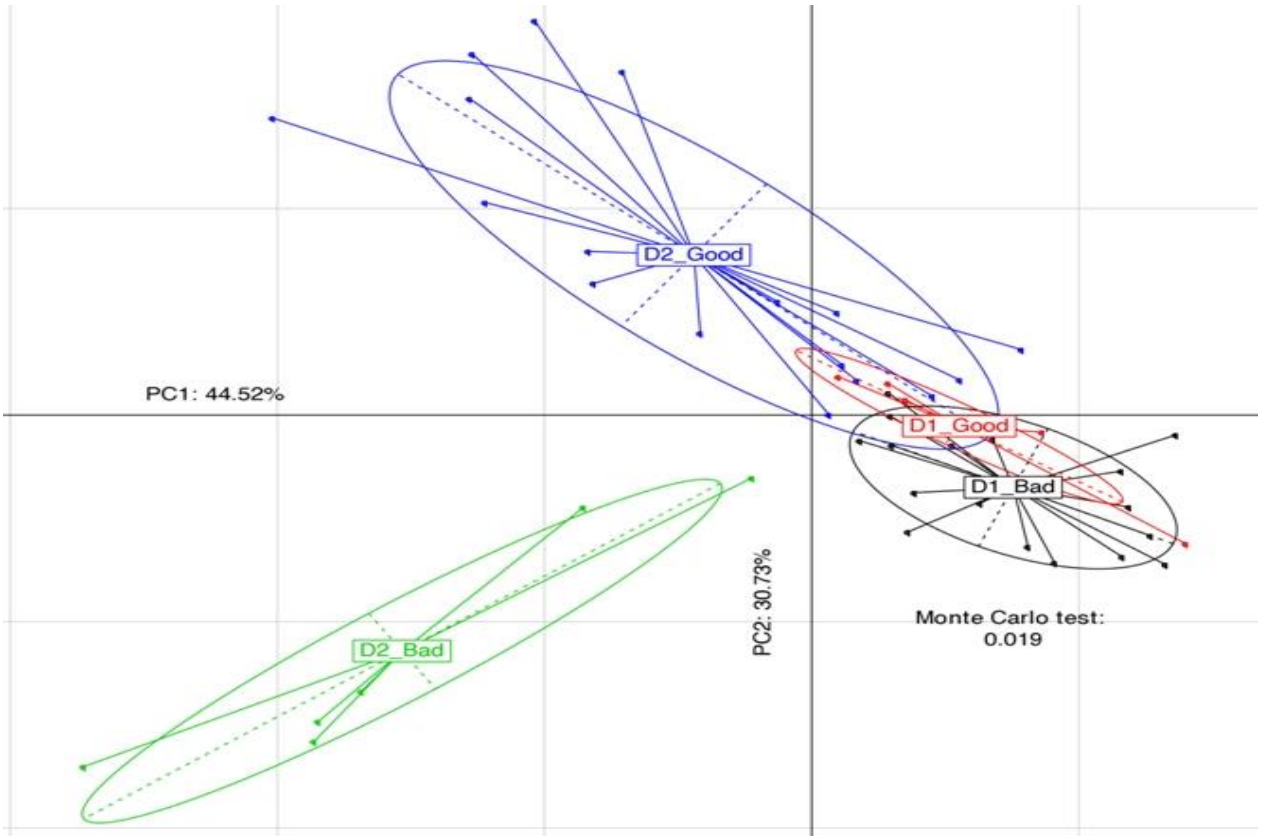


Figure 2.5. 3D PCA plot generated using Ade4 phylogenetic R package. The plot shows the distance between the groups (large dots) and the relationship between groups and OTUs (small dots) based on multivariate analysis. OTUs selected in multivariate analysis as a source of between group variance are coloured according to the colour of the group they separate, while non-significant OTUs are shaded black. D1 is control diet from Trial 1, D2 is zinc bacitracin diet from Trial 1. D3 id control diet from Trial 2, D4 is avilamycin diet from Trial 2. D5 is wheat diet from Trial 3, D6 is sorghum diet from Trial 3.



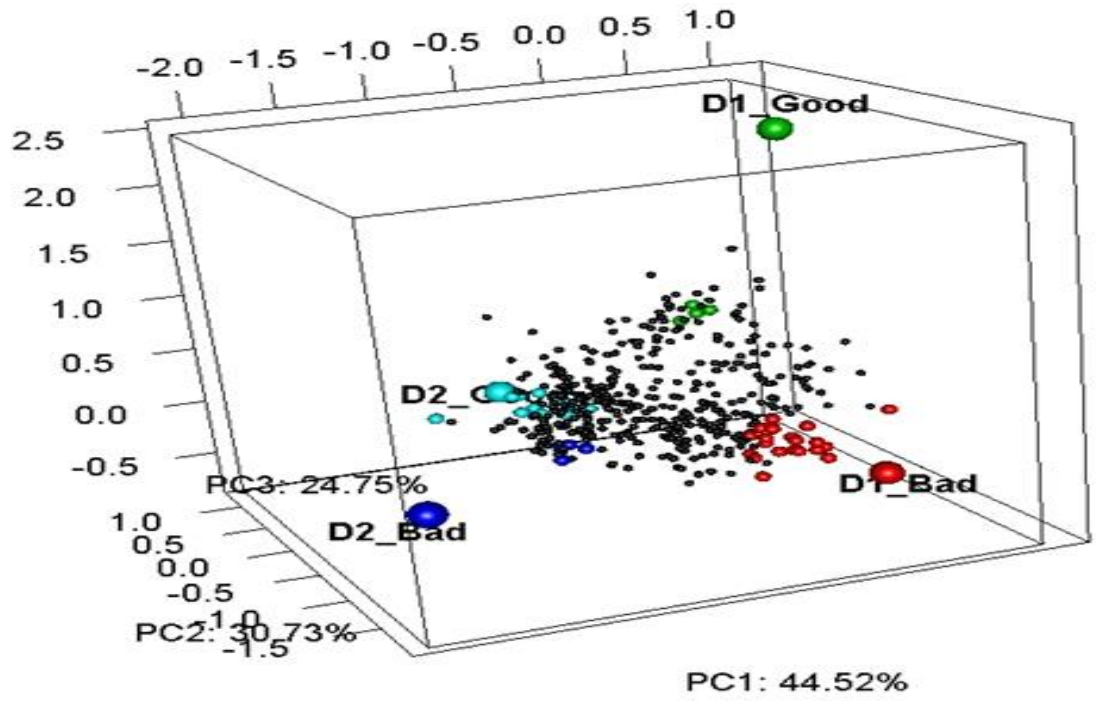


Figure 2.6.1. Monte Carlo analysis and 3D PCA plots of OTU data for Trial 1. D1 is control diet and D2 is zinc bacitracin diet.

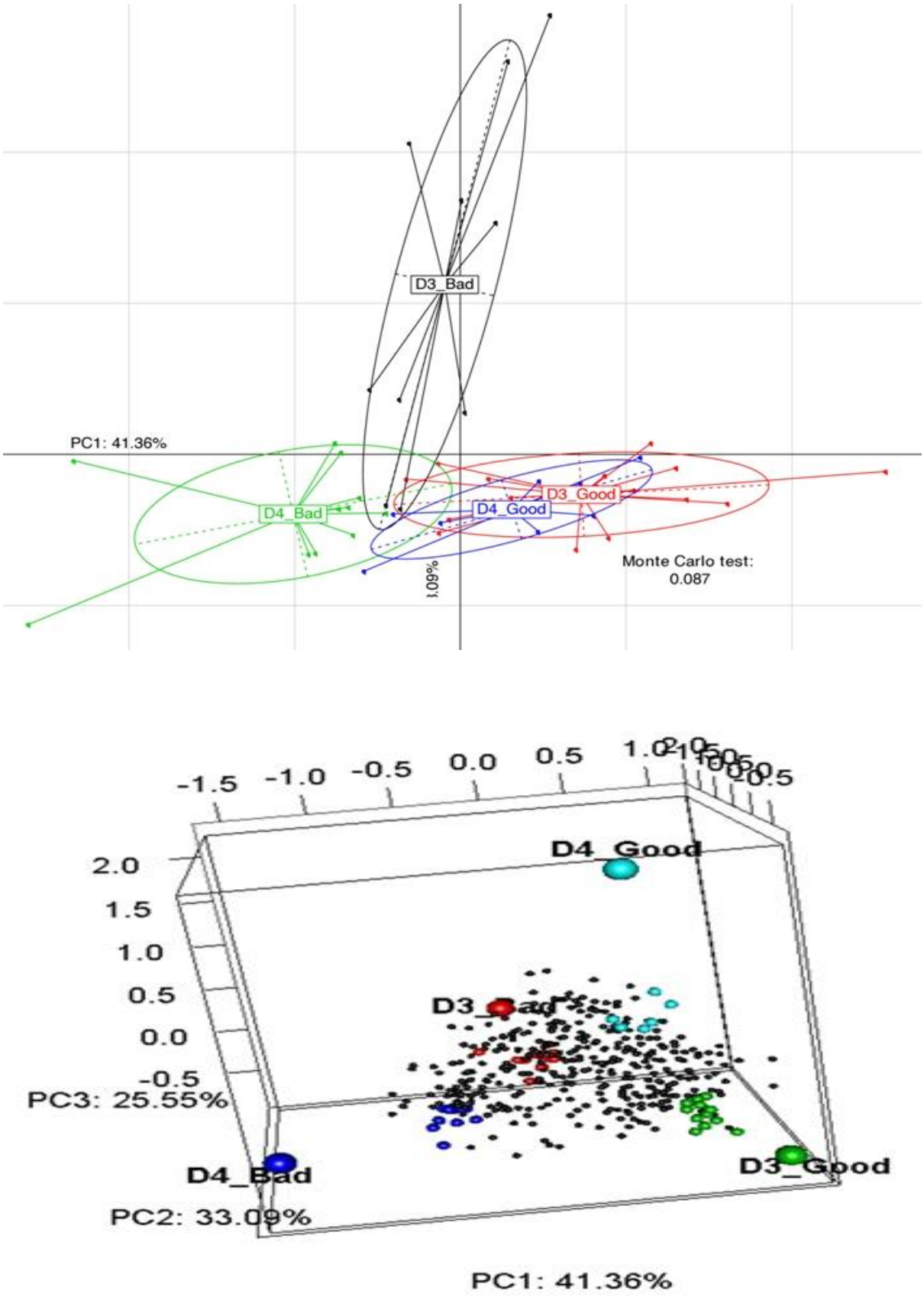


Figure 2.6.2. Monte Carlo analysis and 3D PCA plots of OTU data for Trial 2. D3 is control diet and D4 is avilamycin diet.

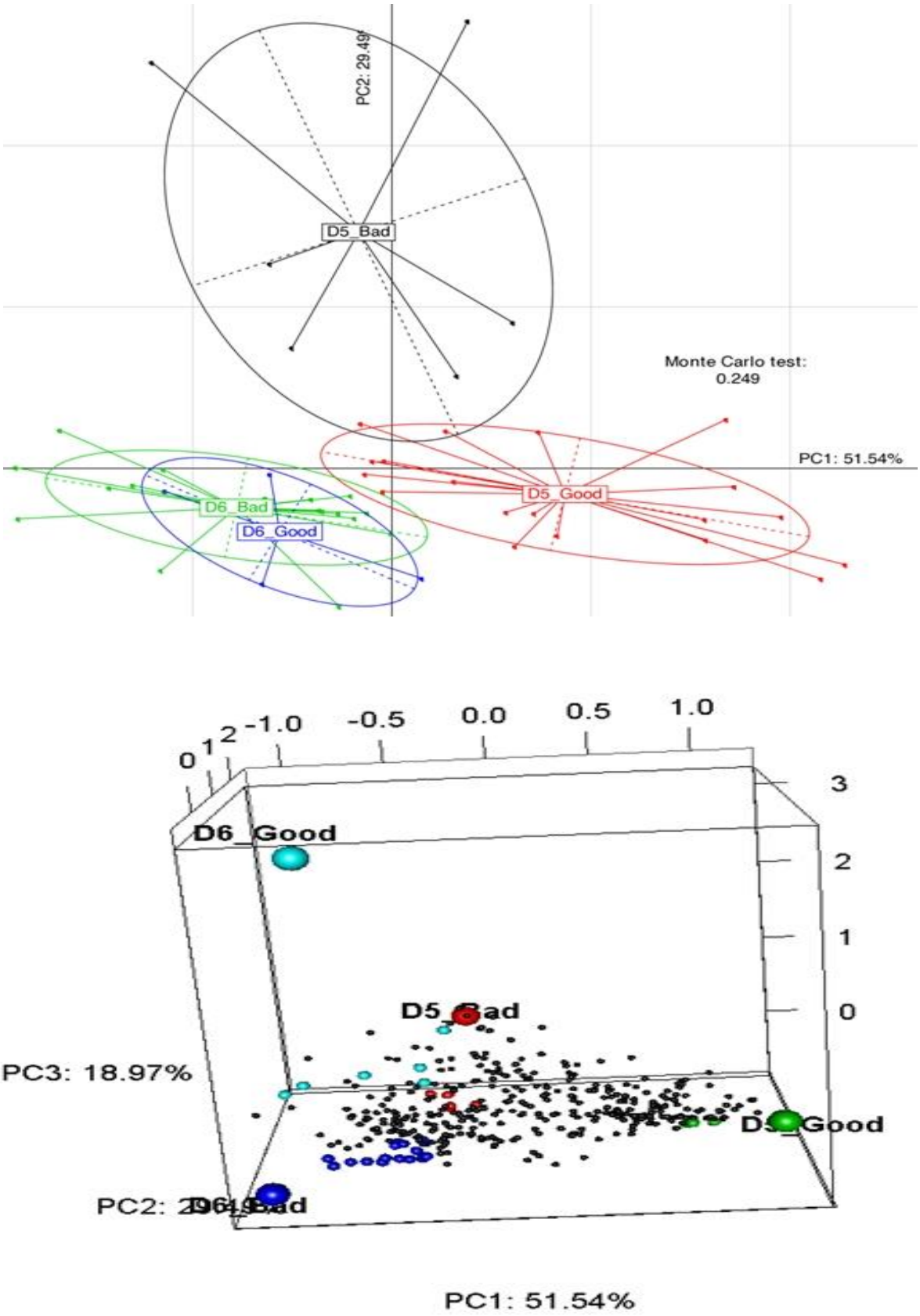


Figure 2.6.3. Monte Carlo analysis and 3D PCA plots of OTU data for Trial 3. D5 is the wheat diet and D6 is the sorghum diet.

Table 2.3. OTUs that had differential abundance between high and low FCR birds in at least one trial. P refers to probability and fold indicates fold difference. Differential values with probability <0.05 are highlighted in grey. The species listed is the nearest match

OTU	P	Fold	P	Fold	P	Fold	P	Fold	P	Fold	P	Fold	Organism
367	0.4182	2.1895			0.3282	0.0000			0.4771	0.5236	0.0035	inf	*Eubacterium hallii*
316	0.8275	1.3750							0.5979	1.9381	0.0102	inf	*Clostridium leptum*
428	0.3282	inf	0.4263	2.0932	0.4949	1.7624	0.5082	1.4722	0.7402	0.7485	0.0241	6.6979	*Lactobacillus helveticus*
103	0.3035	0.2042			0.6984	0.6667	0.8104	1.1925	0.3012	0.5004	0.0264	0.4059	*Oscillibacter valericigenes*
469	0.3299	13.5281	0.3502	inf	0.1491	3.2650	0.8384	0.8656	0.2394	0.3641	0.0488	7.1667	*Lactobacillus reuteri*
185	0.3282	inf	0.1922	0.0000	0.4453	1.2783	0.0333	2.7486			0.0488	inf	*Lactobacillus crispatus*
130	0.2952	4.5404	0.9655	0.9728	0.3539	1.6902	0.0183	inf	0.8995	1.1032	0.0700	inf	*Brevibacillus parabrevis*
369	0.9703	0.9655	0.4681	2.4909	0.1300	0.0534	0.0203	inf	0.1599	9.0000	0.0852	inf	*Fusibacter paucivorans*
746					0.0317	0.0000	0.3282	inf	0.7927	0.6786	0.1171	4.4800	*Clostridium lactatifermentans*
259			0.3071	0.0000	0.3282	inf	0.0384	0.0000	0.4228	0.4185	0.1268	inf	*Clostridium leptum*
379	0.0008	0.0998	0.1752	2.7884	0.5067	0.3846	0.8033	1.2080	0.8891	1.1463	0.1615	inf	*Anaerofilum agile*
719					0.3282	inf	0.4608	0.3797	0.0463	0.2994	0.2879	0.2023	*Blautia hansenii*
125	0.4665	1.5688	0.0801	0.2653	0.7398	0.6739	0.0421	5.7412	0.5634	0.7132	0.3070	1.5844	*Eubacterium fissicatena*
87	0.3287	0.2289	0.4610	1.6858	0.0506	3.5291	0.0315	88.7910	0.0854	0.0881	0.3282	inf	*Halobacillus profundi*
547	0.0407	inf	0.3071	0.0000	0.1532	3.7674	0.4785	2.1556	0.1548	0.0000	0.3282	inf	*Lutispora thermophila*
442	0.2279	0.2957	0.2458	0.2895	0.5308	0.6814	0.0404	11.0000	0.4853	0.4308	0.3282	0.0000	*Clostridium methylpentosum*

Table 2.3 (continued). OTUs that had differential abundance between high and low FCR birds in at least one trial. P refers to probability and fold indicates fold difference. Differential values with probability <0.05 are highlighted in grey. The species listed is the nearest match

OTU	P	Fold	P	Fold	P	Fold	P	Fold	P	Fold	P	Fold	Organism
415	0.3282	inf	0.1285	inf	0.1754	2.5672	0.0284	inf	0.8532	1.2000	0.3282	inf	*Lactobacillus delbrueckii subsp. bulgaricus*
340			0.2549	inf	0.0226	inf	0.1008	inf	0.9177	0.8974	0.3282	inf	
486	0.1501	0.0000	0.7766	1.2861	0.3672	1.8967	0.0114	12.8750			0.3282	0.0000	*Faecalibacterium prausnitzii*
448	0.0161	0.3347	0.2331	0.6444	0.0803	0.1429	0.0610	0.1400			0.3282	inf	*Lactobacillus salivarius*
355	0.0175	3.7989	0.2693	0.6870	0.9372	0.9670	0.9797	0.9860	0.5618	0.7345	0.3317	2.8460	*Butyricicoccus pullicaecorum*
4	0.0300	14.0582	0.7043	1.4332	0.2789	1.9518	0.1537	4.8227	0.5993	1.6560	0.3488	13.0897	*Butyricicoccus pullicaecorum*
197	0.1170	0.0000	0.0202	0.0393	0.6762	1.6087	0.5085	1.6753	0.5725	1.5763	0.3757	6.9532	*Butyricicoccus pcaecorum*
233	0.4538	0.3951	0.3546	0.3419	0.2029	4.0870	0.0380	inf	0.9435	1.0676	0.4025	3.5526	*Blautia hydrogenotrophica*
478	0.3282	inf	0.0326	0.0000	0.3385	1.5104	0.1066	3.6143	0.7750	1.4308	0.4088	1.9559	*Lactobacillus crispatus*
677	0.3282	inf	0.2187	10.0366	0.2876	7.9324	0.0352	18.7059	0.1978	0.2589	0.4285	0.4535	*Lactobacillus helveticus*
16	0.0224	0.1606	0.4378	0.5646	0.6616	0.7617	0.3518	2.1725	0.5332	1.7906	0.4305	2.1677	*Clostridium cellobioparum*
247	0.4903	1.8055	0.0493	0.2780	0.0730	0.3950	0.5561	1.5396	0.8039	0.7425	0.4322	0.4844	*Ruminococcus torques*
199	0.1002	12.8491	0.2568	4.0823	0.0237	4.0887	0.0256	21.3381	0.5980	1.8023	0.4493	4.0729	*Clostridium methylpentosum*
90	0.1473	2.6767	0.0278	0.3139	0.3418	0.5112	0.6927	1.3596	0.2794	9.5161	0.4575	2.4051	*Clostridium methylpentosum*
327	0.4700	1.6072	0.1126	0.3122	0.2827	2.0592	0.0216	12.5517	0.3918	0.3388	0.4948	1.9270	*Ruminococcus albus*
51	0.8455	1.1164	0.0980	0.4323	0.0299	3.2930	0.2973	1.9491	0.5409	0.6880	0.5114	1.7796	*Dorea longicatena*
6	0.0781	0.6267	0.7083	1.1766	0.0965	0.3641	0.0297	0.2385	0.9177	0.8621	0.5126	0.4576	*Lactobacillus salivarius*
20	0.5159	0.7710	0.7973	0.8941	0.5649	1.4365	0.0248	0.0000	0.5267	1.5877	0.5206	0.6731	*Lactobacillus taiwanensis*
41	0.0031	8.8679	0.1588	0.2016	0.4374	0.6173	0.2343	3.5480	0.2551	0.3135	0.5623	0.6954	*Clostridium aldrichii*

Table 2.3 (continued). OTUs that had differential abundance between high and low FCR birds in at least one trial. P refers to probability and fold indicates fold difference. Differential values with probability <0.05 are highlighted in grey. The species listed is the nearest match.

OTU	P	Fold	P	Fold	P	Fold	P	Fold	P	Fold	P	Fold	Organism
410	0.0394	0.3141	0.1103	0.4556	0.0942	3.3978	0.8996	0.9271	0.2311	2.7850	0.5946	0.6778	*Butyricicoccus pullicaecorum*
690	0.2350	6.8252	0.0488	0.2379	0.6627	0.6794	0.2343	3.9799	0.2775	0.0195	0.6136	1.5397	*Anaerofilum agile*
170	0.1390	0.1542	0.0393	0.2306	0.9205	0.9044	0.5045	0.5446	0.2193	inf	0.6495	2.0229	*Clostridium methylpentosum*
311	0.2614	0.4106	0.0844	0.3237	0.0324	6.3158	0.7765	1.2987	0.3001	0.2803	0.6619	1.5652	*Dorea longicatena*
255	0.9564	0.9527	0.0327	0.1205	0.4359	0.3500	0.2432	4.1422	0.1405	3.4286	0.7995	1.2498	*Clostridium termitidis*
368	0.3282	inf	0.3071	0.0000	0.0420	0.2797	0.9818	1.0176	0.3041	0.3646	0.8739	1.1111	*Lactobacillus crispatus*
658	0.0802	0.0000	0.0459	0.0000	0.4110	2.7846	0.2511	inf	0.3282	inf	0.8757	1.2187	*Butyricicoccus pullicaecorum*
112	0.0081	inf	0.1352	4.5356	0.1697	2.2716	0.1370	2.4647	0.2373	inf	0.9055	0.8988	*Subdoligranulum variabile*
105	0.7343	0.8170	0.0145	0.3601	0.3986	0.3132	0.9943	0.9962	0.1358	33.4655	0.9070	0.8828	*Dorea longicatena*
166	0.9854	0.9866	0.9710	1.0247	0.1478	0.2429	0.0078	7.4857	0.8121	0.8125	0.9237	0.8966	*Anaerofilum agile*
687	0.0544	6.4423	0.6676	1.4092	0.3293	2.3011	0.0397	inf	0.3846	0.1078	0.9492	0.9237	*Clostridium leptum*
484	0.9159	0.8592	0.0253	0.0000	0.2162	0.0000	0.1889	3.2063	0.5257	1.9492	0.9943	0.9913	*Eubacterium hallii*
300			0.3071	0.0000	0.3318	0.3940	0.0040	7.1348	0.3624	0.0677			*Lactobacillus crispatus*
384	0.2313	5.4786	0.2493	3.6313	0.0150	10.8170	0.0184	33.8824	0.7111	0.6507			*Clostridium methylpentosum*
241	0.3282	inf	0.9530	0.9572	0.5086	1.5845	0.0145	10.0184	0.7190	0.6567			*Lactobacillus crispatus*
667	0.0271	0.4224	0.6001	1.2771	0.7338	0.8437	0.2468	0.3831					*Lactobacillus salivarius*

Table 2.3 (continued). OTUs that had differential abundance between high and low FCR birds in at least one trial. P refers to probability and fold indicates fold difference. Differential values with probability <0.05 are highlighted in grey. The species listed is the nearest match

OTU	P	Fold	P	Fold	P	Fold	P	Fold	P	Fold	P	Fold	Organism
190	0.0455	0.1366	0.9043	0.9264	0.1123	0.0000	0.3280	0.0530					*Lactobacillus agilis*
256	0.0472	0.1055	0.8636	0.8513	0.1522	0.0000	0.3282	0.0000					*Lactobacillus salivarius*
390	0.0143	0.0284	0.5685	0.4711	0.3213	0.2128	0.3282	0.0000					*Lactobacillus salivarius*
496	0.0294	18.3333	0.9714	0.9500	0.3282	inf	0.3282	0.0000					*Lactobacillus salivarius*
215	0.0455	0.1773	0.9046	1.1324	0.4500	0.2135	0.3282	0.0000					*Lactobacillus salivarius*
201	0.0183	0.0000	0.3071	0.0000			0.3282	0.0000					*Saccharofermentans acetigenes*
94	0.0493	0.0978	0.3502	inf			0.3282	inf					*Subdoligranulum variabile*
739	0.5635	1.4122	0.0398	0.0000	0.3282	inf	0.5572	0.3654					*Syntrophomonas curvata*
286	0.0481	0.2975	0.6487	1.4276									*Lactobacillus salivarius*

Discussion

A series of three experiments were performed. In each experiment two diets were used. Birds were reared on litter, then housed individually in metabolism cages at 15 days of age for monitoring of individual bird performance (FCR and weight gain), and AME. Birds were killed following this AME period and tissue was collected for in-depth analysis of gut microbial profiles. Birds were then classified as high or low-performing birds based on performance data. Microbial profiles were determined by Roche 454 pyrosequencing.

The microbial profiles told a very similar story to that described in Chapter 1. That is, the birds in each trial had distinctly different microbiota and there was a considerable variation from bird to bird within each trial. There were operational taxonomic units (OTUs) that were in differential abundance in the high and low performance birds fed each diet but because of the between trial variation in overall microbiota composition none of these differential OTUs were seen in all diet groups across three experiments conducted in a similar manner.

Conclusion

The results from three similar experiments further demonstrated that there is extensive inter- and intra-trial variation in the microbiota composition of birds, as was the case described in Chapter 1. This required a re-evaluation of the best way to proceed with subsequent experiments described in Chapter 3.

Chapter 3. Inoculation of newly hatched chickens by gross microbial transfer from healthy chickens

Introduction

Colonisation of the gastrointestinal tract is thought to start immediately after hatching and therefore the hatching environment has a major influence on a chicken's microbial profile. Large differences in microbiota profiles has also been reported in chickens (Torok *et al*. 2011) and turkeys (Scupham 2009). This kind of variation has also been found in the GIT microbiota of humans and other animals (Zoetendal *et al.* 1998; Simpson *et al.* 2000) and has been attributed to both host and environmental factors.

Amongst the animal production systems, poultry are somewhat unusual in that the young are generally separated from the parents and hence there is a markedly reduced parental influence on the development of microbiota. Within commercial hatcheries, hygiene measures reduce the bacterial load in the hatching environment to limit the spread of bacterial pathogens. A consequence of this is that newly hatched chicks are exposed to a diverse range of bacteria from environmental sources such as human handlers, bedding material, feed and transport boxes, rather than from parental sources. Widely varying colonisation of the chicken GIT may be a consequence of the high diversity in non-avian bacterial sources and lack of parental bacteria during the first hours and days of life (Fuller 1989; Stanley *et al.* 2013b). This situation is somewhat analogous to the finding that human infants delivered in hospitals can harbour abhorrent microbiota rather than human specialised microbiota (Fryklund *et al.* 1992) and infants delivered by caesarean section have been shown to have different microbiota compared to naturally delivered infants (Dominguez-Bello *et al.* 2010). However, a key difference with chickens is that the eggs are physically separated from hens on breeder farms and hence once eggs have been washed or fumigated prior to hatching, there is no contact with adults during incubation.

Our experiments described in Chapters 1 and 2 demonstrated that there is extensive inter- and intra-flock variation in the gut microbiota composition of birds. In other words, the supposedly sterile guts of the hatchlings initially have been seeded by different bacteria from the environment. Clearly, the "luck of the draw" approach to egg and incubator hygiene in commercial hatcheries does not achieve uniformity or consistency in establishment of a healthy gut microbiota. In these two experiments, we took a more direct approach by coating egg shells during incubation with caecal contents from healthy chickens in experiment described in Chapters 1 and 2. That is, we took a more "natural" approach that simulated how the hen inoculates her eggs during incubation and her chicks post-hatch in an attempt to achieve consistent gut microbiota within and between

batches of chickens. Two experiments designated P3E1 and P3E2 were conducted in this series. The same inoculation treatments of fertile eggs were applied in both experiments, as were the same procedures for incubation, inoculation, rearing of chickens, energy metabolism and tissue collection.

Materials and methods

Animal ethics

The animal ethics committees of The University of Adelaide and Primary Industries and Regions South Australia approved this study.

Experimental design and statistical analysis

The metabolism component of Experiment P3E1 involved three inoculation treatments in a completely randomised design (n = 32 cages per treatment. These were (1) control phosphate buffered saline (PBS) solution, (2) caecal inoculum #1, and (3) caecal inoculum #2. The sex of each chicken was determined by visual observation of gonads when dissected to obtain tissue samples. Experiment P3E2 was a repeat of the first experiment.

SAS for Windows version 9.4 software package (SAS Institute Inc., Cary, NC, USA) was used to determine whether data were normally distributed (Univariate procedure; Shapiro-Wilk test), then two-way analysis of variance with the General Linear Model (GLM) procedure was used to examine the factors inoculation treatment and sex of chicken, and the interaction between treatment and sex. Significant differences between treatments and sex were determined by Duncan's Multiple Range Test.

Incubation and inoculation of fertile eggs

Cobb 500 fertile eggs (total 150) obtained from the Baiada Hatchery, Willaston, South Australia were incubated for 18 days in a single incubator (IM Incubators model IM288; 38ºC, 55% relative humidity, turning hourly). Fertile eggs were allocated by weight to three inoculation treatments and transferred to three separate incubators (Intensive Farming Supplies, Cavan, SA 5094, model MPS24 A; 36.7ºC and 66% relative humidity). On the day before hatching, 12-15 eggs at a time were removed from the incubators, wiped with 70% ethanol then inoculated with (1) control PBS solution, (2) caecal contents #1 diluted in PBS, and (3) caecal contents #2 diluted in PBS. The caecal contents were obtained from healthy chickens in experiment described in Chapters 1 and 2, and the inocula differed in microbial composition. Each inoculum (0.3 ml) was placed on a new cotton bud and painted onto the upper portion of the shell (blunt end up). Eggs were returned to the incubator after less than 10 minutes.

Rearing and energy metabolism

Newly hatched chickens were weighed individually then transferred in inoculation treatment groups to three separate floor pens in a temperature controlled room (day 0). All chickens received a proprietary diet formulated for this breed of chicken (starter/grower crumbles from Ridley Agriproducts, Murray Bridge, SA 5253) until 15 days of age, then were transferred in pairs to metabolism cages in in a controlled temperature room. The usual AME procedure commenced on day 17 when birds were weighed then housed singly in metabolism cages. All birds continued to receive the starter/grower diet. Birds had 23 h light for the first 3 days and then 12 h light for the remainder of the experimental period. Birds were fed *ad libitum* and had access to water from drinking nipples at all times.

The apparent metabolisable energy (AME) values of the commercial broiler diet were determined in a classical 7-day AME study involving measurements of total feed intake and total excreta output and subsequent measurement of gross energy values of feed and excreta by isoperibol bomb calorimetry (as described in Chapter 1).

Feed intake was measured during the adaptation and collection phases of the study. All excreta were collected daily during the 4-day collection phase and dried overnight at 90°C. Birds were weighed at the start and end of the 7-day period then retained in the cages until 27 days of age.

Feed consumption was measured by weighing the feed at the start (day 17) and end (day 24) of the AME period. On day 24, feed was weighed into hoppers and the residue weighed on day 27 in order to provide feed intake and feed conversion ratio data up until the time of kill. All 96 birds were killed by cervical dislocation and tissue samples were collected for analysis of microbial communities and gut histology.

Tissue collection

All 96 birds were killed at a time away from other birds and weighed. A cloacal swab was taken from each bird. Domestic cotton buds were used in the first experiment and micro swabs were used in second experiment.

Tissue scrapings by glass slides (experiment 1) and micro swabs (in experiment 2) were taken from the following organs after rinsing in cooled PBS solution:-

- Crop
- Proventriculus
- Gizzard
- Duodenum (mid section)

- Jejunum (mid section)
- Ileum (mid section)
- Caecum (mid section)
- Rectum (mid section)

A small amount (1 ml) of contents from each caecum was collected, with one sample clearly identified as the one which provided the scraping or swab. Remaining contents from that caecum were retained for future experiments. In addition to the above samples, 2 cm sections were taken from duodenum, jejunum, ileum and caecum directly distal to the section used for tissue scraping or swabbing. The sections were rinsed with cooled PBS and a 1 cm section was placed in 10% buffered for histological analysis, and the other 1 cm section was placed in a 5 ml tube and into liquid nitrogen for enzyme assays and immunohistochemistry**.** Samples intended for DNA sequencing by Dr Rob Moore at CSIRO were stored in ice initially, then frozen and transported in dry ice to the CSIRO Animal Health Laboratories, Geelong. Following extraction of DNA, aliquots were sent to the Ohio State University for further examination by PITChip technology discussed in Chapter 10.

DNA preparation, sequencing, and data analysis

DNA was prepared, sequenced and analysed as detailed in Chapters 1 and 2 and by Stanley *et al*. (2013b) in Appendix 1. Basically, the primers used to PCR amplify the V1-V3 region of the 16S rRNA gene were (forward primer, 5' AGAGTTTGATCCTGG 3'; reverse primer), 5' TTACCGCGGCTGCT 3'). Sequencing was performed using a Roche/454 FLX Genome Sequencer. Sequences were analysed using PyroBayes, pintail chimera detection algorithm and Qiime v1.6.0. Sequences were quality trimmed with length from 300 to 600 nt, no ambiguous nucleotides and maximum 6 homopolymers. OTU picking was done at 3% divergence level, de-noising error-correction was done using Acacia software, abundance and 3 amplicon estimation with UCLUST algorithms. OTUs represented with less than 10 sequences and present in less than 5 samples were filtered out of the analysis. Qiime generated abundance table based data were visualised in Calypso.

Results

Live weight, feed intake, feed conversion and metabolisable energy

The effects of inoculation treatments and sex of chicken on live weight, feed intake, feed conversion ratio and apparent metabolisable energy of the diet are summarised in Tables 3.1 and 3.2 for experiments P3E1 and P3E2, respectively.

Table 3.1. Experiment P3E1 - Effects of inoculation treatments and sex of chicken on live weight at the start of the 7-day metabolism study (BW15, in g/bird), live weight at the end (BW22, in g/bird), feed intake (FI, in g/bird), feed conversion ratio (g feed: g gain) and apparent metabolisable energy of the diet (AME, in MJ/kg dry matter basis)

Summary of analysis of variance

	BW15	BW22	FI	FCR	AME
Treatment (T)	***	**	ns	ns	ns
Sex (S)	*	***	**	***	ns
T x S	ns	ns	ns	ns	ns
Mean	621	1149	741	1.412	15.38
CV	7.6	9.3	10.3	5.1	2.8

*** P < 0.001, ** P < 0.01, * P < 0.05, ns P > 0.05, CV is the coefficient of variation

Effects of inoculation treatments

Treatment	Chickens	BW15	BW22	FI	FCR	AME
Control	30	638 a	1189 a	762 a	1.387 b	15.46 a
Inoculant #1	32	596 b	1105 b	720 b	1.426 a	15.31 a
Inoculant #2	31	630 a	1154 ab	742 ab	1.422 ab	15.37 a

Means within the same column with a common letter are not significantly different P > 0.05

Effects of sex of chicken

Sex	Chickens	BW15	BW22	FI	FCR	AME
Female	48	613 b	1115 b	720 b	1.440 a	15.42 a
Male	45	630 a	1184 a	763 a	1.383 b	15.33 a

Means within the same column with a common letter are not significantly different P > 0.05

Table 3.2. Experiment P3E2 - Effects of inoculation treatments and sex of chicken on live weight at the start of the 7-day metabolism study (BW15, in g/bird), live weight at the end (BW22, in g/bird), feed intake (FI, in g/bird), feed conversion ratio (g feed: g gain) and apparent metabolisable energy of the diet (AME, in MJ/kg dry matter basis)

Summary of analysis of variance

	BW15	BW22	FI	FCR	AME
Treatment (T)	ns	ns	ns	ns	ns
Sex (S)	***	***	***	***	ns
T x S	ns	ns	ns	ns	*
Mean	624	1190	759	1.346	14.51
CV	9.2	8.7	9.6	4.3	1.7

*** P < 0.001, ** P < 0.01, * P < 0.05, ns P > 0.05, CV is the coefficient of variation

Effects of inoculation treatments

Treatment	Chickens	BW15	BW22	FI	FCR	AME
Control	32	612 a	1176 a	755 ab	1.342 a	14.56 a
Inoculant #1	32	616 a	1167 a	735 b	1.339 a	14.53 a
Inoculant #2	32	644 a	1226 a	789 a	1.358 a	14.44 a

Means within the same column with a common letter are not significantly different P > 0.05

Effects of sex of chicken

Sex	Chickens	BW15	BW22	FI	FCR	AME
Female	43	593 b	1109 b	704 b	1.368 a	14.52 a
Male	53	649 a	1256 a	804 a	1.329 b	14.50 a

Means within the same column with a common letter are not significantly different P > 0.05

Effect of the interaction between treatment and sex on AME of the diet

Sex	Chickens	Control	Chickens	Inoculant #1	Chickens	Inoculant #2
Female		14.50 ab		14.51 ab		14.57 a
Male		14.60 a		14.55 a		14.35 b

Means with a common letter are not significantly different P > 0.05

Microbiota in tissue samples

The birds were sampled at multiple time points from day 0 to day 23. However, the microbiota detected in cloacal swabs on day 0 were very low in concentration and most of the samples failed to sequence. Therefore only results from day 1 to day 23 are presented here. There were no significant differences in community diversity between control and two inoculation treatments in either experiment (data not presented), hence the research team decided that no further analyses of data would be undertaken at this point. The timelines for community development in caecal swabs from all three treatments were similar and showed significant differences in diversity as chickens aged from 1 to 23 days.

Species richness (number of different species in the samples) and evenness (how close were numbers of each species in the samples) are summarised in Figures 3.1 and 3.2, respectively.

Community structure underwent enormous perturbations during the first few days after hatch, especially in the first 48 hours. Figure 3.3 shows these changes simplified to a genus level. In both experiments, *Enterococcus* predominated in the cloacal samples in the first 24 hours post hatch then *Lactobacillus* dominated in the next 24 hours (Figure 3.3). *Faecalibacterium* appeared as the community matured.

Overall, only a few genera significantly altered in relative abundance in the period day 1 to day 23. These were *Lactobacillus*, *Enterococcus* and *Faecalibacterium* in Experiment 1, and *Enterococcus* and *Faecalibacterium* in Experiment 2.

Discussion

Inoculation treatments had significant effects on live weight, but not on feed intake, feed conversion or apparent metabolisable energy value of the diet in the first experiment. In contrast, in the second experiment, inoculation treatments had no effect on live weight, feed intake, and feed conversion, but appeared to have a small but significant effect on apparent metabolisable energy with inoculant #2 depressing AME value by 1.6% for male chickens. In both experiments, male chickens were heavier, ate more feed and converted less efficiently than female chickens, as was expected, but there were no differences in AME values.

There were no significant differences in community diversity between control and two inoculation treatments in either experiment, but there were significant changes in diversity of microbiota with time. Community richness increased the most rapidly in the first three days, then continued to grow, but at a lower rate in subsequent days. In the same time, while the richness was increasing, new bacterial species became evident as these successfully colonised and proliferated in the gastrointestinal tract of newly hatched chickens.

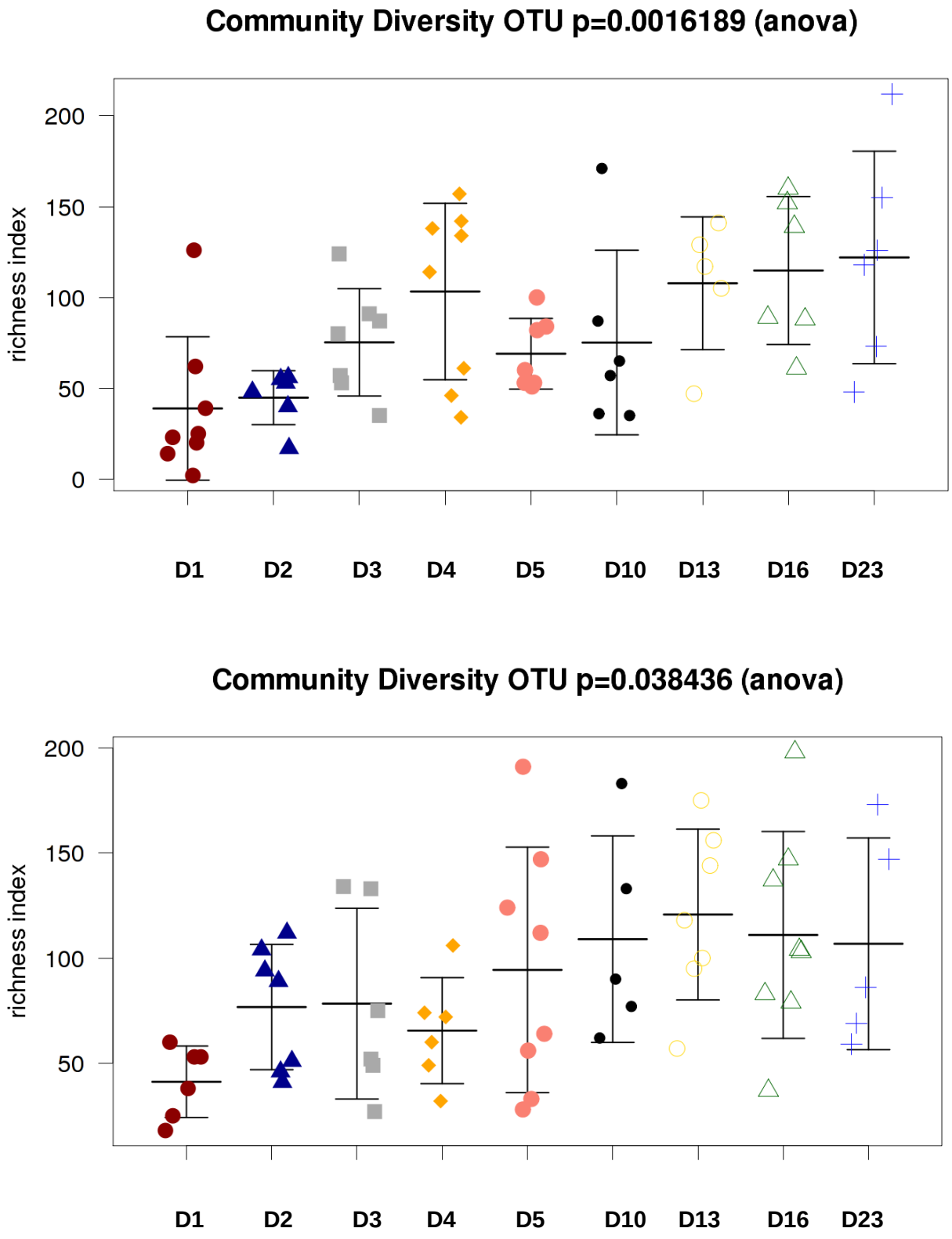


Figure 3.1. Changes in community richness in the first experiment (top section) and second experiment (bottom section) as chickens aged. Richness is defined as the number of different species in cloacal samples

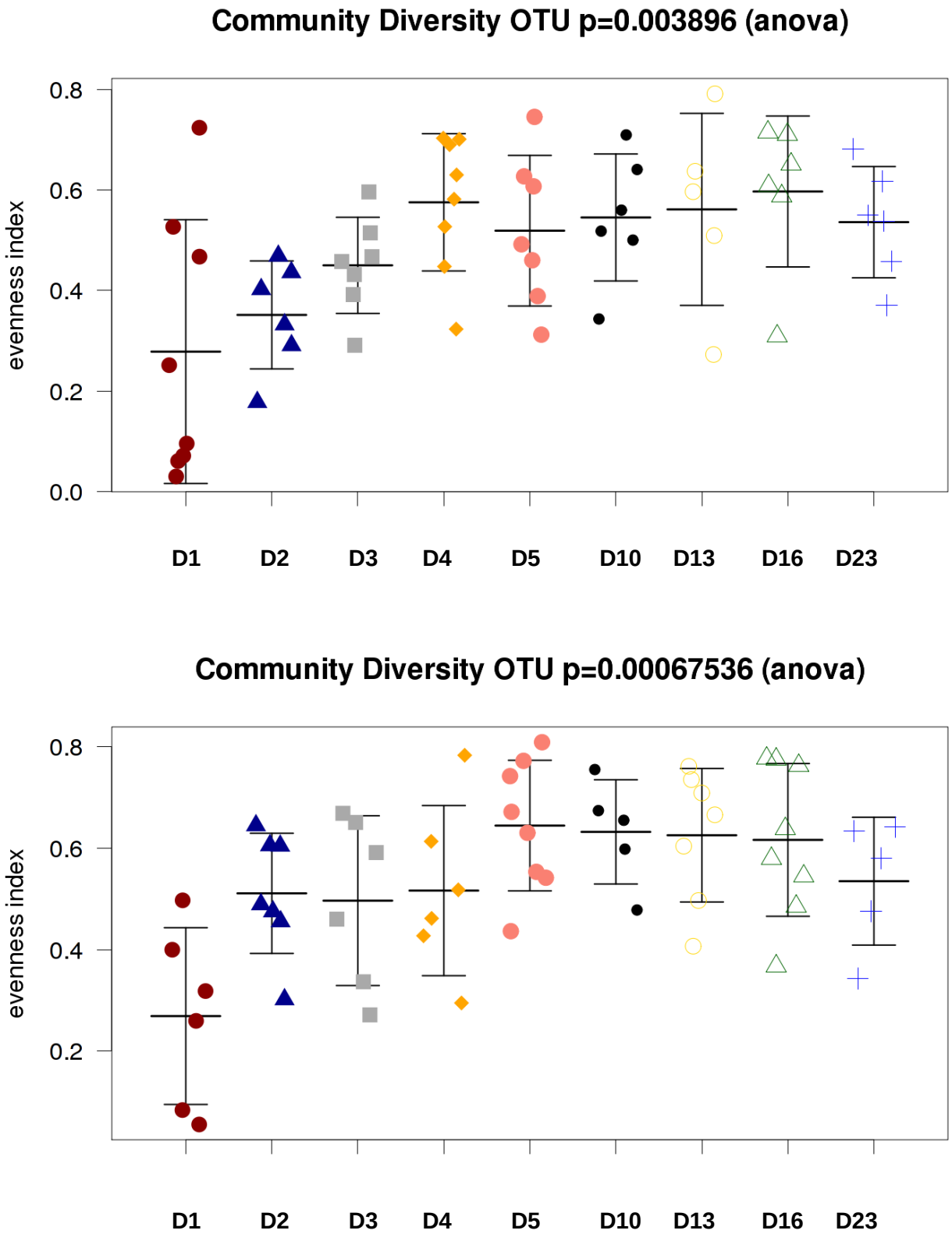


Figure 3.2. Changes in evenness in the community in the first experiment (top section) and second experiment (bottom section) as chickens aged. Evenness is defined as closeness in the numbers of each species in cloacal samples.

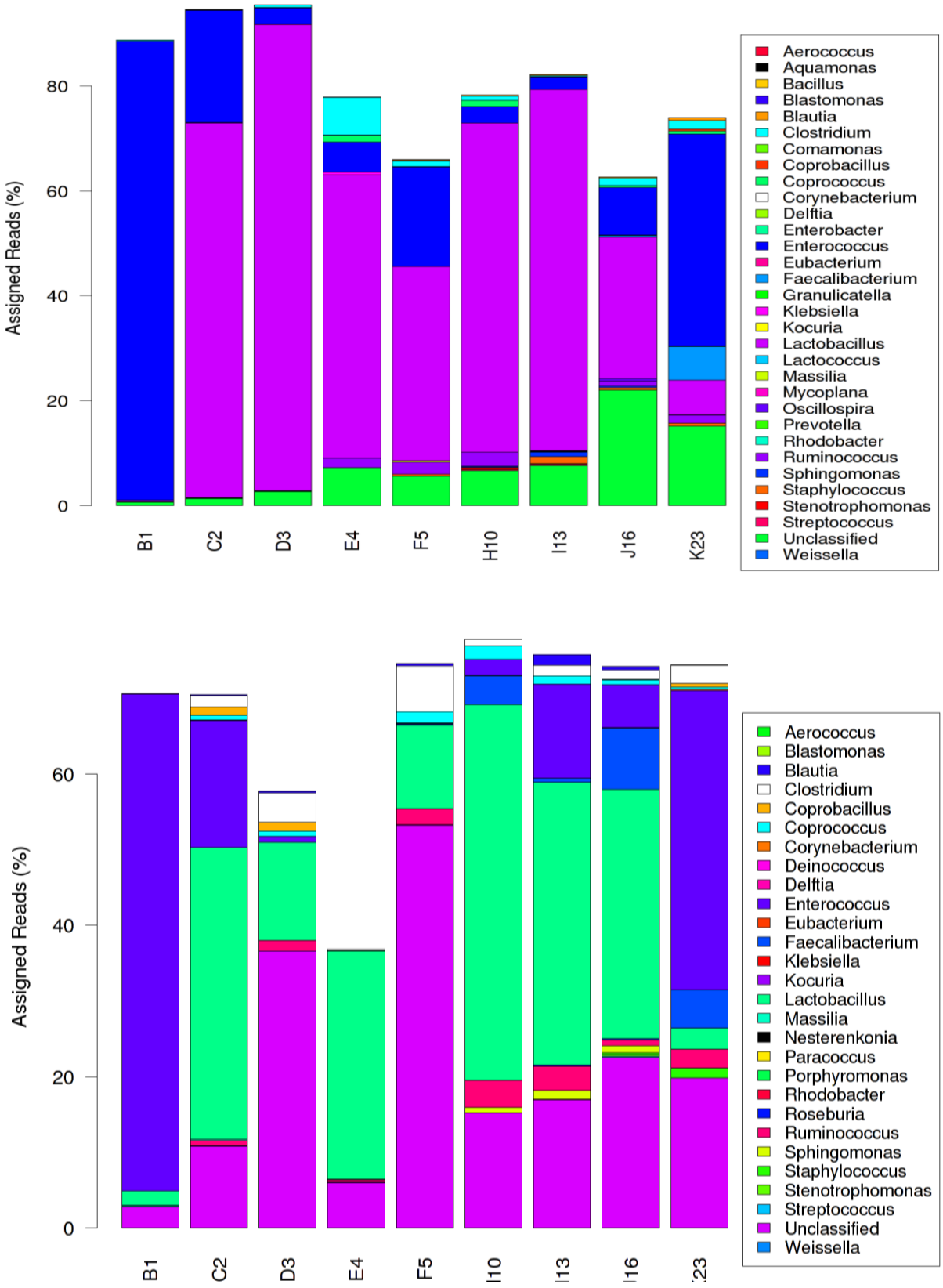


Figure 3.3. Changes in bacterial genera in cloacal samples in the first experiment (top section) and second experiment (bottom section) as chickens aged. Only the most abundant genera (>2% of total abundance) are presented.

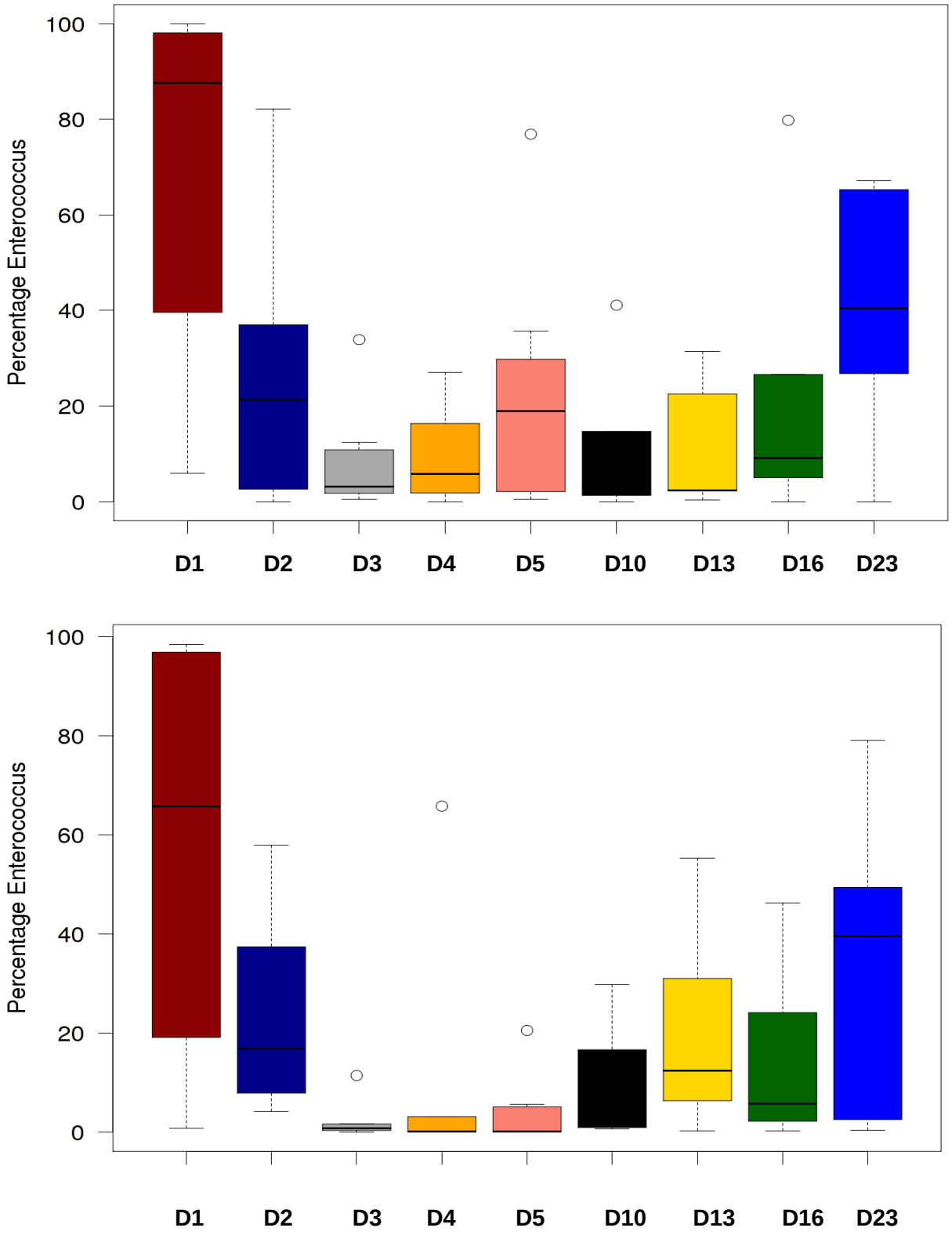


Figure 3.4. Changes in abundance of genus *Enterococcus* in cloacal samples in the first experiment (top section) and second experiment (bottom section) as chickens aged.

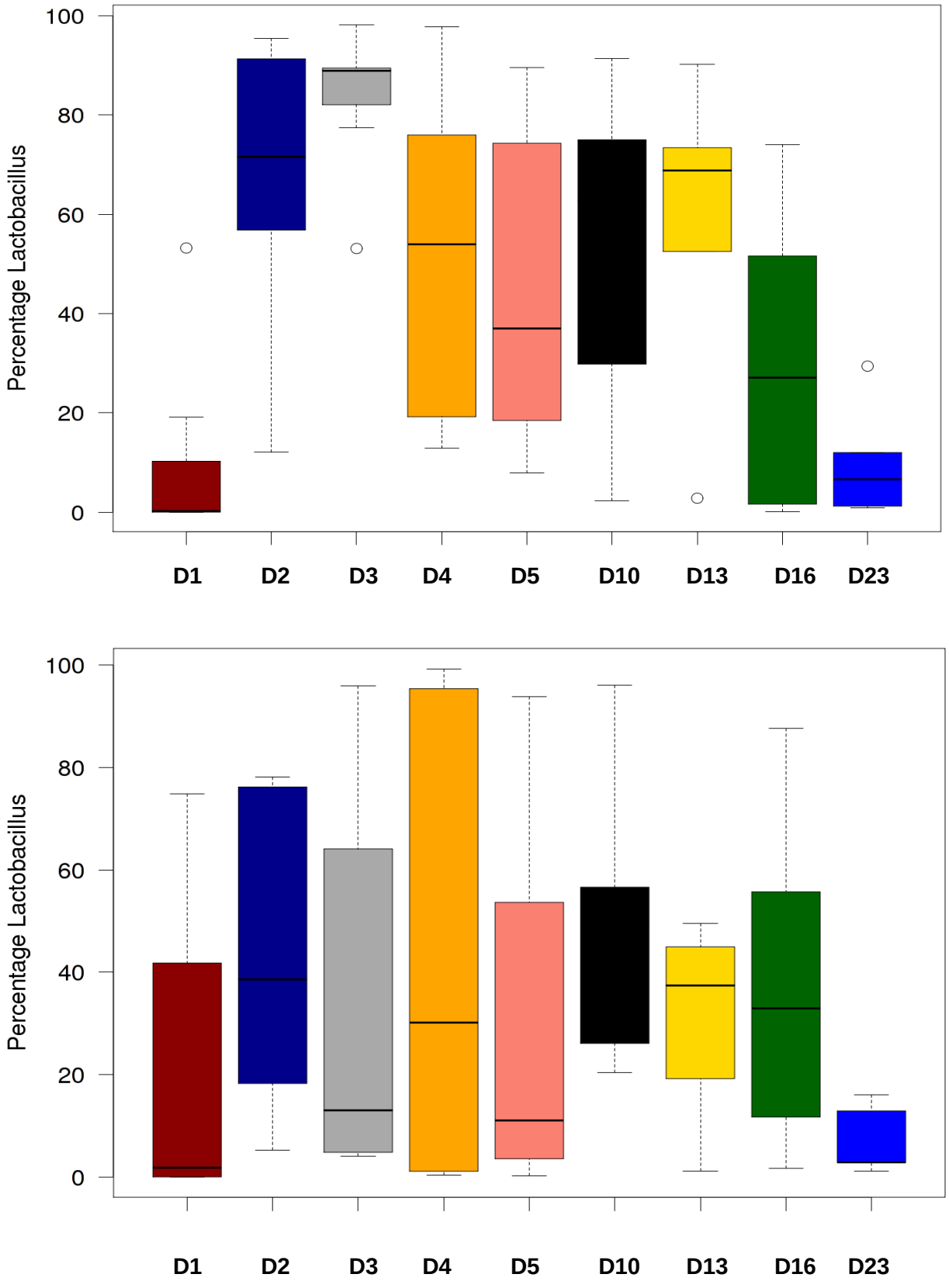


Figure 3.5. Changes in abundance of genus *Lactobacillus* in cloacal samples in the first experiment (top section) and second experiment (bottom section) as chickens aged.

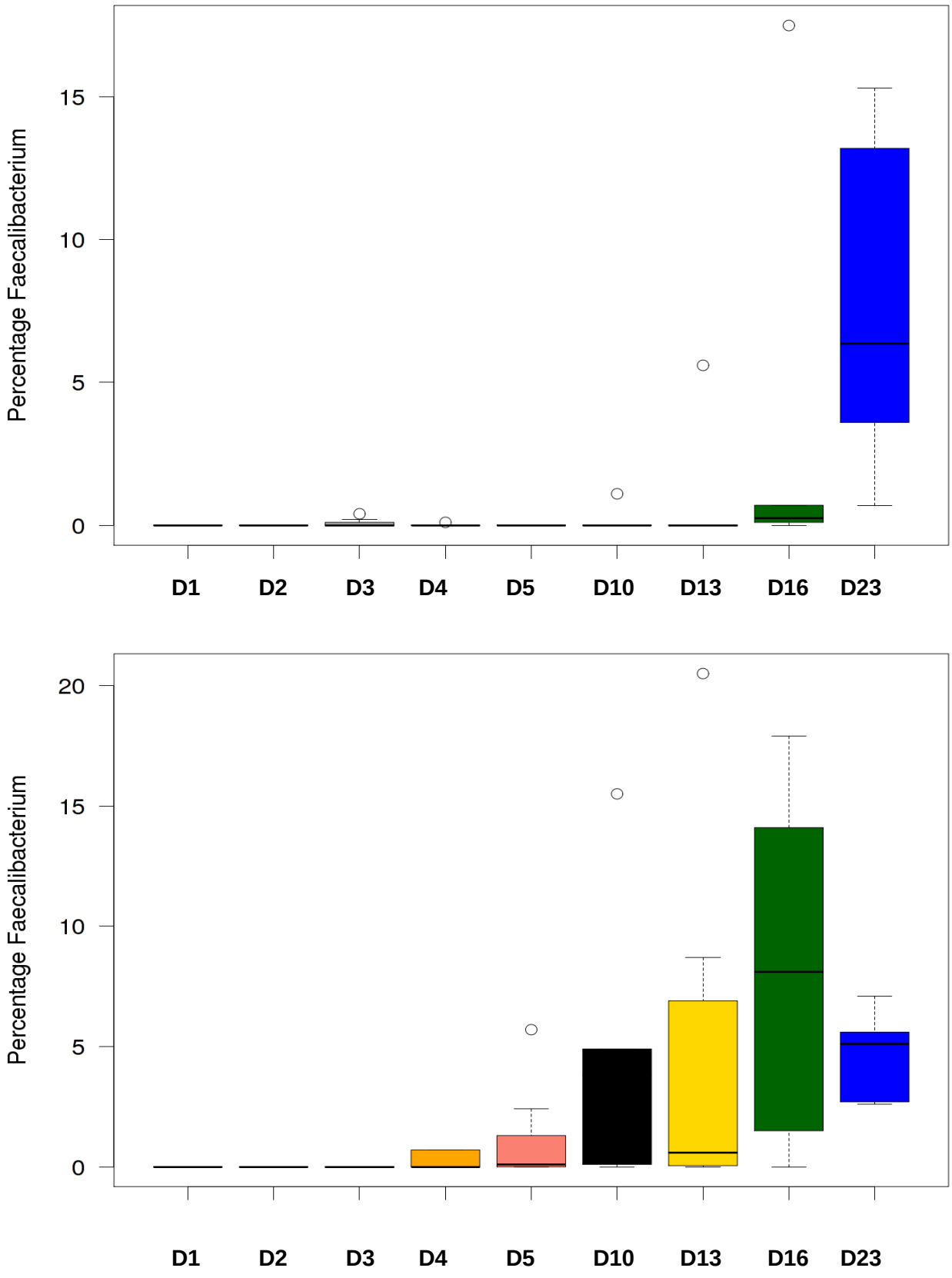


Figure 3.6. Changes in abundance of genus *Faecalibacterium* in cloacal samples in the first experiment (top section) and second experiment (bottom section) as chickens aged.

Overall, only a few genera significantly altered in relative abundance after hatch and during the period that the gut microbiota matured. These were *Enterococcus* and *Faecalibacterium* in both experiments, and *Lactobacillus* in first experiment. Enterococcus were the most abundant species in the day 1 community, followed by Lactobacillus from day 2 which showed a high level of variation in subsequent samplings. *Faecalibacterium*, a genus known to be associated with more mature microbiota, began to appear from day 16 in the first experiment but much earlier (day 4) in the second experiment.

Conclusions

In general, attempts to influence colonisation of the gut microbiota by presentation of caecal organisms from healthy chickens to hatching chickens were unsuccessful. A possible explanation for failure is that bacteria in frozen and thawed caecal contents were not viable. More direct methods of inoculation of newly hatched chickens require further study. Further, *in ovo* inoculation should be investigated. Whatever approaches that may be taken, these must utilise highly characterised species of bacteria known to colonise and proliferate in newly hatched chickens, and which have beneficial effects on chicken performance, health and welfare. These aspects became the main focus of CRC project 2.1.5 and are discussed further in Chapters 4 and 5.

Chapter 4. Comparison of faecal and caecal microbial communities reveals qualitative similarities but quantitative differences

Extracted from an original article by Stanley et al. (2015)

Introduction

The avian caecum generally has a more important role in digestion than the caeca in most mammals. Caeca are usually finger-shaped blind pouches, presenting as lateral extensions at the junction of the small and large intestine and are commonly present in pairs. In birds they range from very long, such as in most domestic poultry, to, very rarely, completely absent (Clench and Mathias 1995). In the chicken they reach 16-18 cm long in adult birds. It is a multi-purpose organ vital to the bird's physiology, a complex system inhabited by a very dense microbial community that converts the caecal pouches into fermentation powerhouses. Members of the caecal microbiota have the ability to digest cellulose, starch and other resistant polysaccharides (Mead 1989; Clench and Mathias 1995). Caeca are not only a major site of water absorption (Gasaway *et al.* 1976, Clench and Mathias 1995) but are also a site of nutrient transport and absorption (Obst and Diamond 1989).

Recent advances in culture-free technologies for microbiota characterization have facilitated an increasing number of studies investigating changes in chicken microbiota within the GIT following manipulation of feed, environment, or health. It has been documented that chicken microbiota responds to changes in feed (Siragusa *et al.* 2008; Amerah *et al.* 2011; Jozefiak *et al.* 2011), litter composition (Cressman *et al.* 2010), antibiotics (Lin *et al.* 2013) and probiotic addition to feed (Lee *et al.* 2011; Nakphaichit *et al.* 2011), disease (Stanley *et al.* 2012; Juricova *et al.* 2013) and stress (Lan*, et al.* 2004; Burkholder;*, et al.* 2008). Recently the use of next generation sequencing techniques to study microbiota composition has been extended beyond phylogenetic analysis to also include functional analysis using metagenomics of whole caecal microbiota (Sergeant et al. 2014).

The importance of the caecum has resulted in it being the major focus of research into the roles of chicken microbiota in bird health and productivity. The caeca sample microbes from both descending and ascending microflora via normal peristalsis and retrograde gut movements (Sklan *et a*l. 1978). The caecal content is emptied several times per day into the gut lumen and then regrows to fill the caeca. This cyclic emptying of the caeca means that large elements of the faecal microbiota must be directly derived from the caeca. We were interested to examine the relationship between the population structure of caecal and faecal microbiota to determine if faecal sampling is

an effective proxy for caecal sampling. Faecal sampling has the great advantage that it is easy to obtain a series of samples from the one bird over time, unlike the case with caecal samples where it is usual to sacrifice the bird to recover a sample and so only a single time-point snapshot can be obtained for any one bird. This has meant that temporal studies of caecal microbiota have had to rely on population sampling with different birds studied at each time point. The analysis of such data is complicated by the large inter-bird variation in microbiota structure that has recently been documented (Stanley et al., 2014). If the relationship between faecal and caecal samples was understood and consistent then faecal samples could be used to determine at least some elements of the microbiota present in the caeca.

The faecal and caecal microbiota profiles of mice have been compared using a gel based analysis method and no obvious correlations were noted (Pang *et al*. 2012) however the biology of the chicken caeca is very different to that of the mouse and so it is likely that this study gives little indication of the relationship of the two sites in chickens. Sekelja *et al*. (2012) used conventional sequencing of cloned 16S fragments to investigate the variability of chicken faecal microbiota and how this was related to emptying of different regions of the GIT. Here we report a larger study using next generation sequencing technology. The study builds on previously reported work that analysed the caecal microbiota of birds in a series of production efficiency trials. Here we analyse further samples and extend the microbiota analysis to generate a statistically powerful data set to compare faecal and caecal sample pairs from 161 birds to determine the level and nature of similarity in microbiota structure between the two sample sites. The relationship between the microbiota in the two caecal compartments within each bird was also investigated.

Materials and methods

Animal ethics

The Animal Ethics Committees of the University of Adelaide (Approval No.S-2010-080) and the Department of Primary Industries and Resources, South Australia (Approval No. 08/10) approved this study. All animal work was conducted in accordance with the national and international guidelines for animal welfare.

Chicken trials

The bird trials were performed as previously described in Chapter 1. Briefly, male Cobb 500 broiler chickens were reared on food comprised of wheat, soybean, barley, canola, peas, meat, tallow, limestone and vitamin mix with free access to food and water. Birds were culled on day 25 and samples collected for microbial analysis. Three independent replicate trials were performed. Cloacal (faecal) swabs and caecal contents were collected from an average of 70 birds from each

trial. Faecal swabs and caecal content were snap frozen and transported to the laboratory for processing.

DNA preparation, sequencing, and data analysis

DNA was prepared as described before in Chapter 1, following the method detailed in Yu and Morrison (Yu and Morrison 2004). The primers used to PCR amplify the V1-V3 region of the 16S rRNA gene were (forward primer (Lane 1991), 5' AGAGTTTGATCCTGG 3'; reverse primer (Felske *et al.* 1997)), 5' TTACCGCGGCTGCT 3').. Sequencing was performed using a Roche/454 FLX Genome Sequencer. Sequences were analysed using PyroBayes (Quinlan *et al.* 2008), pintail chimera detection algorithm (Ashelford*et al.* 2005) and Qiime v1.6.0 (Caporaso *et al.* 2010). Sequences were quality trimmed as described before (Stanley *et al.* 2012). OTU picking was done at 3% divergence level, denoising error-correction, abundance and amplicon estimation by employing USEARCH and UCLUST algorithms (Edgar 2010; Edgar *et al.* 2011). OTUs represented with less than 10 sequences and present in less than 5 samples were filtered out of the analysis. Samples represented by fewer than 1000 quality trimmed and filtered sequences were removed from the analysis and to maintain a paired data design the other sample of the rejected faecal/caecal sample pair was also removed, resulting in a total of 322 sequenced samples, from 161 birds, across three independent trials (50, 54 and 57 birds for trial 1 to trial 3 respectively), each bird represented with both faecal and caecal sample. Normalization of OTU tables was done by performing multiple rarefactions 100 times and averaging counts using a custom Perl script. OTUs were matched to their closest culturable isolate using EzTaxon (Chun *et al.* 2007). R statistical software was used to inspect the correlation between caecal and faecal samples.

Results

Caecal microbiota was richer in OTUs and had fewer dominant OTUs compared to faecal microbiota

A number of alpha diversity measures were inspected to compare within sample diversity of caecal and faecal samples. All of the diversity indicators showed statistically significant differences, all with $P < 0.001$, the lowest possible p-value based on the 999 Monte Carlo permutations used (Qiime). Both non-phylogenetic estimator Chao1 (Figure 4.1A) and phylogenetic diversity (PD) indicator whole tree analysis showed higher diversity in caecal samples relative to faecal samples. Caecal richness, based on number of observed species, was significantly higher than richness in faecal communities. The singles and doubles estimator suggested more rare OTUs in caecal samples. Simpson's evenness (Figure 4.1B) and Strong's dominance index confirmed that faecal samples have more dominant OTUs. Shannon entropy (Figure 4.1C) and the Equitability indices (Figure 4.1D) showed caecal phylotypes as more evenly distributed within samples.

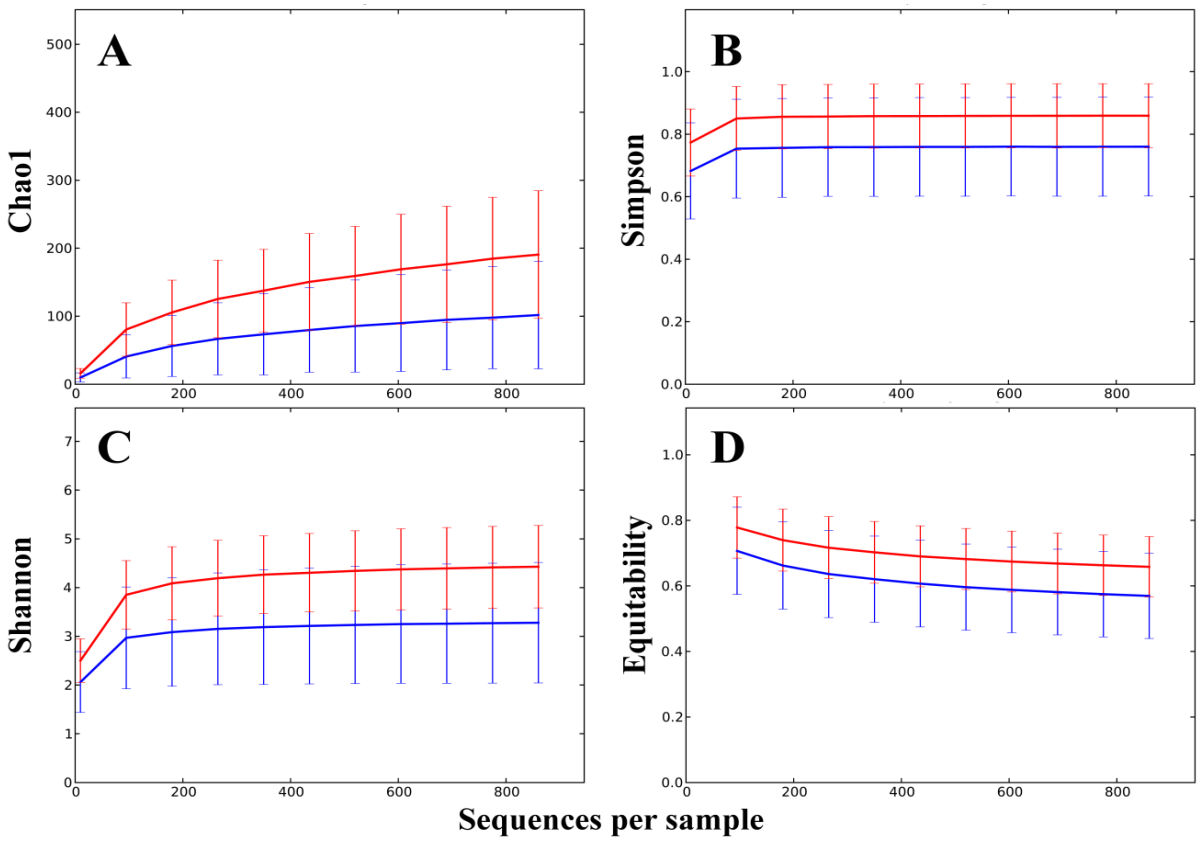


Figure 4.1. Alpha diversity indicators of caecal (red) and faecal (blue) samples all showed significant (*P* < 0.001) difference based on 999 Monte Carlo permutations.

A range of Beta diversity measures were inspected (Jaccard, Bray Curtis, Canberra, Chord, Euclidean, Manhattan, Pearson, Soergel,) and, similar to alpha diversity, they indicated significant (*P* < 0.001) differences between the faecal and caecal samples. Unweighted and weighted UniFrac (Figure 4.2) both showed differences in microbial communities with ADONIS statistics *P*-values lower than 0.001 based on 999 permutations. Separation in both weighted and unweighted UniFrac indicated that the microbiota profiles group on origin (faecal or caecal) based on presence/absence as well as abundance. OTU network analysis confirmed strong community differences (Figure 4.3) and also indicated a few birds where faecal and caecal samples had very similar profiles.

Members of caecal and faecal microbiota

Most of the OTUs were present in both caecal and faecal samples (Figure 4.4). The shared OTUs, represented by 88.54% of all OTUs, accounted for 99.25% of all sequences. The 7.4% of OTUs that were exclusive to caecal samples comprised only 0.27% of all sequences, indicating that they are mostly rare, low abundance OTUs. Fewer OTUs were exclusive to faecal samples (4.05%), however they made up a greater proportion of all sequences (0.45%) than the caecum exclusive OTUs, indicating that on average OTUs exclusive to faeces are 3 times more abundant than

caecum exclusive OTUs. OTUs present only in caecum were all, with the exception of one OTU,

unknown and uncultured relatives of *Bacteroides fragilis, Faecalibacterium prausnitzii* and a number of unknown *Lactobacillus* and clostridia. On the other hand, 27% of OTUs exclusive to faecal origin shared sequence similarity higher than 97% to known type strains of bacteria not commonly found in chicken caecum such as *Streptococcus minor, Vagococcus fluvialis, Streptococcus henryi, Staphylococcus gallinarum* and *Staphylococcus aureus,* all with 100% sequence identity to type strains.

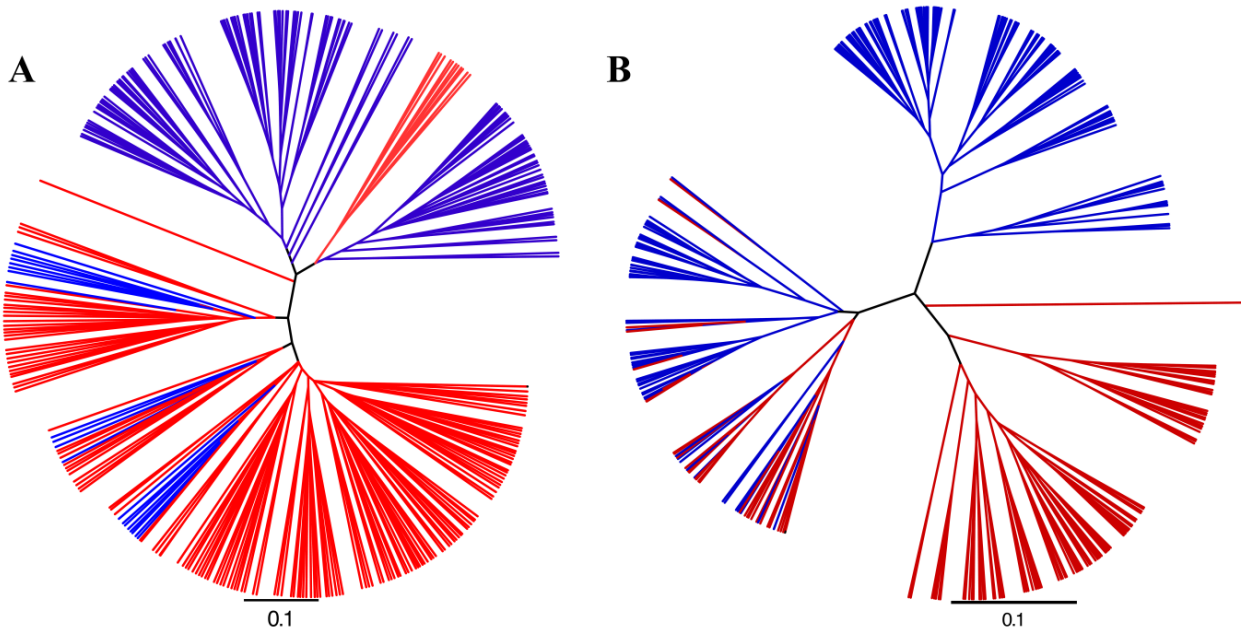


Figure 4.2. A tree representing caecal (red) and faecal (blue) samples. The sample clustering was based on jacknifed weighted (A) and unweighted (B) UniFrac. Tree files were generated in Qiime and visualised in FigTree. The two communities were significantly (*P* < 0.001) different based on both weighted and unweighted UniFrac and ADONIS statistics using 999 permutations.

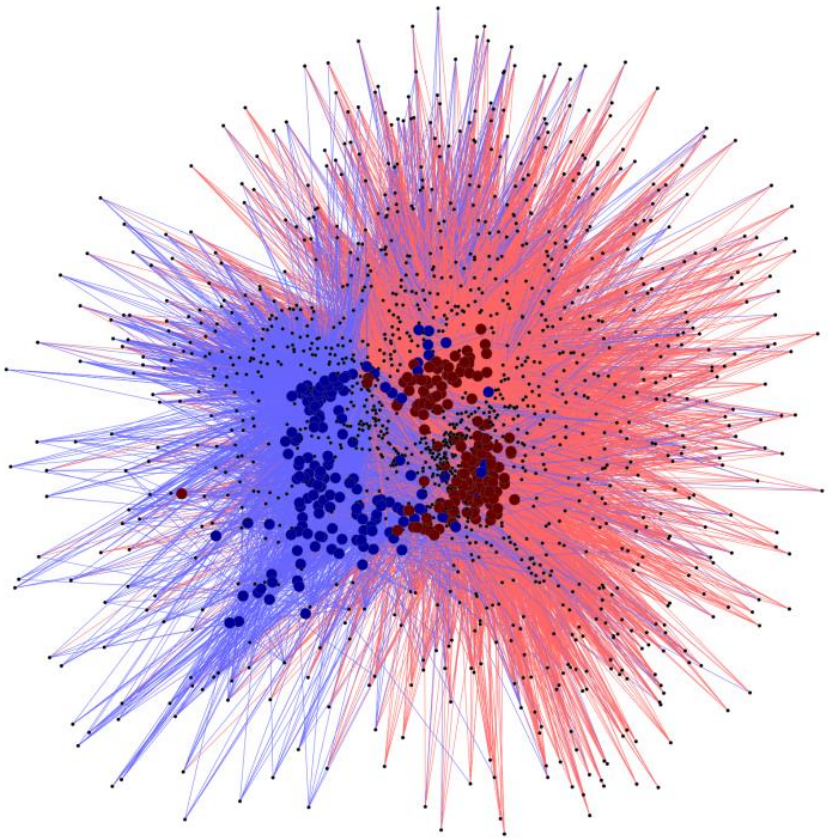


Figure 4.3. OTU network map generated in Qiime. The network demonstrates interactions between caecal (•) faecal (•) samples and OTUs (•). Edges (lines) connecting from samples to OTUs that are present in that sample are colored red for caecal and blue for faecal samples. The distance from an OTU to a sample (length of the edge line) is proportional to the OTU abundance, with OTUs closer to the sample being more abundant. There were a few faecal samples clustered with caecal and vice versa showing the presence of outlier birds in which faecal and caecal communities were more similar than in most other birds as also evident in Figure 4.2.

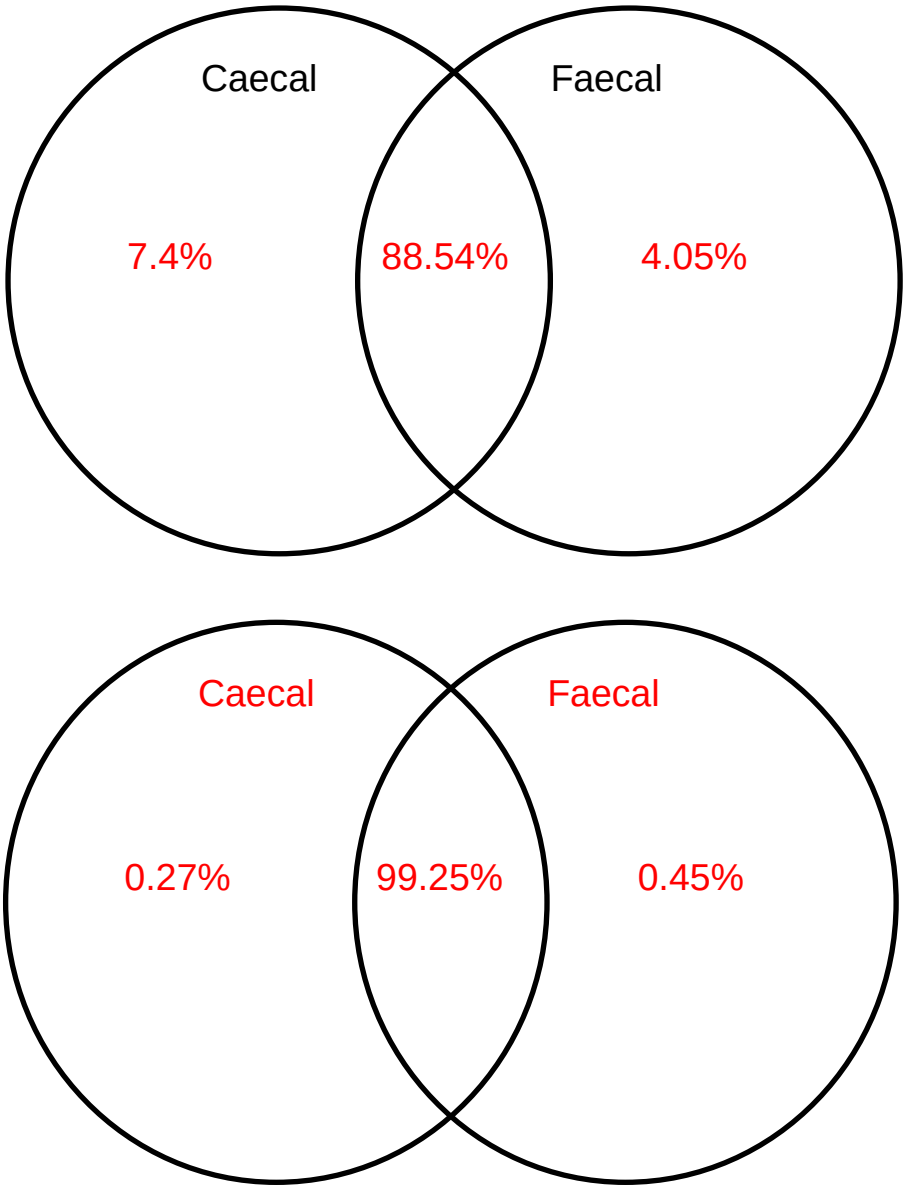


Figure 4.4. Venn diagrams showing percentage of shared OTUs (top) and the percentage of sequence reads that they represented (bottom) of caecal and faecal origin at 3% divergence. 88.54% of all OTUs are shared between the sections and those OTUs represented 99.25% of all sequences.

Tables 4.1 and 4.2 show the 20 most abundant OTUs from caecal and faecal samples respectively, classified using EzTaxon to their nearest culturable isolate. The most abundant OTU in chicken caecum, across the 3 trials, was 99.6% identical to a type strain of *Bacteroides fragilis* and represented 14.1% of all caecal sequences based on the 100-times rarefied data (Table 4.1). This OTU was the top most abundant only in trial 1 with 20.3%, third most abundant in trial 2 (8%) and second most abundant in trial 3 with 14.4% of total sequences. The next 4 most abundant OTUs were confidently (>99% similarity) classified as *Lactobacillus crispatus*, *Lactobacillus johnsonii*, *Lactobacillus salivarius* and *Lactobacillus reuteri*. There were 3 more OTUs in the caecal top 20 classified with sequence similarity >97% to *Lactobacillus helveticus*, *Lactobacillus vaginalis* and *Parabacteroides distasonis*. The remaining OTUs, except for *Lactobacillus helveticus* (96.6%) were of unknown species with sequence similarity to the closest known isolates in the range of 91.9-78.3%. Caecal microbiota contained more unknown OTUs (similarity to known isolate <97%) than the faecal microbiota; those OTUs comprised 47% of caecal microbiota as opposed to 33.2% in faecal samples, based on total caecal and total faecal sequences.

Faecal microbiota were dominated by *Lactobacillus* with the 5 most abundant OTUs making up 54.5% of all faecal sequences. The most abundant classified with >97% sequence similarity to type strains were *L. crispatus, L. salivarius, L. johnsonii, L. helveticus, L. reuteri* and *L. vaginalis*. Moreover, only 4 out of 20 most abundant OTUs were not *Lactobacilli*: *Bacteroides fragilis*, *Candidatus Arthromitus*, *Clostridium perfringens* and unknown clostridium similar to *C. lituseburense*. The remaining *Lactobacilli* OTUs, which showed sequence similarity to the closest culturable isolates of between 89.4 and 94.4%, most likely comprise a number of novel members of this genus. There were 406 OTUs out of 1282 total faecal and caecal, classified as most closely related to *Lactobacillus* strains, 351 of those with sequence similarity <95% to known culturable isolates.

Faecal samples contained *Clostridium perfringens* at an average level of 4.5%, the 6th most abundant OTU classified, with 99.6% similarity to the type strain. However, caecal samples contained on average only 0.14% of this known chicken pathogen. The carriage of *C. perfringens* varied markedly across the 3 trials; in Trial 2 the faecal samples carried 12.8%, Trial 3 had 0.5% and none was detected in Trial 1. Although *C. perfringens* carriage was lower in the caecal samples the same relative trend between trials was noted with 0, 0.3 and 0.1% detected in trials 1-3 respectively.

Table 4.1: The 20 most abundant OTUs in chicken caecum. The representative sequences for all 20 OTUs are publically available in EMBL database with OTU ID as identifier with accession numbers HG810851 - HG810882.

Closest culturable isolate	Isolate accession number	% similarity	% in caecal	% in faecal	OTU ID
Bacteroides fragilis	CR626927	99.62	14.1	1.2	4
Lactobacillus crispatus	Y17362	99.81	11.3	15.4	1
Lactobacillus johnsonii	ACGR01000047	99.61	9.6	12.2	2
Lactobacillus salivarius	AF089108	99.43	5.4	12.7	3
Lactobacillus reuteri	AP007281	99.40	4.3	5.6	5
Acholeplasma palmae	L33734	79.53	3.9	0.5	8
Lactobacillus helveticus	ACLM01000202	97.62	3.5	8.6	133
Butyricicoccus pullicaecorum	EU410376	82.18	3.4	0.2	9
Faecalibacterium prausnitzii	AJ413954	94.71	2.3	0.3	12
Lactobacillus vaginalis	AF243177	99.60	1.3	0.8	10
Lactobacillus helveticus	ACLM01000202	96.67	1.2	2.7	874
Pontibacillus litoralis	EU583724	78.46	0.9	0.2	13
Exiguobacterium acetylicum	X70313	78.33	0.9	0.1	14
Ruminococcus albus	L76598	82.62	0.9	0.1	21
Ruminococcus albus	L76598	89.88	0.8	0.1	16
Parabacteroides distasonis	CP000140	97.31	0.8	0.0	18
Ruminococcus flavefaciens	X83430	86.85	0.8	0.1	15
Clostridium cellobioparum	X71856	83.27	0.7	0.1	17
Clostridium termitidis	FR733680	80.37	0.7	0.1	23
Clostridium symbiosum	M59112	91.97	0.7	0.1	25

Table 4.2: The 20 most abundant OTUs in chicken faeces. The representative sequences for all 20 OTUs are publically available in EMBL database with OTU ID as identifier with accession numbers HG810851 - HG810882.

Closest culturable isolate	Isolate accession number	% similarity	% in caecal	% in faecal	OTU ID
Lactobacillus crispatus	Y17362	99.81	11.3	15.4	1
Lactobacillus salivarius	AF089108	99.43	5.4	12.7	3
Lactobacillus johnsonii	ACGR01000047	99.61	9.6	12.2	2
Lactobacillus helveticus	ACLM01000202	97.62	3.5	8.6	133
Lactobacillus reuteri JC	AP007281	99.40	4.3	5.6	5
Clostridium perfringens	CP000246	99.60	0.1	4.5	6
Lactobacillus crispatus	Y17362	91.98	0.3	2.9	2234
Clostridium lituseburense	M59107	96.91	0.4	2.8	7
Lactobacillus salivarius	AF089108	93.01	0.3	2.8	2200
Lactobacillus helveticus	ACLM01000202	96.67	1.2	2.7	874
Candidatus Arthromitus sp.	X80834	100.00	0.0	2.1	11
Lactobacillus salivarius	AF089108	94.44	0.1	2.1	1905
Lactobacillus crispatus	Y17362	94.50	0.1	1.2	1845
Lactobacillus johnsonii	ACGR01000047	89.41	0.2	1.2	2251
Bacteroides fragilis	CR626927	99.62	14.1	1.2	4
Lactobacillus pontis	AJ422032	92.01	0.1	1.0	2154
Lactobacillus helveticus	ACLM01000202	98.59	0.2	0.9	254
Lactobacillus vaginalis	AF243177	99.60	1.3	0.8	10
Lactobacillus crispatus	Y17362	93.60	0.1	0.7	1557
Lactobacillus gallinarum	AJ417737	90.77	0.1	0.6	1814

Low abundance faecal OTUs more closely correlated with caecal levels than high abundance OTUs

The main question we aimed to answer in this study was whether faecal samples could provide a reliable snapshot of caecal community structure. The high number of sequences and samples across the three trials provides sufficient statistical power to attempt to make these predictions. The three trials were inspected separately and as a whole set for correlations between faecal and caecal abundance of all shared OTUs present in both caecal and faecal samples across all of the birds. All 3 trials showed positive correlation between caecal and faecal abundances (Figure 4.5). To inspect if the level of correlation is influenced by abundance, i.e., if more abundant OTUs show better correlation, we inspected correlations for subsets of data with different caecal and separately faecal minimal abundance for all trials. Although we expected that more abundant OTUs would have higher caecal-faecal correlation, we found the opposite trend for both caecal and faecal abundances (Figures 6-8). Rare OTUs seem to be of similar low abundance in both caecal and faecal while higher abundance taxa tended to differ more. This is especially notable in the faecal dominant OTUs. OTUs comprising more than 50% of caecal sequences in one bird (Figure 4.6A) had wide range of faecal abundances. The number of birds with caecal microflora dominated by more than 50% was much lower than in faecal samples. Figure 4.8 simplifies the message from detailed Figures 4.6 and 4.7; shared OTUs present in lower abundance correlated better, while correlation for more abundant OTUs was very low.

The microbiota of caecal pairs were similar

The interpretation of caecal microbiota data could be influenced by sampling procedure if the pair of caecal pouches within a bird had radically different microbiota populations. To address this issue, in an independent experiment, each of the pair of ceca within 24 birds were sampled and analysed. There was no statistically significant difference in alpha diversity between caecal pairs with Shannon, Simpson or Observed Species indices (P-values of 0.98, 0.77 and 0.56 respectively). Beta diversity was also unaffected by choice of ceca; Unweighted UniFrac differences using ADONIS statistics showed a P-value of 0.81 and Unweighted Unifrac P-value of 0.72. In Figure 4.8 it can be seen that the pairs of caecal samples from each bird tended to be closely related to each other and hence map close to each other in both the unweighted (Figure 4.8A) and weighted (Figure 4.8B) UniFrac plots.

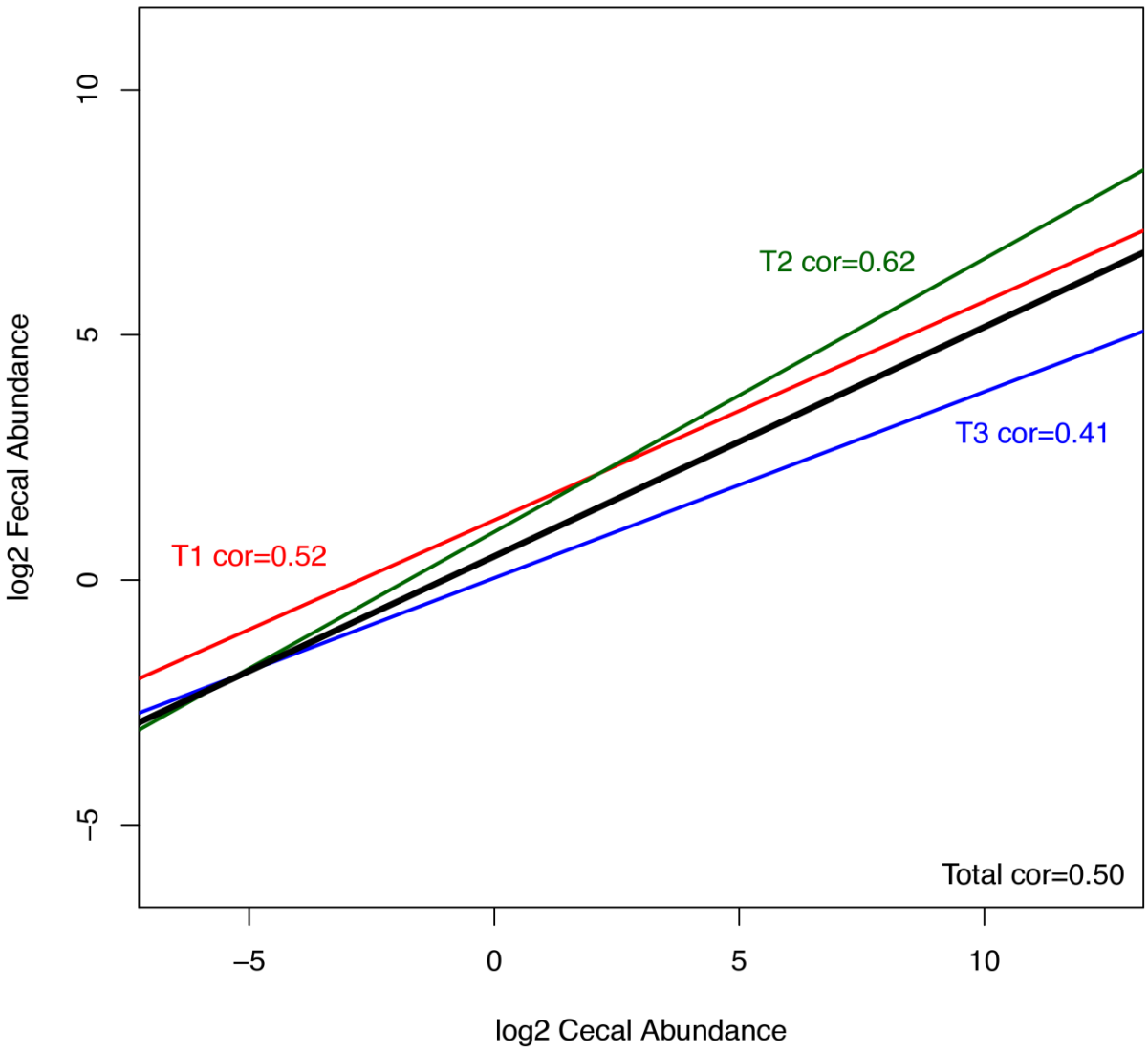


Figure 4.5. Line graph representing correlations between caecal and faecal abundance in trial 1 (red), trial 2 (green), trial 3 (blue) and complete set (black line). There was a reproducible positive correlation across the 3 trials. All individual OTUs present in both caecal and faecal sample for all 161 birds were used in calculation, however, due to a very high number they are not all displayed in the plot.

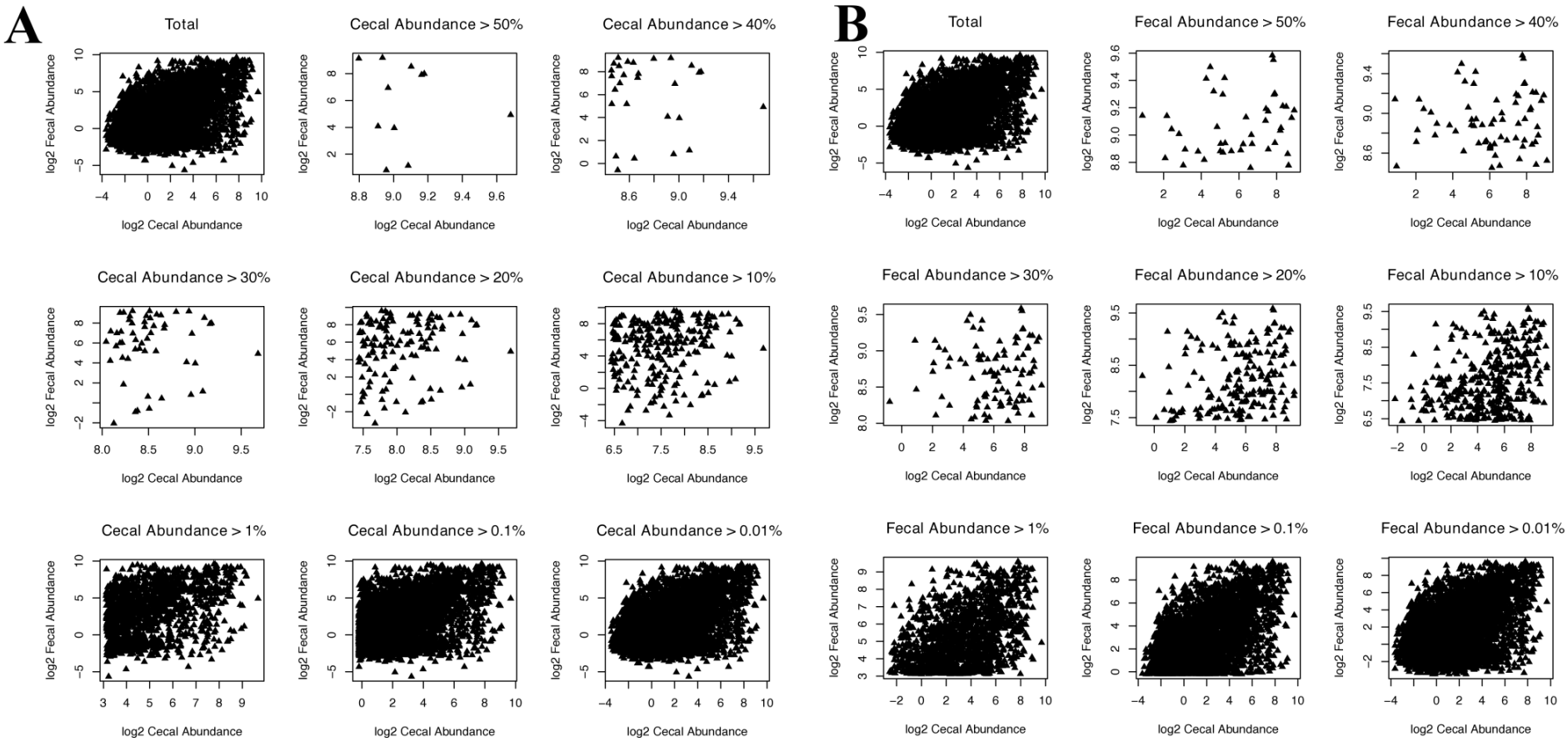


Figure 4.6. Correlation plots for the different minimal caecal (A) and faecal (B) abundances. Note that each shared OTU was plotted for all 161 birds and may be highly abundant in many of the birds, thus total percentages are not expected to add up to 100. For example, there were 11 birds that had OTUs with caecal abundance higher than 50% vs 43 birds that contained faecal OTUs with abundance over 50%.

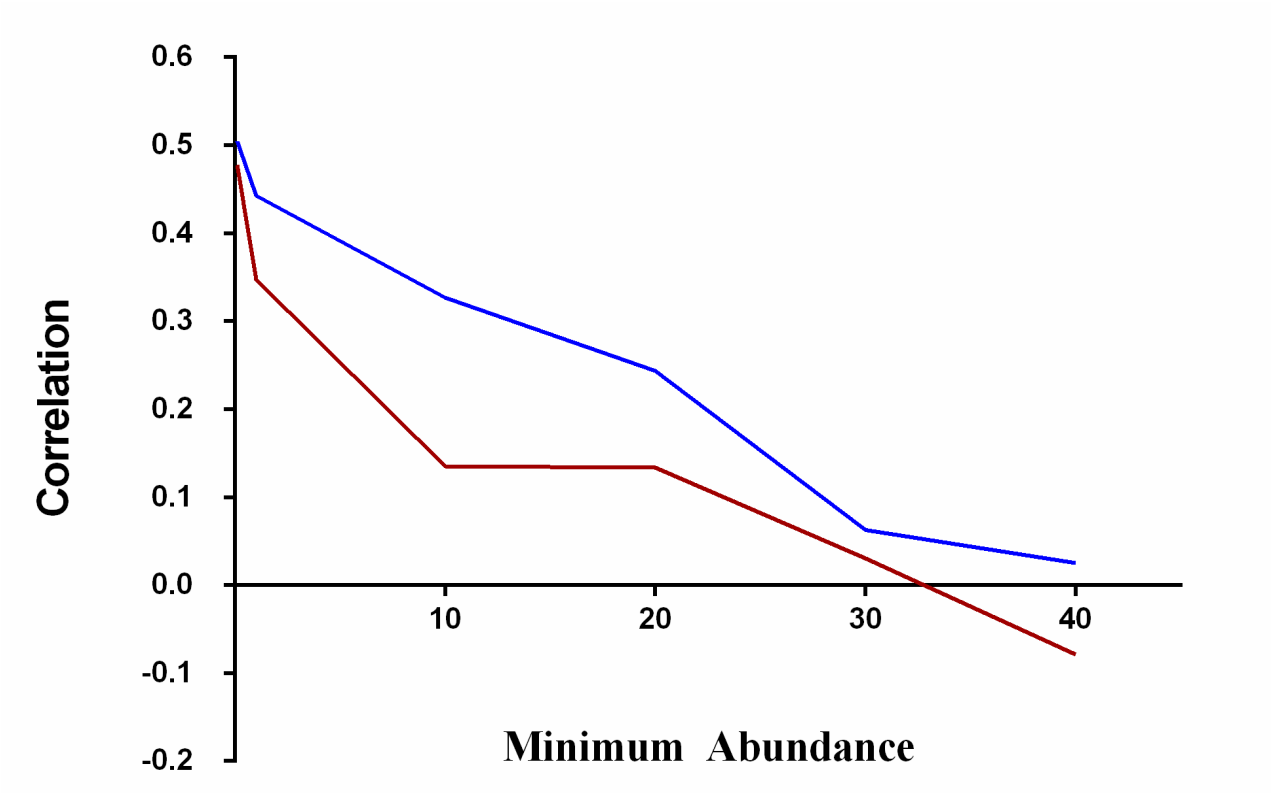


Figure 4.7. Relationship between faecal/caecal correlation and levels of OTU abundance. Correlations were plotted for both caecal (red) and faecal (blue) samples across the range of OTU abundances. There was linear relationship between correlation and minimum abundance showing higher correlation for low abundance OTUs and a very low correlation for high abundance OTUs.

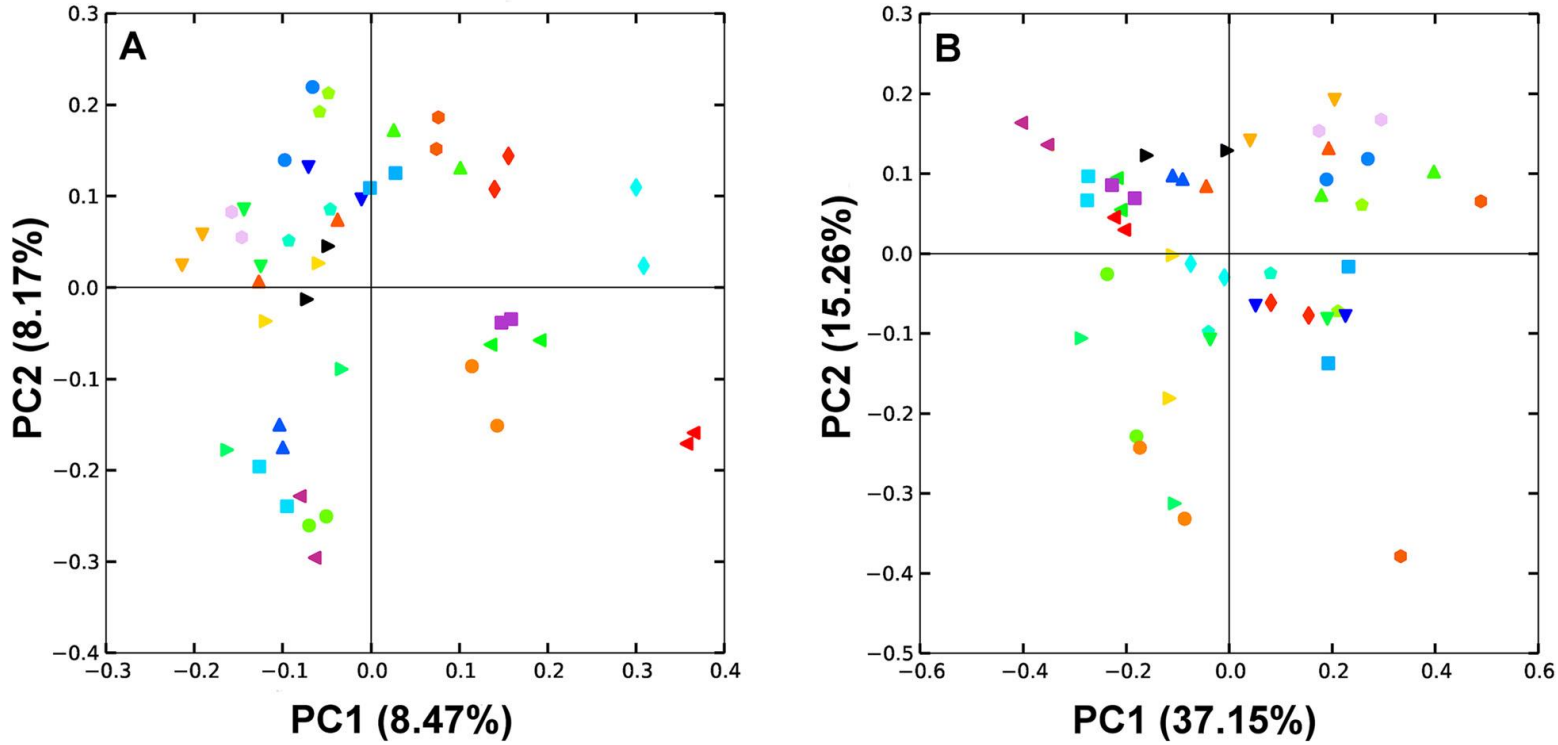


Figure 4.8. Differences between left and right caeca samples from the same bird represented using UniFrac distance. The unweighted (A) and weighted (B) UniFrac PCoA plots demonstrate that left and right caeca are harbouring similar bacterial communities. Left and right caeca from each bird are represented with the same unique symbol and colour combination.

Discussion

Chicken caecal microbiota has been widely investigated due to the significant role of the caecum in heath and disease (Mead 1989; Clench and Mathias 1995). The first insights into caecal microbiota that suggested the complexity of the microbial populations came from culture-based studies. It has been suggested that extreme microbial diversity of chicken caecum has been altered with common use of antibiotics in the feed worldwide and that it has lost much of its natural, chicken specialised microflora and with it the microbial potential and metabolic capabilities (Thomas 1984; Clench and Mathias 1995).

Caeca were found to harbour microbiota capable of degrading cellulose and other indigestible carbohydrates and producing high amounts of beneficial metabolites such as short chain fatty acids (SCFA) (Clench and Mathias 1995). Along the GIT the caecal community takes the most time to develop and mature (Mead 1989) and it contains the highest microbial diversity within the GIT (Gong *et al.* 2002; van der Wielen *et al.* 2002; Zhu *et al.* 2002; Lu *et al*, 2003; Stanley *et al.* 2012; Sergeant *et a*l. 2014). Although it was reported that differences between trials and flocks in chickens can be extensive, overall caecal composition identified in the present study agrees with previously published data. Lu et al. (2003) found clostridia to dominate chicken caecum in their trial. In the present study 8 of the 20 most abundant OTUs are most closely related to the *Clostridiales* order. However all of these had <95% sequence similarity to known culturable isolates, indicating that there is a high degree of caecal metagenomic diversity that is yet to be revealed. In general the clostridia have a reputation as bad, undesirable members of the microbiota, based on pathogenic potential of strains of *C. perfringens*, *C. difficile*, *C. tetani* and a few others. However, this order also encompasses many beneficial bacteria such as cellulose and starch degraders; Clostridium clusters IV and XIV have been linked to prevention of inflammatory bowel disease (IBD) and maintenance of mucosal homeostasis (Frank *et al* 2007; Sokol *et al.* 2009) and clostridia protect from allergy and autoimmune disorders (Atarashi *et al.* 2011). Atarashi *et. al*. (2011) reported the colonisation of germ free mice with a mix of Clostridium strains and also inspected mice enriched in *Clostridium* abundance. *Clostridium* strains promoted T_{reg} cell accumulation. Oral administration to conventionally grown mice, to increase *Clostridia* abundance, was beneficial for mice health, for example in inducing resistance to colitis by the means of suppressing weight loss, bleeding, colon shortening, oedema and other symptoms of colitis allowing Clostridia supplemented mice to show mild, if any, symptoms. Chicken caecum is a major source of uncultured Clostridia that may represent enormous microbial potential.

Abundance of *Bacteroides, Lactobacillus* and *Clostridiales* in the caecum in the present study is in broad agreement with previous studies (Gong *et al.* 2007; Torok *et al.* 2008; Giannenas *et al.* 2010). The benefits of *Lactobacillus* are well known, caecal samples in this study have shown high

diversity in this genus and indicated potential for further probiotic research by estimating 351 potentially novel *Lactobacillus*-related species with similarity to the closest *Lactobacillus* database matches of <95%.

The most abundant OTU in the caecal microbiota was *B. fragilis* due to extreme values in one of the trials. *Bacterioides* are known for beneficial effects on the host, being effective degraders of indigestible carbohydrates, especially cellulose and starch (Al-Sheikhly and Al-Saieg 1980). *B. fragilis* produces SCFA (Choct *et al.* 1996) and when colonising germ free animals aids T_{reg} differentiation and IL10 production (Round and Mazmanian 2010). Similarly another abundant phylotype related to *Faecalibacterium prausnitzii* belongs to a butyrate producing cluster (Louis and Flint, 2009). Absence of *Faecalibacterium prausnitzii* is linked to Crohn's disease (Sokol *et al.* 2009) while caecal richness in *Ruminococcus* is in line with the previously reported (Clench and Mathias, 1995) cellulose degrading potential (Morrison and Miron 2000) of the caeca.

Faecal microbiota was found to be dominated by *Lactobacillus*. This is consistent with previous studies (Wise and Siragusa 2007). The high abundance of *C. perfringens* was attributed to one trial with extreme values and *B. fragilis* was present but at much lower abundance than in the caecum. Surprisingly we identified an abundant faecal OTU, comprising 2.1% of faecal sequences, as 100% identical to *Candidatus Arthromitus* sp. LSFO1.94,LSFO2.94 (EzTaxon database type strain). Gong *et al*. (2007) reported high abundance of Candidatus division Arthromitus in chicken jejunum and ileum representing 34% and 28% of sequences in these regions respectively. They are found in gut microbiota of humans, chicken, rodents and fish, where they anchor to the intestinal epithelial cells in the ileum to act as immune system modulators (Thompson*et al.* 2013). They are major activators of T-cells (Gaboriau-Routhiau *et al.* 2009, Ivanov *et al.* 2009), epithelial lymphocytes, (Umesaki *et al.* 1995, Gaboriau-Routhiau *et al.* 2009) and IgA (Talham *et al.* 1999) and could thus also be important in chicken health and performance. Until recently *Candidatus arthromitus* was the term used for two physiologically similar groups of segmented filamentous bacteria (SFB), one commonly found in the gut of arthropods and others commonly found in GIT of vertebrates (Thompson *et al.* 2012). Although there were striking similarities between the two groups, Thompson et al. (2012) demonstrated them to be distinct and unrelated. Arthropod inhabiting filamentous bacteria have yet unknown function while the ones inhabiting GIT of vertebrates, including chicken, play absolutely critical roles in immune function of the host (Ivanov and Littman 2010). Based on SSU rRNA gene sequence analysis, Thompson suggested that GIT originating SFB form a monophyletic group in the *Clostridiaceae* based on 16S sequence analysis, are now renamed "*Candidatus savagella*" (Thompson *et al.* 2012). However, arthropods specific filamentous bacteria were identified as members of a *Lachnospiraceae arthromitus* cluster and should keep the name "*Candidatus arthromitus*" (Thompson *et al.* 2012). The abundance of

Candidatus savagella in chicken faecal microbiota is not reproducible across the studies. In our own opinion its identification is influenced by the taxonomic databases and algorithms used and in many cases they fall under unknown and uncultured bacteria. It is possible that their role in chicken health, especially immunity is significant therefore more attention should be given to deeper taxonomic identification of significant OTUs in chicken studies.

Sekelja *et al*. (2012) inspected the influence of other GIT sections on chicken faecal microflora over 16 days. They detected massive temporal variations in faecal microflora and different profiles to match different sections of GIT. They proposed that the major reason for the temporal variation was periodic emptying of different GIT sections. The emptying of different GIT areas may influence faecal profile but faecal analysis still remains a useful and powerful approach for microbiota studies in animals with proven success record in human and mammalian studies. It is possible that the timing of sampling and caecal emptying is the reason for the existence of a number of outlier birds with higher similarity between faecal and caecal communities. In an auxiliary study we found that there were strong similarities between the microbiota compositions of pairs of caeca from birds, indicating that the choice of caeca is unlikely to be a concern in sampling procedures. Perhaps a major lesson to be learnt is that it may be misleading to draw conclusions from just a few samples; a large number of samples will represent a range of different emptying events from different regions of the GIT and may allow a more complete and representative overall picture to emerge of microbiota composition.

In the present study we compared faecal and caecal microbiota across many birds and three independent trials in order to investigate the relationship between the two most commonly used and reported sample types for microbiota analysis in birds. We were particularly interested in determining the extent to which the faecal microbiota may reflect the content of the caecal microbiota. From this extensive sample set we can conclude that for the majority of OTUs (88.54%), comprising 99.25% of sequences, presence in faecal implies presence in caecal community.

Our results show highest the correlation between faecal and caecal samples within the rare biosphere species, the correlation analysis however, included only shared OTUs, but all OTUs that were not shared (i.e., present only in faecal or only in caecal samples) were also from the low abundance group. Therefore high correlation among rare OTUs cannot be used to exactly predict caecal from faecal abundance since some rare OTUs are also likely not to be detected in the other community at all. The data shows that OTUs exclusive to caecum, which would be missed if faecal origin was sampled instead, were all of low abundance.

Based on the positive correlation for nearly all shared OTUs it is possible that the low abundance OTUs that appear to be unique to the caecal or to faecal samples would be identified in the opposite group if the depth of sequencing was higher as they are near the detection limit and the limits of the exclusion criteria used in quality filtering parameters to generate the data set. Although the alpha diversity plots indicated satisfactory sequence coverage an increase in sequencing depth may identify more shared OTUs but is also likely to detect additional rare OTUs. There is a positive overall correlation between caecal and faecal abundance as shown in Figure 4.5; this correlation is however negligible in the more dominant OTUs comprising over 10% of total sequences. This trend was found to be reproducible over the three trials that harbour very different microbial communities.

Based on the findings of the present study, we anticipate that with enough sequencing depth, faecal samples can be used to reflect the presence and absence of the vast majority of the members of the caecal community and vice versa. However, the two communities are generally very distinct based on alpha and beta diversity. Positive correlation cannot be used to accurately predict OTU counts in the low abundance part of the microbiota due to it also carrying the highest number of unshared OTUs, nor in high abundance OTUs where correlation is minimal. However, regardless of community structure differences, i.e., species showing different abundance, dominance and equitability, both caecal and faecal microbiota analyses are likely to accurately report if a treatment or condition has induced changes in microbiota. This conclusion is based on the high number of shared species that represent 99.25% of all community members, which would be part of the community response to treatment. Although control/treatment differences could be detected using either caecal or faecal samples, conclusions drawn from the separate analyses are likely to differ. Hence choice of sampling site remains critical in experimental design as faecal microbiota do not provide a complete indication of caecal community structure.

Conclusions

There was a positive correlation between caecal and faecal abundance in the shared sequences, however the two communities differed significantly in community structure, represented as either alpha or beta diversity. The microbial populations present within the paired caeca of individual birds were also similar. Faecal sample analysis captured a large percentage of the microbial diversity present in the caeca. However, the qualitative similarities in OTU presence were not a good representation of the proportions of OTUs within the microbiota from each sampling site. The faecal microbiota were qualitatively similar to caecal microbiota but quantitatively different. Faecal samples can be effectively used to detect some shifts and responses of caecal microbiota.

Chapter 5. Isolation, culture and characterisation of potential probiotic organisms

Introduction

It is clear from the work presented in earlier chapters and from the scientific literature that there are correlations between microbiota composition and complexity and the growth performance of birds. A direct way to positively influence this host/microbiota interaction is to use probiotic strains of bacteria. In this section of the project our aim was to isolate bacteria from high performance birds and then test their probiotic potential. In previous chapters a number of different genera of bacteria were found to be represented by OTUs that were correlated with superior performance. For the work in this chapter we chose to concentrate on the assessment of lactic acid bacteria (LAB) because of their wide acceptance as probiotic bacteria.

Most of the current first generation batch of probiotics that are reported in the scientific literature and used in industry have been initially selected on the basis of results from *in vitro* screening methods such as adhesion to cultured cells, bile and acid resistance, and *in vitro* effects on specific pathogens – usually mediated by acid production but sometimes due to specific antimicrobial proteins. A review of the literature indicates that there is very little definitive evidence to indicate that these *in vitro* tests really have any predictive value with regard to their suitability in identifying probiotic strains. In most studies the *in vitro* assays have been used to direct the choice of a few strains which were then tested *in vivo*, in animals. In no cases have negative control strains, i.e., strains that did not perform well in the *in vitro* tests, been used in the animal experiments. Therefore, there is no formal proof that the *in vitro* testing identifies valuable strains at any rate greater than random choice would. The fact that most current probiotics must be continually dosed tends to indicate that the strains aren't particularly suitable for purpose as a highly functional probiotic should be able to exert effects with less frequent dosing.

In the course of our work presented here we have taken a fundamentally different approach to the initial screening and identification of probiotic strains, compared to the usual methods described in the scientific literature. We have hypothesised that direct *in vivo* screening for colonisation and persistence in the natural milieu of the gut microbiota may offer a more powerful means of identifying probiotic bacteria than the traditional *in vitro* screening methods.

Our previous work had indicated that bacteria capable of reliably colonising and persisting within the gut microbiota are relatively rare. Work on *Lactobacillus* and *Escherichia coli* strains has shown that only approximately 5% of the strains tested, all derived directly from healthy chickens, could

reliably recolonise birds (Stephenson et al., 2010; Sheedy, 2006). Our goal was to develop the strain isolation, characterisation, handling, and storage methods to facilitate large scale screening of new LAB strains for colonisation ability and then subsequently efficacy as probiotics.

Our basic approach to strain isolation and *in vivo* testing for persistence is shown in Figure 5.1.

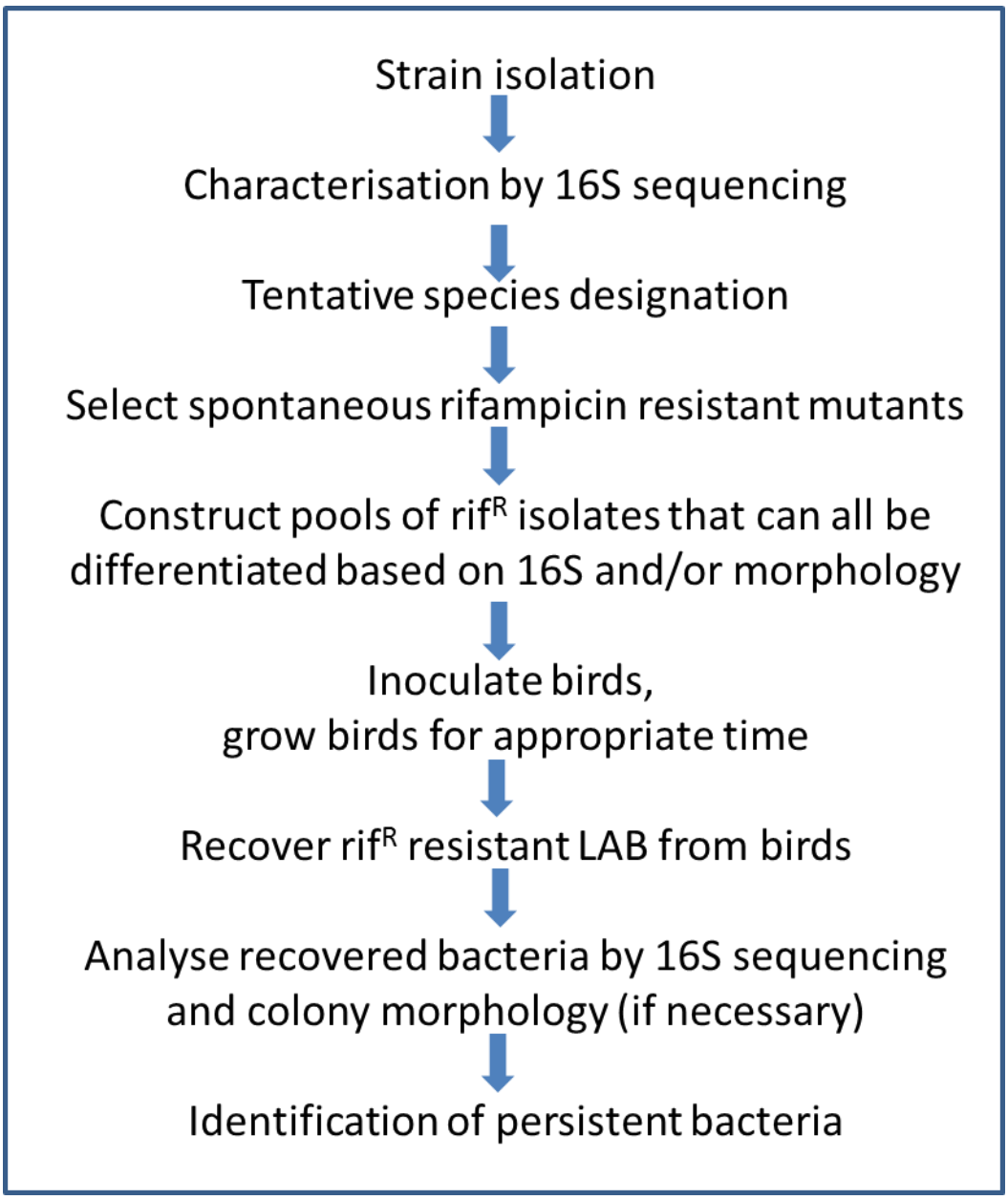


Figure 5.1. Outline of method for identification of persistent bacterial isolates.

Materials and methods

Animal ethics

The Animal Ethics Committees of RMIT University (Approval No. 1508) and Central Queensland University (Approval No. A1409-318) approved these studies. All animal work was conducted in accordance with the national and international guidelines for animal welfare.

Isolation of LAB strains

The LAB strains were derived from the caecal material collected from birds used in the three trials described in Chapter 1. The caecal samples at been stored at -80^{o}C for several years. Samples from birds with the highest growth performance were used. Because all strains were derived from material that had been frozen and stored for several years we could be assured that all the isolates that we investigated had excellent biotechnological characteristics in terms of storing, handling and long term survival.

The caecal content samples were diluted in de Man, Rogosa, Sharpe (MRS) broth and plated out onto MRS agar plates at a level to give approximately 100 colonies per plate – a density which allows the easy picking of individual colonies. From each caecal sample 8 colonies were picked and streaked out onto MRS agar and then re-streaked again to ensure a pure culture. Colonies were not picked at random, rather we aimed to identify and pick as many morphologically distinct colony types as possible. From each caecal sample we aimed to pick large, small and intermediate colony sizes, and colonies with different colours or appearances (e.g. shape of colony, sheen or glossiness or dullness of colony). To further differentiate isolates we found that incorporation of dye into the MRS media helped. Different strains took up differing levels of dye such that strains that looked identical in size and shape could be differentiated on the dye media. We investigated bromocresol green and bromophenol blue at various levels and found that bromophenol blue incorporated at 0.005 g/L gave the best differentiation of strains. Our aim in colony picking was to capture as much variety as possible.

Handling and storage of LAB strain collection

To facilitate the handling of large numbers of LAB isolates we developed methods for growth and analysis in 96-well microtitre plates. We optimised a range of factors including the type of plate, media volumes, how to incubate, best sealing films, etc.

Sequencing of 16S ribosomal RNA genes of LAB isolates

The V3-V4 regions of the 16S ribosomal RNA (rRNA) genes of each of our candidate LAB isolates were sequenced in order to determine the species of each. Some of the isolates with identical 16S sequences could be differentiated by differences in colony morphology. As with the development of

methods for the handling and growth of the strains we also undertook a lot of work to determine the most efficient, cost effective and rapid means to prepare DNA from each of the isolates to use for PCR amplification of the 16S genes. The LAB isolates were grown in 96-well plates, DNA prepared and then 16S amplified over the V3-V4 region – all in 96-well plates. Each of the 16S amplicons were uniquely bar-coded following the method detailed by Fadrosh et al. (2014) so that a large collection, up to several thousand strains, could be sequenced in a single Illumina MiSeq DNA sequencing run using 2 x 300 bp paired-end sequencing. The quality filtered sequences were analysed in Qiime 1.8 software (Caporaso et al. 2010) using Qiime default parameters unless stated otherwise. OTUs were picked using Uclust algorithm (Edgar 2010). Unlike the general implementation of this pipeline for microbiota analysis the OTU picking parameter was set at 100% identity so that only identical sequences were grouped together, rather than the clustering of similar sequences that are grouped together under the usual 95 or 97% similarity parameters used for general microbiota OTU picking. The derived 16S sequences were compared to the Genbank 16S database to determine the probable species designation of each isolate.

Antibiotic resistance marking of LAB isolates

To assist with the recovery and enumeration of the LAB strains used to inoculate birds we isolated antibiotic resistant mutants of each strain by selecting spontaneous mutants resistant to 10 µg/µl or rifampicin. This was easily achieved by streaking the wild type strain onto Rif10 plates; for most strains the resistant isogenic strain could be isolated at the first or second attempt. For a few strains we were unable to isolate resistant mutants after several attempts and hence they were not used in the animal trials. All the strains selected for use in the animal trials were Rif10 marked.

Animal trial at RMIT to identify persistent LAB isolates

One day old Cobb 500 male chicks from a commercial hatchery were randomly assigned to 4 groups, with 5 birds in each group. Each group was housed in a pen with wood shavings litter and water and organic grower feed, with no antibiotics or coccidiostats, were available *ad libitum*. The birds were inoculated by direct oral gavage with 0.5 ml of a mixture of Rif10 marked LAB strains. Each group of birds received a different collection of LAB strains. The birds were maintained for 3 weeks and then euthanised and guts sampled to monitor colonisation of the LAB strains.

Preparation of bacterial inoculum

Because of wide variability in growth rates and the poor growth of some isolates in liquid culture the cells for inoculation were harvested from plates. A 10 µl loop was used to take cells from streak plates and cells were resuspended in MRS broth. Samples of each bacterial strain in a pool were added to a single tube of MRS broth. The OD_{600} of the pooled strains was measured and then adjusted to give a final OD_{600} of 4; an expected cfu of $2x10^9$/ml. Preliminary work had indicated that an OD_{600} of 1 was equivalent to $5x10^8$ cfu/ml. An OD_{600} of 4 cannot be accurately read directly from

the spectrophotometer so a dilution was necessary – a 1 in 4 dilution was used to obtain an accurate measurement. The actual cfu of each bacterial pool was checked by plating dilutions onto MRS agar.

Microbiological analysis of inoculum treated birds

Retention of the inoculated isolates was monitored by plating diluted samples, derived from various regions of the gut, onto MRS rif10 agar. To determine which of the inoculated strains were retained we took a number of approaches. For some samples we individually characterised up to 48 colonies to determine the predominant retained isolates. For all samples we washed off all the colonies from a dilution plate and performed 16S rRNA gene population analysis and for each raw sample from the gut we also performed whole microbiota analysis.

Animal trial at CQU to determine effects of potential probiotics on gut microbiota

Fertilised eggs (Ross 308) were obtained from Bonds hatchery in Toowoomba, Queensland, and incubated in a clean, but not sterilised or fumigated hatchery. The hatchery was prepared by careful cleaning of the room with general detergents and the eggs were not cleaned or fumigated. The incubator (Figure 5.2) was new and not previously used (Kunshan Yunboshi Electronic Technology Co., Ltd., China, model YBS-FD-440). Hatchlings were retained for approximately 1 hour in the hatching tray to fluff-up and then taken out of the incubator and inoculated with either 1ml of sterile PBS or 1ml of inoculum mixture. The inoculum mixture contained equal amounts of freshly plate-grown strains of *L. ingluviei*, *L. agilis* and *L. reuteri*, initially isolated at RMIT by Professor Rob Moore. The birds were then placed in pens on wood shavings in a temperature controlled room (Figure 5.1). The temperature in the room was set as per Ross Broiler Handbook (Aviagen). The initial number of birds per group was 10 for control and 11 for probiotic mix group, each group was placed into a pen (1.2x1.2m, Figure 5.1). Feed used was commercial Blue Ribbon chicken starter crumbles. One bird in the control group died at 14 days. The trail was continued for 28 days with individual birds weighed daily and pen feed intake also measured daily. Faecal samples were taken for 16S community analysis at week 2 and before slaughtering of the birds at 28 days of age. In addition, caecal contents and ileal mucosa scrapings were collected at week 2 and 4 and prepared for sequencing.

DNA preparation, PCR amplification of 16S rRNA gene sequences, and bioinformatic analysis

DNA was extracted using Bioline ISOLATE Faecal DNA Kit (#BIO-52038) according to the manufacturer's instructions and 16S variable regions V3-V4 were amplified. DNA was amplified using Q5 DNA polymerase (New England Biolabs). Sequencing was performed on an Illumina MiSeq system (2 x 300 bp) using the dual-indexing, variable spacer, method detailed by Fadrosh *et al*. (2014). The quality filtered sequences were analysed in Qiime 1.9.1 software (Caporaso *et al.*

2010) using Qiime default parameters unless stated otherwise. OTUs were picked using Uclust algorithm (Edgar 2010) and inspected for chimeric sequences using Pintail (Ashelford *et al*. 2005) and taxonomy assigned with GreenGenes database (DeSantis *et al*. 2006). Additional taxonomic assignments were done using blastn against NCBI 16S database. Data were visualised using Calypso (http://cgenome.net:8080/html/wiki/index.php/Calypso).

Figure 5.2. The animal trial setup at CQU. Panel A, view of the hatchery showing the incubator used. Panel B, inside the incubator. Panel C, pen setup in temperature controlled room. Panel D, a pen with 3 weeks old chickens.

Results

Collection and analysis of LAB isolates at RMIT

Approximately 650 LAB strains, derived from 80 caecal samples, were colony purified and arrayed into 96-well plates for further analysis by 16S sequencing. For this work we restricted the isolates to those that could grow readily on MRS agar. We found that incorporation of dye into the media increased the ability to resolve different colony types as different colonies either excluded or accumulated some dyes at different levels. We tested media with 0.05g/L bromocresol green and bromophenol blue at 0.002 g/L and 0.005 g/L. We found that media incorporating bromophenol blue at 0.005 g/L gave the greatest level of colony differentiation so we routinely employed such media for the main phase of isolate collection.

A number of different methods for DNA preparation were tested, ranging from simple boiling of cell pellets to chemical cell disruption methods and finally mechanical cell disruption methods. A simple cell pellet boiling technique was suitable for some but not all isolates. Mechanical disruption via bead beating in an MP FastPrep-24 Instrument was reasonably effective but was limited to handling only 24 samples per run. It was clear to us that we had to design methods that were capable of being adapted for high-throughput analysis. Therefore we next attempted to improve the boiling method and transfer to 96-well plates; we pre-treated the cells with lysozyme and proteinase K before boiling but still obtained somewhat variable levels of amplification in the 16S PCR. As bead beating had been the most successful method RMIT invested in a new instrument, a SPEX Mini-G tissue homogeniser, capable of handling 96-well plates. With this instrument we were able to produce high quality DNA preparations that gave good, reproducible, consistent, levels of 16S gene PCR amplification across all the strains in our collection.

The 16S amplicons from our strain collection were sequenced and analysed in QIIME software with 100% identity for picking of operational taxonomic unit (OTU) picking. Such analysis identifies all the unique 16S sequences present in the collection and, in most cases, allows the determination of the species of each isolate as well as picking up minor sequence variations (i.e. one nucleotide difference) that allow the differentiation of some isolates of the same species. The classification of isolates into sequence types can be complicated because most LAB genomes have multiple ribosomal RNA operons. Each isolate can carry between 1 and 4 variants of 16S sequence. In some cases strains with the same basic sequence complement can be differentiated by differences in the proportion of each sequence type.

To confirm the validity of the results obtained with Illumina sequencing of the short amplicon covering the V3-V4 region of the 16S genes we took a sub-sample of the isolates and compared the V3-V4 Illumina sequence results to the Sanger sequencing of longer 16S amplicons and the

species assignment as determined by sequencing of the pheS gene. In most cases the results were concordant.

For the animal trial we constructed four pools of LAB isolates, each containing up to 25 isolates, each of which could be differentiated from all other strains in the group based on 16S sequencing and colony morphology. Therefore, any strains that colonised and persisted in the chickens could be uniquely identified. The strains used in each group are shown in Table 5.1A – 5.1D.

Identification of persistent strains in animal trial at RMIT

We aimed to standardise the number of bacteria used to inoculate. To achieve this we measured the OD_{600} of each pool of bacteria and adjusted to 4.0. However, despite this standardisation the actual measured viable cell counts of the four pools of bacteria used to inoculate birds turned out to be quite variable across the groups. Pool 1 was 1.6 X 10^{11} cfu/ml; Pool 2 was 1.2 x 10^{9} cfu/ml; Pool 3 was 1.9 x 10^{11} cfu/ml; and Pool 4 was 1.2 x 10^{10}cfu/ml.

The chickens in Group 4 were ethanased at 2 weeks of age, one week ahead of the original plan because one bird in the group was very low weight. We made the decision to cull the whole group to provide samples to test, validate, and refine the methods we intended to apply for the microbial analysis of the output strains from the trial. The chickens in Groups 1, 2 and 3 were euthanased at 3 weeks of age and recovery of rifampicin resistant LAB isolates from various sections of the gut was measured (Table 5.2).

The trial was designed to investigate bacterial carriage but we also looked at the growth performance of birds by measuring their final weights at necropsy for Groups 1, 2 and 3 (Table 5.2). Of course with such small numbers of birds there are no statistically significant differences in average body weights across the groups but there were numerical differences between the groups with Group 3 being the heaviest.

The recovery of rif^R colonies from the birds at the end of the trial is recorded in Table 5.3. Samples were taken from the mucosal layer of the mid-points of the duodenum, jejunum and ileum and from the caecum luminal content. High level recovery was recorded from all gut sections and from all birds. As expected, LAB carriage levels were much higher in the caecum compared to that seen in the different sections of the small intestine.

Table 5.1A. Bacterial strains included in Group 1

Strain ID	Species[a]	Colony Morphology
3a 2.8	*Enterococcus cecorum*	Small
3a 4.12	*Enterococcus cecorum*	Normal
84.5	*Enterococcus faecalis*	Normal
3a 40.8	*Enterococcus gallinarum*	White, small
3a 4.15	*Enterococcus hirae, etc.*	Normal
62.4	*Enterococcus hirae, etc.*	Very small
3a 31.2	*Lactobacillus agilis*	Normal
3a 1.15	*Lactobacillus crispatus, etc.*	Small
3a 2.2	*Lactobacillus crispatus, etc.*	Normal
3a 4.14	*Lactobacillus fermentum*	White
3a 25.5	*Lactobacillus ingluviei*	Green, fuzzy, irregular
3a 72.1	*Lactobacillus ingluviei*	Green, fuzzy, big
3a 11.1	*Lactobacillus johnsonii*	Normal
5.4	*Lactobacillus johnsonii*	Very small
3a 4.6	*Lactobacillus kitasatonis, etc.*	Very small
62.5	*Lactobacillus kitasatonis, etc.*	Normal
3a 2.4	*Lactobacillus pontis*	Very small
3a 1.1	*Lactobacillus reuteri*	Normal
3a 1.9	*Lactobacillus reuteri*	Very small
3a 72.7	*Lactobacillus saerimneri*	Green, fuzzy, medium
3a 11.2	*Lactobacillus salivarius*	Normal
94.3	*Lactobacillus salivarius*	Very small
3a 6.2	*Pediococcus stilesii, etc.*	White
3a 1.3	*Streptococcus gallolyticus, etc.*	Very small
3a 4.10	*Streptococcus gallolyticus, etc.*	White

[a]The “etc.” after some species names indicates that the species of the organism is uncertain from the 16S sequence – there are a number of related species that have identical or very similar sequences over the 16S rRNA gene region that was sequenced.

Table 5.1B. Bacterial strains included in Group 2

Strain ID	Species[a]	Colony Morphology
3a 4.8	*Enterococcus cecorum*	Small
10.11	*Enterococcus cecorum*	Normal
84.6	*Enterococcus faecalis*	Normal
12.5	*Enterococcus gallinarum*	Normal
66.7	*Enterococcus gallinarum*	Very small
62.1	*Enterococcus hirae, etc.*	Very small
61.1	*Lactobacillus agilis*	Normal
3a 4.9	*Lactobacillus crispatus, etc.*	Light colour
7.14	*Lactobacillus crispatus, etc.*	Rough edge
34.3	*Lactobacillus ingluviei*	Fuzzy, blue
40.3	*Lactobacillus ingluviei*	Flat, fuzzy, white
3.12	*Lactobacillus johnsonii*	Normal
96.4	*Lactobacillus johnsonii*	Small
7.7	*Lactobacillus kitasatonis, etc.*	Small
47.4	*Lactobacillus kitasatonis, etc.*	Normal
3a 5.2	*Lactobacillus pontis*	Very small
3a 1.16	*Lactobacillus reuteri*	Normal
3a 4.3	*Lactobacillus reuteri*	Fuzzy
31.1	*Lactobacillus salivarius*	Normal
59.7	*Lactobacillus salivarius*	Small
3a 6.3	*Pediococcus stilesii, etc.*	White, small
3a 1.13	*Streptococcus gallolyticus, etc.*	Very small, white
2.6	*Streptococcus gallolyticus, etc.*	Small/normal

[a]The “etc.” after some species names indicates that the species of the organism is uncertain from the 16S sequence – there are a number of related species that have identical or very similar sequences over the 16S rRNA gene region that was sequenced.

Table 5.1C. Bacterial strains included in Group 3

Strain ID	Species[a]	Colony Morphology
8.12	*Enterococcus cecorum*	Small
69.3	*Enterococcus gallinarum*	Normal
66.4	*Enterococcus hirae, etc.*	Very small
59.6	*Lactobacillus agilis*	Big, blue centre
86.1	*Lactobacillus agilis*	Normal
89.5	*Lactobacillus crispatus, etc.*	Very small, light colour
91.2	*Lactobacillus crispatus, etc.*	Normal
40.4	*Lactobacillus ingluviei*	Flat, fuzzy, blue
67.5	*Lactobacillus ingluviei*	Very small
59.5	*Lactobacillus johnsonii*	Medium
95.5	*Lactobacillus johnsonii*	Flat
72.8	*Lactobacillus kitasatonis, etc.*	Small, dark blue
25.6	*Lactobacillus salivarius*	Big, blue centre
60.1	*Lactobacillus salivarius*	Small
4.13	*Lactobacillus reuteri*	Wet
91.4	*Lactobacillus reuteri*	Normal
3a 6.9	*Pediococcus stilesii, etc.*	White
7.9	*Streptococcus gallolyticus, etc.*	Small
9.14	*Streptococcus gallolyticus, etc.*	Very small, white

[a]The “etc.” after some species names indicates that the species of the organism is uncertain from the 16S sequence – there are a number of related species that have identical or very similar sequences over the 16S rRNA gene region that was sequenced.

Table 5.1D. Bacterial strains included in Group 4

Strain ID	Species[a]	Colony Morphology
9.15	*Enterococcus cecorum*	Normal
12.6	*Enterococcus gallinarum*	Normal
25.3	*Enterococcus hirae, etc.*	Normal
59.2	*Lactobacillus agilis*	Medium
60.3	*Lactobacillus agilis*	Big
86.4	*Lactobacillus crispatus, etc.*	Normal
89.7	*Lactobacillus crispatus, etc.*	Small, light colour
68.7	*Lactobacillus ingluviei*	Very small, light colour
69.7	*Lactobacillus ingluviei*	Light colour, flat
47.7	*Lactobacillus johnsonii*	Green, fuzzy
94.6	*Lactobacillus johnsonii*	Small, light colour
72.4	*Lactobacillus kitasatonis, etc.*	Blue, small
6.8	*Lactobacillus reuteri*	White
87.5	*Lactobacillus reuteri*	Normal
5.5	*Lactobacillus salivarius*	Normal
40.6	*Lactobacillus salivarius*	Medium, blue centre
8.9	*Pediococcus stilesii, etc.*	White
9.11	*Streptococcus gallolyticus, etc.*	Normal

[a]The “etc.” after some species names indicates that the species of the organism is uncertain from the 16S sequence – there are a number of related species that have identical or very similar sequences over the 16S rRNA gene region that was sequenced.

Table 5.2. Bird weights at end of trial

Group 1, Bird identification	Weight (g)	Group 2, Bird identification	Weight (g)	Group 3, Bird identification	Weight (g)
8512	336	8517	483	8524	583
8513	277	8518	441	8491	579
8514	593	8519	362	8492	528
8515	546	8520	541	8493	604
8516	511	8521	608	8494	580
Group average	453		487		575
Standard Deviation.	123		84		25

Table 5.3. Recovery of rifampicin resistant LAB from inoculated chickens at 21 days of age.

	Duodenum	Jejunum	Ileum	Caecum
	Colony forming units per gram of sample			
Group 1				
8512	1.5×10^6	4.6×10^5	9.7×10^5	4.6×10^9
8513	2.5×10^6	6.2×10^6	4.8×10^6	4.9×10^9
8514	5.3×10^6	2.4×10^7	1.3×10^7	4.9×10^9
8515	3.4×10^5	4.2×10^5	2.9×10^6	1.1×10^9
8516	6.8×10^5	6.7×10^5	2.9×10^5	9.7×10^8
Group 1 average	2.1×10^6	6.4×10^6	4.4×10^6	3.3×10^9
Group 2				
8517	8.3×10^5	1.2×10^7	2.6×10^7	3.0×10^9
8518	1.8×10^5	2.1×10^5	1.2×10^6	1.8×10^9
8519	1.4×10^6	4.1×10^6	2.4×10^7	2.7×10^9
8520	1.1×10^6	2.2×10^6	2.9×10^6	2.3×10^9
8521	2.7×10^5	1.6×10^5	7.4×10^6	1.4×10^9
Group 2 average	7.6×10^5	3.7×10^6	1.2×10^7	2.1×10^9
Group 3				
8524	7.5×10^6	1.1×10^7	9.0×10^7	1.2×10^9
8491	2.7×10^6	2.2×10^6	3.8×10^7	1.6×10^9
8492	2.5×10^6	1.1×10^6	7.2×10^6	3.2×10^9
8493	1.9×10^6	1.7×10^6	3.0×10^6	1.7×10^9
8494	1.4×10^6	2.5×10^6	2.8×10^7	2.6×10^9
Group 3 average	3.2×10^6	3.7×10^6	4.6×10^7	2.1×10^9
Group 4				
8495	5.4×10^5	1.7×10^5	2.3×10^6	7.2×10^9
8496	1.7×10^7	4.1×10^7	2.3×10^7	1.8×10^9
8497	1.2×10^6	1.2×10^8	1.1×10^7	3.2×10^9
8498	4.8×10^6	2.5×10^6	6.5×10^7	1.2×10^9
8499	2.9×10^6	3.8×10^7	1.7×10^6	7.4×10^9
Group 4 average	5.3×10^6	4.0×10^7	2.1×10^7	4.2×10^9
Average across all groups	3.7×10^6	1.3×10^7	2.1×10^7	2.9×10^9

Table 5.4. Identification of bacterial colonies recovered from inoculated birds

	1	2	3	4	5	6	7	8	9	10	11	12
A	*L. reuteri*	*L. crispatus*	*E. cecorum*	*L. reuteri*	*L. ingluviei*	*L. agilis*	*L. crispatus*	*L. reuteri*	*E. cecorum*	*L. crispatus*	*L. reuteri*	*L. crispatus*
B	*L. crispatus*	*E. cecorum*	*L. agilis*	*E. coli test*	*E. cecorum*	*L. agilis*	*L. crispatus*	*L. crispatus*	*L. crispatus*	*L. agilis*	*E. cecorum*	*L. reuteri*
C	*L. salivarius*	*L. crispatus*	*E. coli test*	*E. cecorum*	*L. agilis*	*L. crispatus*	*E. cecorum*	*L. reuteri*	*L. agilis*	*L. crispatus*	*L. crispatus*	*L. crispatus*
D	*L. crispatus*	*L. crispatus*	*L. reuteri*	*E. cecorum*	*L. agilis*	*L. crispatus*	*E. cecorum*	*L. crispatus*	blank	blank	blank	blank
E	*L. reuteri*	*L. salivarius*	*L. ingluviei*	*L. reuteri*	*L. ingluviei*	*L. salivarius*	*L. reuteri*	blank	*L. reuteri*	*L. ingluviei*	*L. ingluviei*	*L. ingluviei*
F	*L. salivarius*	*L. crispatus*	*L. ingluviei*	*L. reuteri*	*L. crispatus*	*L. crispatus*	*L. ingluviei*	*L. reuteri*	*L. reuteri*	*L. ingluviei*	*L. crispatus*	*L. reuteri*
G	*L. salivarius*	*L. ingluviei*	*L. salivarius*	*L. reuteri*	*L. salivarius*	*L. salivarius*	*L. ingluviei*	*L. crispatus*	*L. reuteri*	*L. reuteri*	*L. reuteri*	*L. reuteri*
H	*L. salivarius*	*E. cecorum*	*L. crispatus*	*L. reuteri*	*L. crispatus*	*L. ingluviei*	*L. ingluviei*	*L. ingluviei*	*L. reuteri*	*L. reuteri*	*L. reuteri*	*L. ingluviei*

The colonies used to produce the data in Table 5.4 were derived from the caecum samples from two birds (Chicken 8516 from Group 1 for samples in rows A to D and Chicken 8517 from Group 2 for samples in rows E to F). It can be seen that of the 42 colonies analysed for the first sample 17 were *L. crispatus*, 8 were *E. cecorum*, there were 7 for each of *L. reuteri* and *L. agilis*, and I colony was identified as *L. ingluviei*. Forty seven colonies from the second sample were analysed and found to comprise of 17 *L. reuteri*, 14 *L. ingluviei*, 8 *L. salivarius*, 7 *L. crispatus*, and 1 *E. cecorum*. These bacterial species were represented in the pool of strains used to treat each group by two representatives that could be differentiated based on colony morphology. Therefore the next step was to determine, based on the appearance of streak colonies, whether one or both representatives of each species were present. So for example, with the *L. reuteri* clones in the bottom half of the above table 1 of the 17 clones was identified as isolate 3a 4.3 ("fuzzy" colony) and the other 16 were from isolate 3a 1.16 ("normal" colonies). This type of analysis was completed across the trial to produce a list of persistent strains of LAB which had colonised and survived as dominant members of the microbiota over the course of the trial (Table 5.5).

Table 5.5. Persistent strains of LAB identified in RMIT Trial 1

Colony name	Plate position	Species	Morphology
72.8	P9c1-C1	*Lactobacillus kitasatonis etc.*	Small, dark blue
8.12	P11-H5	*Enterococcus cecorum*	Small
3a 31.2	P9c1-B10	*Lactobacillus agilis*	Normal
3a 25.5	P9c1-B11	*Lactobacillus ingluviei*	Green, fuzzy, irregular
3a 1.16	P11-E4	*Lactobacillus reuteri*	Normal
59.7	P11-B5	*Lactobacillus salivarius*	Small
40.3	P9c1-C5	*Lactobacillus ingluviei*	Flat, fuzzy, white
10.11	P11-H3	*Enterococcus cecorum*	Normal
31.1	P9c1-B9	*Lactobacillus salivarius*	Normal
91.2	P11-C1	*Lactobacillus crispatus*	Normal

Performance of the control and probiotic treated chickens at CQU

The rate of weight gain was in favour of the control group in the first week, similar for the following two weeks, then appeared comparable until the fourth week of the trial when probiotic inoculated birds started to grow faster and consume more feed in the fourth week (Table 5.6 and Figure 5.3A and 5.3B). When a t-test was performed for each day, the results showed slight but significant advantage of control in the first days (Figure 5.3C) and significant advantage of probiotic inoculated birds in the last days of the trial (Figure 5.3D and Table 5.5). Unfortunately, it was not possible to continue the trial beyond day 28 due to constraints imposed by the initial animal ethics approval.

The ability of the strains to colonise in the CQU experiment

The three inoculated strains were identified in the sequenced inoculum formulation, however, only one OTU was able to persist until the end of the trial while others were not detected at any stage of sampling (Figure 5.4).

Influence of administration on diversity

Richness index was significantly different ($P < 0.05$) between the groups. Initially, 2 weeks after inoculation, the probiotic group showed higher community richness, with this advantage washed-out by week 4 (Figure 5.5A). Richness index was however higher in probiotic inoculated ileal mucosa and in caecum of probiotic inoculated birds. The evenness was even more affected ($P < 0.0001$) with probiotic groups showing higher evenness than control in ileal mucosa (Figure 5.5B). Individual t-tests showed that the ANOVA differences were driven by origin of sample and not the treatment.

Multivariate redundancy analysis (RDA) additionally showed significant differences between the groups (RDA P<0.001, Figure 5.6A). However, individual significance analysis between control and probiotic groups faecal samples at week 2 (Figure 5.6B) or week 4 (Figure 5.6C) shows better separation between control and probiotic groups at week 4 ($P = 0.058$) than in week 2 ($P = 0.2$). When comparing faecal samples at both weeks 2 and 4 (Figure 5.6D) the differences were highly significant ($P < 0.001$) with strong overlap between control and probiotic at week 2 and segregation between the control and probiotic at week 4. Finally, comparing caecal and ileal mucosal samples from control and probiotic birds, there was numeral by statistical differences between the groups (Figure 5.6E and 5.6D)

Similar phylotypes were significantly altered ($P < 0.01$) in caecal microbiota between control and probiotic groups (Figure 5.8). Three OTUs most similar to *Bacteroides uniformis* emerged while a number of *Alistipes* and *Ruminococcus*-like species were reduced in probiotic caeca samples. Microbiota in ileal mucosal samples had only one OTU significantly reduced ($P = 0.0052$) in probiotic mucosa samples. This OTU was removed from the mucosa of probiotic inoculated birds and showed 97% sequence identification with both *Shigella dysenteriae* Sd197 strain and *Escherichia fergusonii* ATCC 35469 (Figure 5.9).

OTUs significantly altered by probiotic inoculation at 4 weeks

There were a number of phylotypes altered between control and probiotic inoculated groups in faecal (Figure 5.7), caecal (Figure 5.8) and ileal mucosa samples (Figure 5.8). Probiotic treatment significantly reduced the number of related *Ruminococcus* species and increased abundance of *Bacteroides uniformis* completely identical with the *Bacteroides uniformis* strain JCM 5828 across amplified sequence section.

Table 5.6. Mean daily growth rates of birds in control and probiotic treated groups

Day	Control	Probiotic	Probability
1	49.1	49.1	NS
2	54.0	49.0	**
3	66.1	56.7	***
4	81.7	69.4	***
5	103.6	88.0	**
6	140.6	124.5	**
8	166.9	152.4	NS
9	194.6	176.8	NS
10	228.2	211.3	NS
11	266.9	247.6	NS
12	298.8	281.6	NS
13	370.3	363.6	NS
14	405.4	404.1	NS
15	448.0	452.4	NS
16	498.7	510.2	NS
17	550.0	568.6	NS
18	600.9	626.4	NS
19	738.6	775.6	NS
20	835.7	888.5	NS
21	855.2	938.3	NS
22	961.0	1039.8	NS
23	1043.4	1135.4	NS
24	1124.2	1235.6	*
26	1270.6	1391.9	NS
27	1339.7	1499.0	*

NS not significant $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

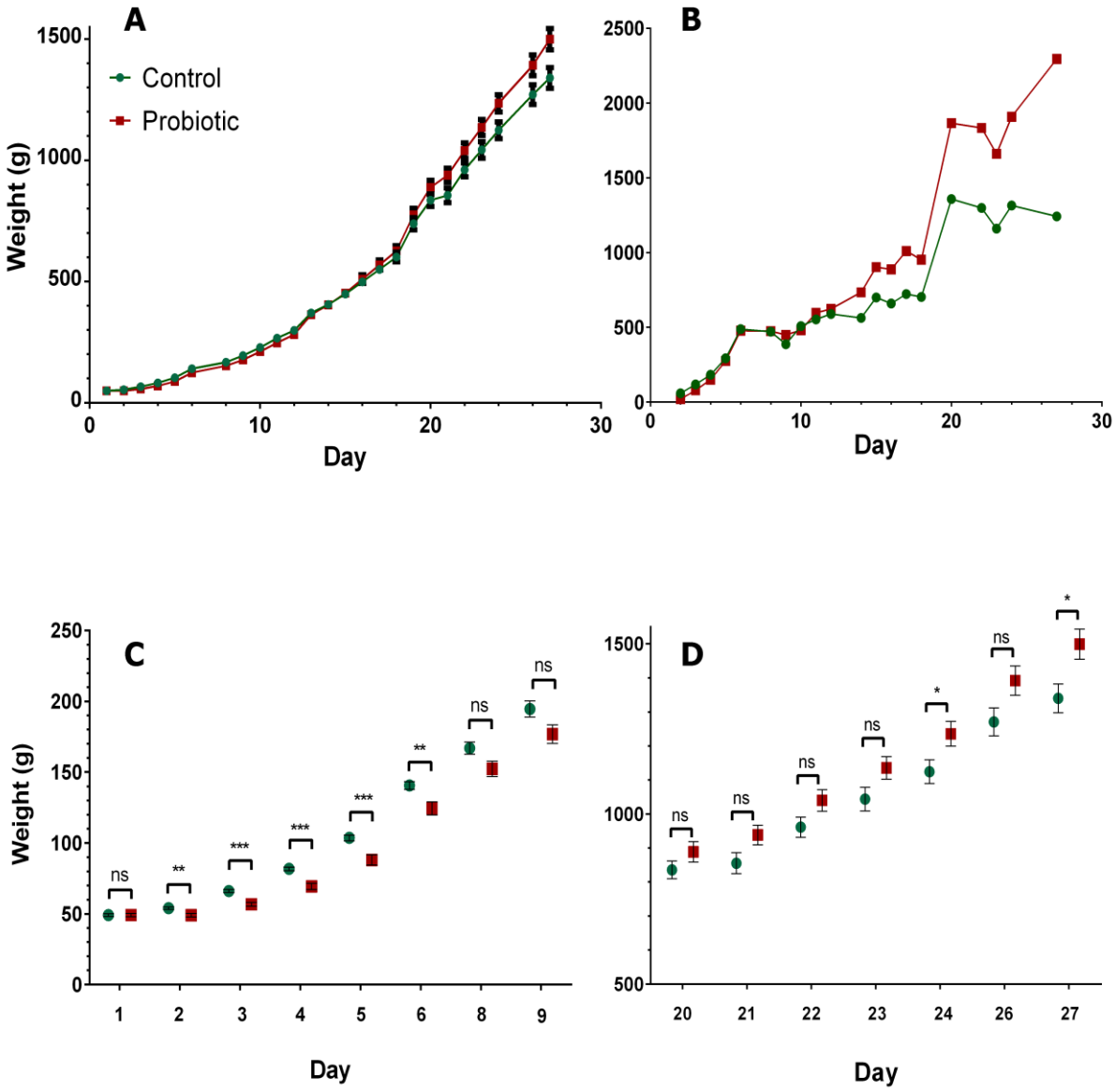


Figure 5.3. The weights of the PBS inoculated control (green) and probiotic inoculated group (red) (panel A).

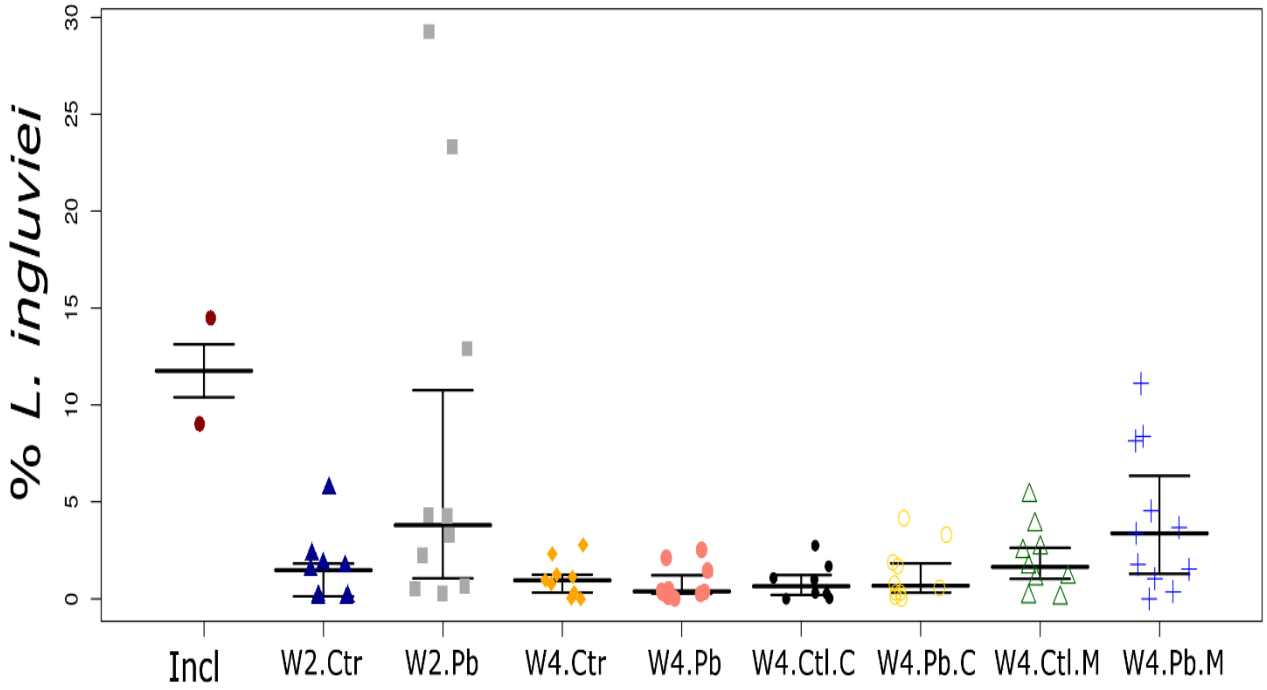


Figure 5.4. *L. ingluviei* was the only one of the 3 species inoculated to be found in birds at detectable concentrations. In week 2, this OTU was strongly present in bird faeces of birds in the probiotic group, however, it was washed out by week 4. The same OTU of *L. ingluviei* showed strong presence in th eileal mucosa of inoculated bird at week 4. Legend, W = week, Pb = probiotic, Ctr = control, .C= caecum and .M = ileal mucosal scrapings.

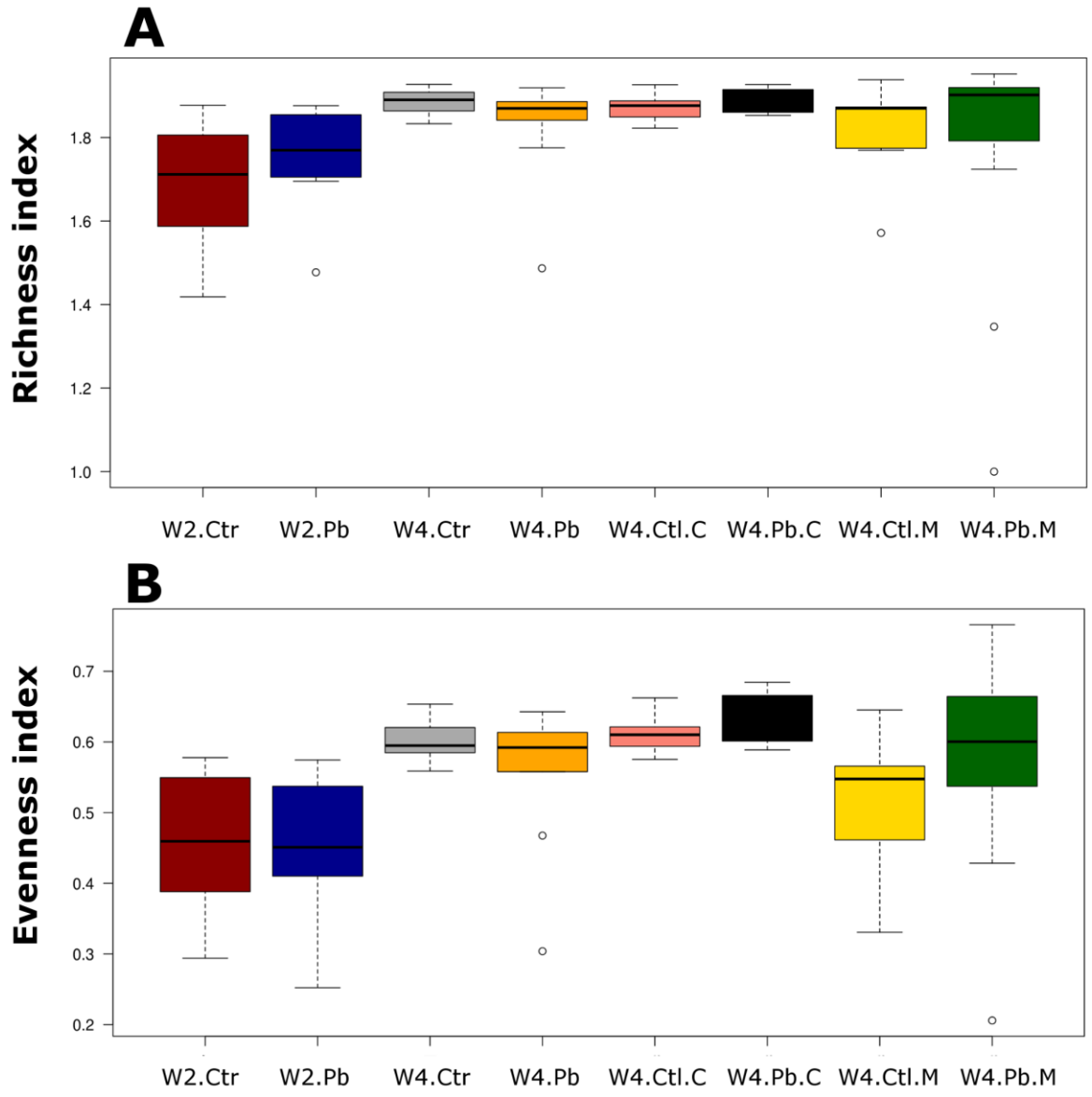


Figure 5.5. Diversity indicators show significant differences in richness and evenness indices. However, the individual t-tests show no significance between control and probiotic in either faecal, caecal or mucosal samples. The ANOVA differences in diversity were driven mostly by between origin differences, i.e., faecal – caecal - mucosal) while other comparisons were not significantly different (*P* > 0.05).

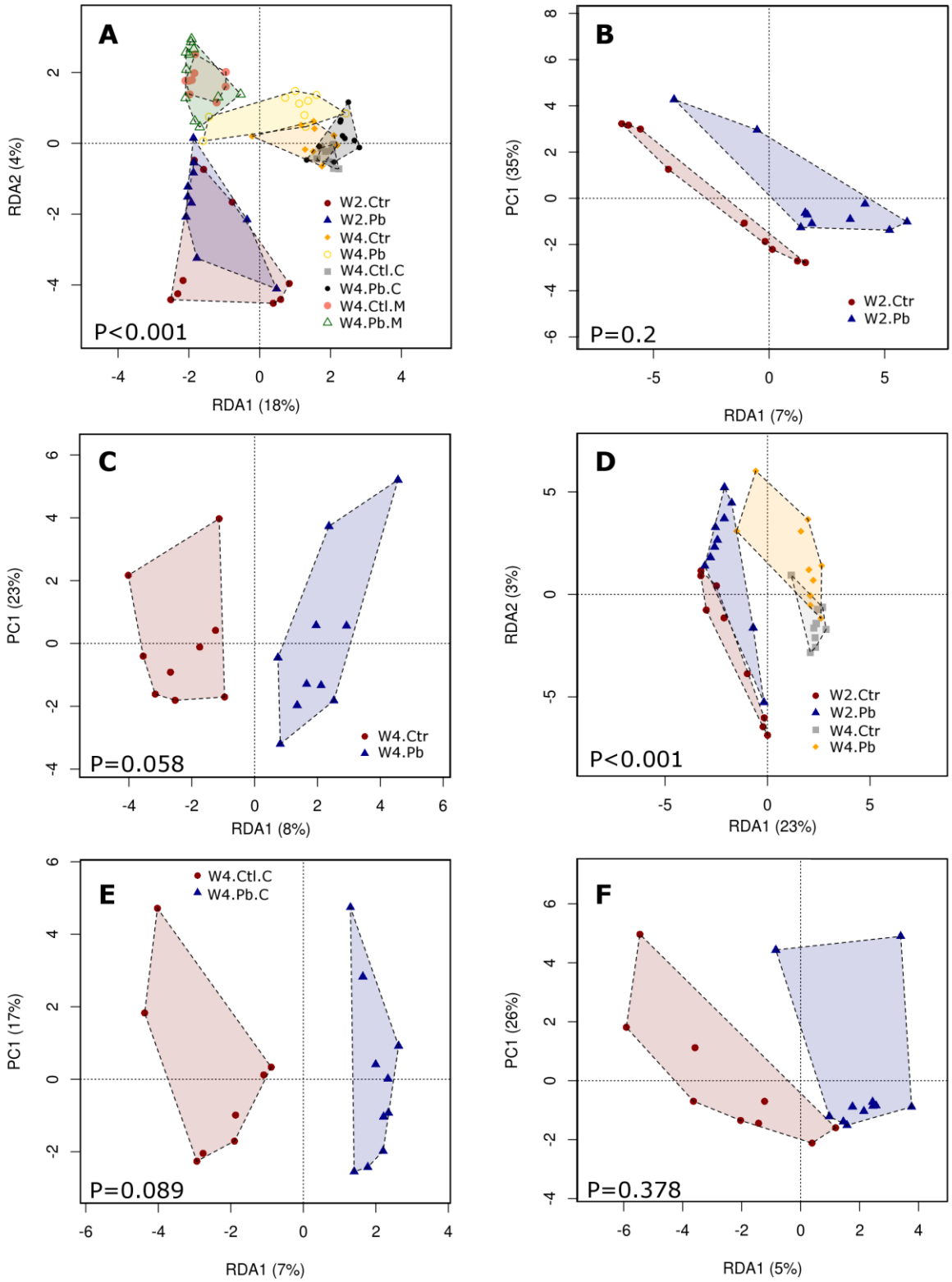
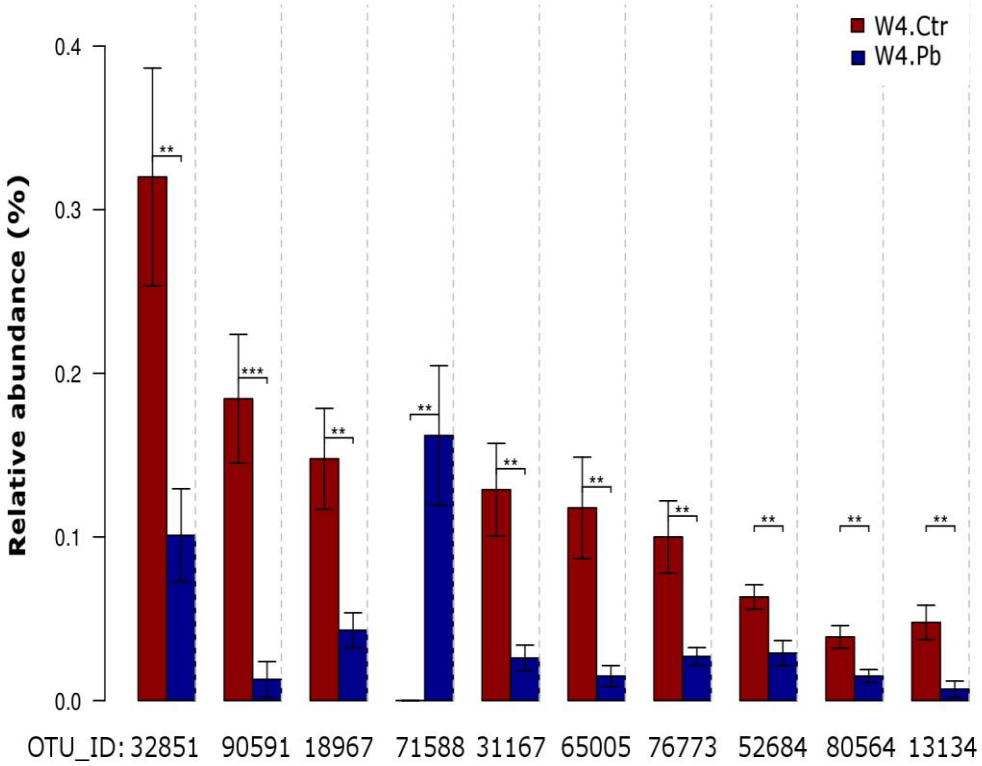
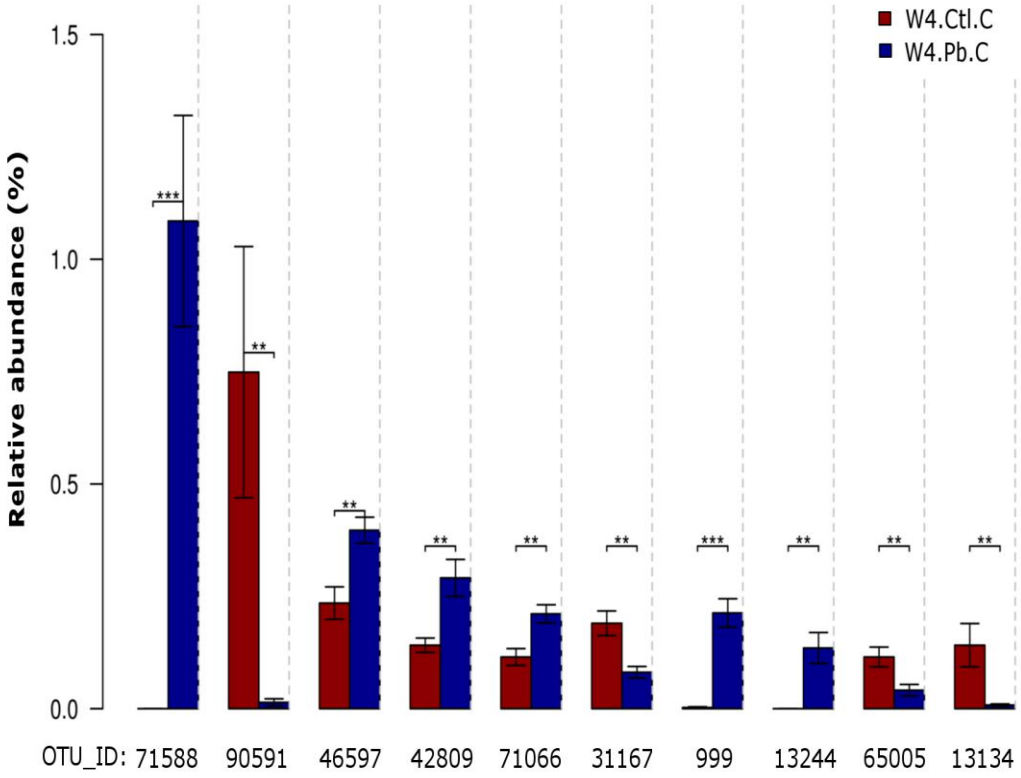


Figure 5.6. Redundancy analysis (RDA) plot showing group to group microbial community differences between all treatments (A), faecal samples at 2 (B) and 4 weeks (C) as well as at both 2 and 4 weeks (D). Difference between control and probiotic in caecal microbiota is shown in panel E and in ileal mucosa in panel F.



OTU_ID	Blast hit	%ID
32851	Ruminococcus torques strain VPI B2-51	95
90591	Alistipes onderdonkii strain WAL 8169	100
18967	Ruminococcus lactaris strain ATCC 29176	96
71588	Bacteroides uniformis strain JCM 5828	100
31167	Ruminococcus lactaris strain ATCC 29176	95
65005	Blautia luti strain bln9	97
76773	Ruminococcus lactaris strain ATCC 29176	95
52684	Ruminococcus lactaris strain ATCC 29176	94
80564	Ruminococcus lactaris strain ATCC 29176	97
13134	Alistipes onderdonkii strain WAL 8169	95

Figure 5.7. T-test (*P* < 0.01) barchart of the most significantly probiotic altered OTUs. Probiotic administration influenced faecal microbiota members of species closely related to *Ruminococcus* and *Alistipes* (reduced in the probiotic group) and *Bacteroides uniformis* – like strain that significantly increased.



OTU_ID	Blast hit	%ID
71588	*Bacteroides uniformis* strain JCM 5828	100
90591	*Alistipes onderdonkii* strain WAL 8169	100
46597	*Clostridium citroniae* strain RMA 16102	96
42809	*Clostridium saccharolyticum* WM1	96
71066	*Ruminococcus torques* strain VPI B2-51	96
31167	*Ruminococcus lactaris* strain ATCC 29176	95
999	*Bacteroides uniformis* strain JCM 5828	97
13244	*Bacteroides uniformis* strain JCM 5828	96
65005	*Blautia luti* strain bln9	97
13134	*Alistipes onderdonkii* strain WAL 8169	95

Figure 5.8. OTUs significantly (*P* < 0.01) altered in caecal microbiota between control and probiotic groups.

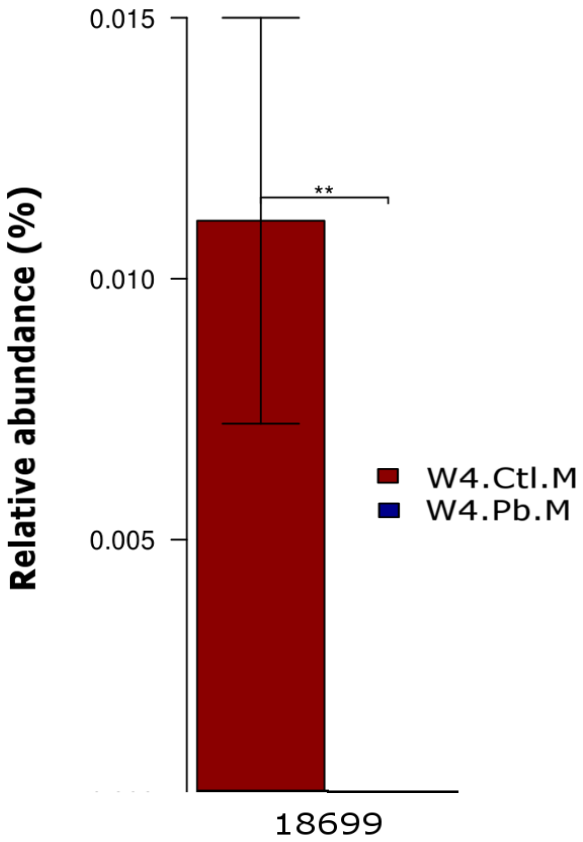


Figure 5.9 Ileal mucosa OTU18699 was significantly absent (P = 0.0052) from the probiotic mucosa. This OTU showed 97% sequence identification with both *Shigella dysenteriae* Sd197 strain and *Escherichia fergusonii* ATCC 35469.

Discussion

We isolated and characterised approximately 650 LAB strains from the caecal contents of high performance chickens derived from six experiments conducted by SARDI (Chapters 1 and 2). We were somewhat disappointed at the species diversity that we captured and there may be opportunities in the future to vary the culture conditions to increase the number of species that can be recovered and hence subsequently assessed for probiotic potential.

We chose a number of groups of strains, constructed based on the ability to differentiate amongst all strains within the group, to inoculate birds and then monitored the recovery of the strains 3 weeks later. We were able to identify strains that persisted in birds over the life of the trial – such strains are good candidates for use as probiotics.

To test a small selection of these strains further a small chicken trial was performed independently at CQU. The probiotic growth study at CQU was the prelude to the larger study described in Chapter 6. After an initial lag in growth rate compared with the control group, the probiotic administered group recovered and proceeded to significantly overtake the control group in the last few days of the 28-day trial. This was preceded by a week of increased feed intake in birds given the probiotic mix. Although only one of the inoculated strains was detected in birds, it needs to be noted that 16S sequencing based microbiota profiling studies can detect OTUs present in faecal material as higher than 10^6 cell per g (Hiergeist *et al.* 2015) and that based on changes observed in total microbiota profiles between the control and probiotic administered group, it is very likely that the inoculated species remained to some extent in the community, and that deeper sequencing is needed to determine the degree of success in colonisation. The differences in weight between treatment groups only became apparent between week 3 and week 4 when the orally inoculated group of birds increased feed intake. This is consistent with our previous reports which showed that an increase in *Lactobacillus* species significantly increased, and was correlated with, feed intake in meat chickens (Stanley *et al*. 2016). RDA multivariate analysis also shows that the control and probiotic groups differed at week 4 but not at week 2, consistent with live weight and feed intake data.

Richness was not significantly affected by probiotic, only slight insignificant increase in richness and evenness (higher evenness = reduced dominance) were noted in ileal mucosa at 4 weeks of age. In addition, same or comparable phylotypes were changed in the probiotic inoculated group in both faecal and caecal samples. These changes in the probiotic group consisted of reduction in *Alistipes* and *Ruminococcus* similar species. Not much is known about the roles of *Alistipes*, while *Ruminococcus* are generally considered as good bacteria. However, most of the *Ruminococcus*–similar OTUs were also highly similar with *Clostridium* species from *Ruminococcaceae* family,

these are often difficult to resolve with 16S based taxonomy and may require additional identification. Probiotic also increased *Bacteroides uniformis*–like species in both faecal and caecal samples; these OTUs were completely absent from control faeces and caeca. *Bacteroides uniformis* is involved in degradation of the isoflavones in the gut (Renouf and Hendrich 2011), and significantly improves metabolic and immunological dysfunction in mice with diet induced obesity (Gauffin *et al*. 2012), while at the same time, some antibiotic resistant strains may have pathogenic potential (Zar and Bond 1985). Also, probiotic ileum mucosa was completely depleted of an OTU most similar to both *Shigella dysenteriae* and *Escherichia fergusonii*. The present results indicate that early inoculation of fresh probiotic strains does influence intestinal microbiota and has a potential to improve weight gain via increase in food consumption. These effects/outcomes will be different for the same strains inoculated into the birds on different feeds (especially starter) as feed is expected to be a major factor in the ability of the strains to colonise and establish themselves in the gut of meat chickens. The way of preparation (fresh vs freeze-dried for example), chicken breed and shed/farm resident microbiota are all expected to play a role in the outcome of probiotic treatment.

Conclusion

We isolated and identified many hundreds of different gut LAB strains from healthy chickens (from experiments described in Chapters 1 and 2), and have shown that some of these can colonise and proliferate in the gut of naive chickens vaccinated orally on day of hatch, then can be recovered from gut contents taken from these chickens at 3 or 4 weeks of age, in separate experiments at RMIT and CQU. The *in vivo* (i.e. chicken) based screening method represents a new advance over the methods that have been traditionally used as reported in the scientific literature for the identification of potentially probiotic strains of bacteria. Rather than rely on *in vitro* surrogate measurements of factors which may affect colonisation we have used real colonisation in the chicken as our primary screen. Furthermore, there were encouraging signs that some of the identified strains promoted higher feed intake and faster growth in the final days of the 28-day experiment at CQU.

The isolation and characterisation of a collection of LAB strains on a small scale is relatively straight-forward, however, to handle and analyse large numbers we needed to develop and refine high throughput methods for culturing, storage and processing of the samples. The know-how developed places us in a good position to dramatically increase the scale on which we can undertake future studies to undertake a more exhaustive search for reliable probiotic strains of bacteria.

Chapter 6. Influence of potential probiotic organisms on growth rate and feed efficiency of meat chickens

Introduction

Fontana *et al*. (2013) indicated that “a useful probiotic must fulfil the following criteria; (1) have a demonstrated beneficial effect on the host, (2) be non-pathogenic, non-toxic and free of significant adverse side effects, (3) be able to survive through the gastrointestinal tract, (4) be present in the product in an adequate number of viable cells to confer the health benefit, and (5) be compatible with product matrix, processing and storage conditions to maintain the desired properties, and labelled accurately”.

We have isolated and identified many hundreds of different gut bacterial specimens from healthy chickens (from experiments described in Chapters 1 and 2), and shown that these can colonise and proliferate in the gut of naive chickens vaccinated orally (as described in Chapter 5) without adverse effects on health of the host. A further test of a potential probiotic is that it can confer a health benefit to the host. Our current objective is more ambitious than this in that we wish to identify consortia of bugs that can enhance productivity, which is possibly more challenging than conferring a health benefit, such as competitive exclusion of a particular pathogen.

Published research in this area features similar combinations of bacterial species rather than pure cultures of a single species, possibly because combinations are better able to colonise and proliferate whereas monocultures tend to become competitively excluded by mixed populations of native species derived randomly from the chickens' environment. For this experiment we examined two live bacterial cultures each comprised of five different *Lactobacillus* species and one *Enterococcus* species, but different strains of each in each culture (Table 6.3). These organisms were originally isolated from previous experiments described in Chapters 1 and 2, then cultured and identified by sequencing and *in vivo* testing in Chapter 5.

The primary aim of this study was to assess productivity measured as body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR). A secondary aim was to assess the survival and proliferation of organisms introduced by oral gavage, and changes in gut microbial composition during a 6-week growth period.

Materials and methods

Animal ethics

The animal ethics committees of The University of Adelaide (S-2016-054) and Primary Industries and Regions South Australia (15/16) approved this study.

Experimental design and statistical analysis

The study involved four treatments with n = 6 pens per treatment. A total of 360 male Ross 308 newly-hatched chickens were obtained from the Baiada Hatchery, Willaston, S.A. and randomly allocated in groups of 15 to each of 24 pens. Swabs of faecal matter were taken from the boxes used to transport chickens from the hatchery. These samples were stored in ice for transport to RMIT for identification of bacterial species derived from the hatchery environment.

SAS for Windows version 9.4 software package (SAS Institute Inc., Cary, NC, USA) was used to determine whether data were normally distributed (Univariate procedure; Shapiro-Wilk test), then analysis of variance with the General Linear Model (GLM) procedure was used to compare parameters, with significant differences between treatments determined by Duncan's Multiple Range Test.

Feed

Three basal diets were used. Commercial broiler starter diet (Ridley Agriproducts, Murray Bridge, S.A.) was fed from 0 – 13days of age, followed by commercial broiler grower diet (Ridley Agriproducts) from 13 – 27 days, and then commercial broiler finisher diet from 27 – 41 days of age. All feed meet or exceeded the requirements recommended by the breeder.

Housing and environment

The shed heating, cooling and ventilation systems were set to provide an air temperature of 18^{o}C in the room, with individual brooders providing an ideal environment for chickens in each pen. Control setting were adjusted daily. The raised rearing pens hade the following dimensions; 1800 x 1200 mm, with 600 mm high mesh sides and were set 300 mm from the concrete floor. Each pen had a feed hopper and drinker nipples. Allocation of pens to treatment groups was done to avoid cross-contamination of gut microbiota by direct bird-to-bird contact bird. Staff wore disposable gloves when handling birds or gut contents within a treatment group and changed these between groups.

Treatments

Four treatment groups were assessed in this study. The basal diet without added antibiotics or coccidiostats was given to three of the four groups of birds. The same diet with zinc bacitracin (50 ppm active ingredient) was given to the fourth group of chickens. The zinc bacitracin treatment was

included to provide an industry benchmark against which we could compare potential non-antibiotic based alternatives for maintaining high performance without detriment to health and welfare of chickens grown for human consumption.

The four treatments were:-

(a) Sterile culture medium given by feeding tube on day of hatch
(b) Live bacterial culture #1 given by feeding tube on day of hatch (Table 6.1)
(c) Live bacterial culture #2 given by feeding tube on day of hatch (Table 6.1)
(d) Commercial broiler diet + 50ppm zinc bacitracin

To prepare the mixed bacterial cultures each strain was grown separately in 20 ml of MRS broth. The six cultures in each collection were combined and thoroughly mixed and the mixture was used to gavage the chickens.

Table 6.1. Composition of live bacterial cultures

Species	Culture #1	Culture #2
	Strain	
Lactobacillus kitasatonis	P9c1-C1	P11-D1
Lactobacillus agilis	P9c1-B10	P9c1-C12
Lactobacillus ingluviei	P9c1-B11	P9c1-C2
Lactobacillus reuteri	P11-E4	P9c1-B5
Lactobacillus salivarius	P11-B5	P9c1-B9
Enterococcus cecorum	P11-H3	P11-A4

Chickens were given the oral gavage treatment (0.5 ml per bird) via commercially available rodent feeding tubes (Instech Laboratories, Plymouth Meeting, PA, USA; model FTP-18-30) which were 18 g and 30 mm in length. The tip was inserted in the mouth and entered the crop which readily took 0.5 ml of liquid with viscosity similar to water. This very simple procedure took a few seconds per bird without any observable effects on subsequent wellbeing.

Bird weight and feed intake

Individual birds were weighed in tared buckets to avoid damage to legs and wings. Any culled or dead birds were weighed individually. Feed intake on a pen basis was measured weekly at days 6, 13, 20, 27, 34 and 41. Weighed amounts of extra feed were allocated as required.

Tissue collection and analyses

Each week, one bird per pen was killed by cervical dislocation for collection of gut contents from the crop, ileum and caecum. Birds were removed one at a time from the room and killed away from other birds. Samples were stored in ice for transport each week to RMIT for enumeration of the

bacterial species surviving from the initial oral gavage. Similarly, at day 41, two birds from each pen were killed by cervical dislocation for collection of gut contents from the crop, ileum and caecum, and subsequent enumeration.

Results

Live weight gain (g/bird), feed intake (g/bird) and feed conversion ratio are summarised in Tables 6.2 – 6.4. The effects of treatment on live weight gain approached significance ($P < 0.1$) at 6 and 13 days of age, with chickens gavaged with sterile culture medium being the lowest numerically, and chickens given culture #2 to highest (Table 6.2). The only statistically significant effect ($P < 0.05$) was observed at 6 days of age with chickens given sterile culture medium consuming less feed than chickens given culture #2 (Table 6.3). There were no indications at any point that treatments affected feed conversion (Table 6.4).

Losses due to mortality and culling reached 4.7% of all chicks placed, with no discernible differences between treatment groups. Losses among treatments were 3.3, 3.3, 5.6 and 6.7% for culture medium, culture #1, culture #2 and zinc bacitracin, respectively. The majority of losses (3.6%) occurred within the first 20 days.

Live weight uniformity expressed as the coefficient of variation within each pen was unaffected ($P > 0.05$) by treatment (Table 6.5).

Table 6.2. Treatment effects on live weight gain (g/bird)

Age in days	Culture medium	Culture #1	Culture #2	Zinc bacitracin	LSD5%	Probability
6	124.2	125.6	129.0	124.1	4.0	0.07
13	442.8	459.2	460.0	456.5	15.3	0.10
20	1030	1070	1038	1073	54	0.24
27	1723	1727	1745	1775	84	0.56
34	2468	2515	2514	2521	118	0.77
41	3297	3378	3361	3380	149	0.63

Means within a row with the same letter are not significantly different ($P > 0.5$)

Table 6.3. Treatment effects on feed intake (g/bird)

Age in days	Culture medium	Culture #1	Culture #2	Zinc bacitracin	LSD5%	Probability
0 – 6	117.7	121.7	125.1	120.8	4.5	0.02
0 – 13	517.0	530.4	537.9	533.6	20.6	0.21
0 – 20	1267	1316	1310	1308	59	0.32
0 – 27	2368	2415	2459	2485	114	0.18
0 – 34	3917	4015	4089	4041	189	0.31
0 - 41	6028	6189	6328	6171	376	0.62

Means within a row with the same letter are not significantly different ($P > 0.5$)

Table 6.4. Treatment effects on feed conversion ratio (g feed: g gain)

Age in days	Culture medium	Culture #1	Culture #2	Zinc bacitracin	LSD5%	Probability
6	0.947	0.969	0.970	0.974	0.030	0.24
13	1.168	1.155	1.169	1.169	0.027	0.61
20	1.292	1.288	1.323	1.276	0.043	0.16
27	1.414	1.440	1.449	1.439	0.045	0.42
34	1.619	1.628	1.659	1.634	0.058	0.52
41	1.855	1.858	1.911	1.852	0.087	0.46

Means within a row with the same letter are not significantly different ($P > 0.5$)

Table 6.5. Treatment effects on live weight uniformity expressed as the coefficient of variation within each pen

Age in days	Culture medium	Culture #1	Culture #2	Zinc bacitracin	LSD5%	Probability
0	8.1	7.6	7.7	8.8	2.0	0.59
6	8.3	8.2	8.1	8.3	1.8	0.99
13	8.6	8.0	7.5	9.0	2.7	0.68
20	8.7	7.1	8.6	10.0	3.3	0.36
27	9.3	8.5	9.2	9.2	2.7	0.93
34	9.4	7.9	8.9	8.4	2.5	0.62
41	8.7	7.0	9.1	7.7	2.8	0.41

Total counts of gavaged organisms are shown in Table 6.6. It is of note that the rif^R marked LAB strains have spread across the groups and colonised all birds, not just those that were directly treated. Presumably this spread has occurred via aerosols as the birds in each pen were physically separated. It can be seen that the organisms colonised to high levels at day 13 but after that their presence in the caecum markedly declined. The reason for this sudden drop in colonisation is unknown and somewhat unusual in that experience has taught us that if a bacterial isolate becomes established for a significant length of time, i.e. over a week, it generally is able to maintain its position within the complex bacterial populations of the microbiota. The change in colonisation level may have resulted from the change of feed from broiler starter feed to broiler grower feed that occurred at day 13. Dietary changes can have a rapid and profound effect on the bacterial populations that reside in the gut. A check of general LAB levels in the caeca of a sample of birds at day 41 indicated that the overall LAB population was as expected – of the order of 10^8 to 10^9 cfu/g. This demonstrated that the drop in the numbers of the strains we introduced was a specific effect rather than a general LAB wide effect. There is a slight indication that the colonisation levels of the introduced strains may have just been starting to increase again when the trial was terminated.

Analysis of the total microbial populations in the caecum of birds gave interesting results. To a large degree each of the treatment groups were clearly separated, as visualised in the RDA plots (Figure 6.1). Therefore, although the productivity of birds was not influenced by the mixed bacterial cultures used for treatment, the overall composition of the caecal microbiota was. Clearly the different treatments have had differing effects on the caecal microbiota. It is interesting to note that the different treatment groups are reasonably well separated from each other at the sampling points at day 6 and day 13 (Figure 6.1A and B). However, they converge on each other at the day 20 sampling point, apart from the group treated with Culture #1, which are still quite separated (Figure 6.1C). Interestingly that group of birds had a much lower colonisation level of the introduced strains, so is also distinctive on that basis. At day 41 the different groups are all clearly separated in the RDA plot (Figure 6.1D)

Table 6.6. Enumeration of gavaged species resistant to the antibiotic Rifampicin in caecal contents (counts per g).

Pen	Day 13	Day 20	Day 27	Day 34	Day 41
1	$1.04*10^{9}$	$1.6*10^{2}$	$5.4*10^{2}$	$1.02*10^{3}$	$1.34*10^{4}$
2	$6.8*10^{9}$	$4*10^{2}$	$1.34*10^{3}$	$4.48*10^{4}$	$5.4*10^{2}$
3	$5.12*10^{9}$	$8*10^{2}$	$5*10^{3}$	$2.07*10^{4}$	$4.1*10^{3}$
10	$8*10^{8}$	$1*10^{2}$	$1.75*10^{3}$	$6.6*10^{3}$	$4*10^{3}$
11	$7.5*10^{4}$	$4*10^{2}$	$2.5*10^{3}$	$1.12*10^{5}$	$2.61*10^{4}$
12	$6*10^{8}$	$1*10^{1}$	$4.8*10^{3}$	$1.6*10^{3}$	$3.91*10^{4}$
4	$8*10^{4}$	$1.73*10^{4}$	$8.6*10^{3}$	$1*10^{1}$	$3.0*10^{3}$
5	$1.26*10^{10}$	$7.5*10^{3}$	$3*10^{2}$	$4.8*10^{2}$	$2.0*10^{3}$
6	$2.24*10^{10}$	$1.64*10^{5}$	$3*10^{3}$	$8.1*10^{3}$	$3.1*10^{3}$
7	$1.44*10^{10}$	$4.2*10^{3}$	$7*10^{2}$	$1.04*10^{3}$	$1.78*10^{5}$
8	$3.23*10^{5}$	$4.5*10^{4}$	$2*10^{3}$	$7.2*10^{2}$	$9.2*10^{4}$
9	$5.54*10^{9}$	$2.63*10^{4}$	$4.84*10^{4}$	$4.5*10^{2}$	$5.26*10^{4}$
16	$2.15*10^{9}$	$1.3*10^{3}$	$9.6*10^{5}$	$9*10^{1}$	$6.3*10^{3}$
17	$5*10^{3}$	$8.6*10^{3}$	$2*10^{3}$	$7*10^{2}$	$2.6*10^{3}$
18	$2.75*10^{9}$	$7.5*10^{3}$	$3*10^{3}$	$8.4*10^{2}$	$2.3*10^{3}$
19	$4*10^{9}$	$1.7*10^{4}$	$9.44*10^{4}$	$9.5*10^{2}$	$1.76*10^{4}$
20	$1.36*10^{10}$	$4.5*10^{4}$	$1.3*10^{4}$	$2.84*10^{4}$	$3.8*10^{3}$
21	$8.6*10^{6}$	$2.4 *10^{4}$	$5*10^{2}$	$9.8*10^{3}$	$4.0*10^{3}$
13	$2.09*10^{9}$	$2.55*10^{4}$	$9*10^{1}$	$1.2*10^{2}$	$1.5*10^{3}$
14	$2*10^{7}$	$2.05*10^{5}$	$1.12*10^{4}$	$7*10^{1}$	$2.4*10^{3}$
15	$2.33*10^{9}$	$2.91*10^{4}$	$1.5*10^{3}$	$7.5*10^{4}$	$4.1*10^{2}$
22	$3*10^{8}$	$2.9*10^{3}$	$3.6*10^{4}$	$2.4*10^{3}$	$1.32*10^{5}$
23	$6.24*10^{9}$	$6.1*10^{3}$	$1.2*10^{4}$	$1.07*10^{3}$	$4.08*10^{3}$
24	$1.22*10^{10}$	$3.3*10^{4}$	$3.24*10^{4}$	$2.4*10^{2}$	$1.64*10^{4}$

Culture #1; Culture #2; Zinc bacitracin; Culture medium

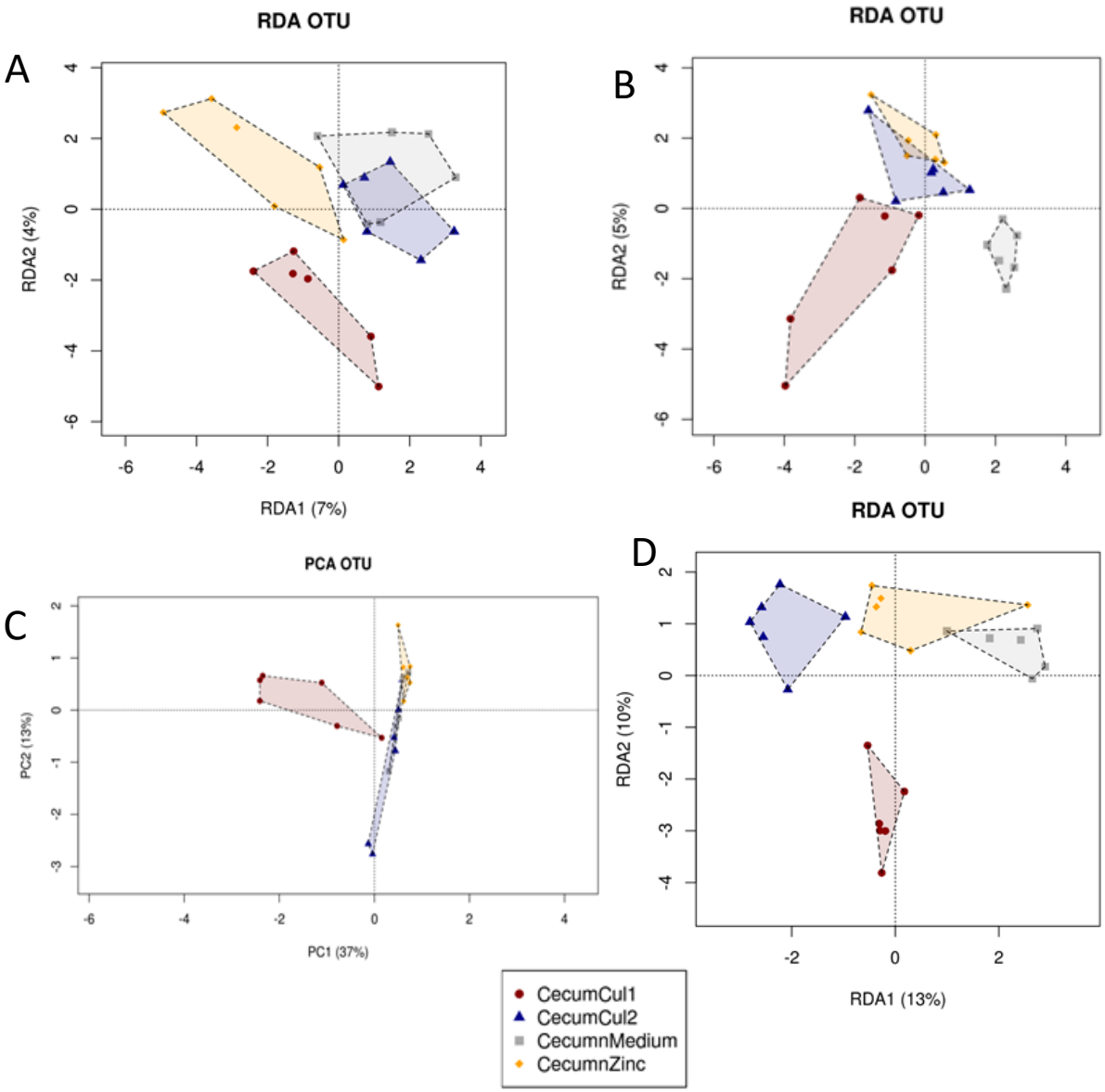


Figure 6.1. RDA analysis of the microbiota composition of caecal samples.

Panel A, 6 day samples; panel B, 13 day samples; panel C, 20 day samples; and panel D, 41 day samples.

Discussion

Growth rate and feed conversion were consistent with expectations of the breeding company (Aviagen 2012). However, there were no discernible differences in live weight, feed intake or feed conversion at any point in this study, although there were tendencies for lower live weight at weeks 1 and 2 for those chickens given sterile culture medium compared with other treatments. It appears that the chosen bacterial gavage cultures colonised and proliferated to high levels in the caeca up to end the second week but then reduced in numbers, to be taken over by the native LAB populations within the birds. The bacteria isolates that were chosen for use in the trial were isolates that had successfully colonised chickens in the trial undertaken at RMIT (Chapter 5), although that trial only went out until the birds were 3 weeks of age. It is possible that the change from starter to grower feed may have caused disruption of the microbiota in this SARDI-based trial.

The relatively uniform spread of labelled gavage species across all pens, including those with birds gavaged with culture medium only, or fed a diet with zinc bacitracin (Table 6.6), suggests either (a) rapid cross-inoculation of birds due to transfer bacteria in the air or on staff during feeding and weighing, and/or (b) proliferation of naturally occurring bacteria already present in the environment. The latter possibility will be examined further by sequencing of bacterial DNA in gut samples.

Because, from early in the experiment, all birds across the trial were colonised with the inoculated strains it is perhaps not surprising that no differences were seen in the productivity measurements of the birds. However, even though there was spread of the bacteria there were still clear differences in the birds microbiota (Figure 6.1) and these differences do not seem to have translated across into productivity differences

Conclusion

The selected combinations of these organisms did not influence productivity, health or welfare of chickens during a 41-day experimental period, possibly because birds were already functioning at peak performance. In addition, it was evident from gut samples that there was cross inoculation of birds with gut bacteria resistant to Rifampicin in all pens including in those birds given a diet containing 50 ppm zinc bacitracin, thus confounding the results. The potential of these probiotic organisms to enhance growth rate and feed efficiency of meat chickens remains to be demonstrated.

Chapter 7. Predisposing factors in a necrotic enteritis challenge model

Extracted from an original article by Rodgers et al. (2015)

Introduction

Necrotic enteritis (NE) is an important infectious bacterial disease in broiler chickens which leads to over $6 billion loss for the global poultry industry annually (Wade and Keyburn 2015). In recent years, more attention has been paid to the control of this disease as the ban on the use of antibiotics in animal feed has been legislated in the European Union (Casewell *et al*. 2003) and pressure from consumers to reduce antibiotic usage in other countries is also increasing. Therefore, the outbreak of NE in clinical or subclinical form has again becomes one of the major concerns in the broiler chicken industry (Dahiya *et al.* 2006). Necrotic enteritis occurrence is the result of the infection by its etiologic agent *Clostridium perfringens*. Recent evidence indicates that the NetB toxin from *C. perfringens* plays a key role in the pathogenesis of NE (Keyburn *et al.* 2008), however, other microbial, host and environmental factors are also important in disease development (van Immerseel *et al*. 2009).

The etiology of NE is highly complex since bacterial infection solely by *C. perfringens* does not necessarily induce the disease (Lu *et al.* 2006; Wu *et al.* 2010). Co-existence of one or more predisposing factors such as nutritional deficiency, stress and/or coccidiosis with the presence of a poultry pathogenic *C. perfringens* strain or strains is necessary for the occurrence of the disease (Timbermont *et al.* 2011). Among those, *Eimeria* infection, consumption of a wheat or barley-based diet, and a high level of protein in the feed have been considered the most critical factors (Annett *et al.* 2002; Cooper and Songer 2010; van Immerseel *et al*. 2004). *Eimeria* infection (coccidiosis) causes intestinal damage that predisposes the intestinal epithelium to *C. perfringens* colonisation. The induced leakage of plasma from ruptured epithelial cells and enhanced mucus production in the intestine brought about by *Eimeria* infection increase available nutrients for fermentation and create a favorable environment for *C. perfringens* proliferation (Timbermont *et al*. 2011). Wheat or barley-based diets containing moderate to high levels of water soluble non-starch polysaccharides (NSP) may produce high intestinal digesta viscosity and thus may slow intestinal digestion leaving undigested nutrients available for microbial propagation (van Immerseel *et al*. 2004). Switching to a high fishmeal diet during the *C. perfringens* challenge has been a feature of a number of successful experimental models for inducing NE under

experimental conditions (George *et al.* 1982; Keyburn *et al.* 2008; Truscott and Al-Sheikhly 1977; Wu *et al.* 2010). The predisposing role of dietary fishmeal in the induction of NE has been considered important in the past. This is because an excessive level of dietary fishmeal increases the amount of biogenic amines in the gut, which is believed to inflame the gut wall. Drew *et al*. (2004) found higher concentrations of methionine and glycine in diets containing fishmeal were associated with elevated levels of *C. perfringens* in ileal and caecal contents of meat chickens. Furthermore, Dahiya *et al.* (2007a) demonstrated that supplementation of diets with glycine increased *C. perfringens* numbers whereas addition of high methionine levels (Dahiya *et al*. 2007b) reduced *C. perfringens* proliferation. The evidence is therefore somewhat mixed and further studies are needed to fully elucidate the roles of individual predisposing factors in the reproduction of NE in broiler chickens.

Hence, NE disposing factors are critical in introduction of the disease in a challenge model and the occurrence of the disease in the field. Fishmeal and *Eimeria* have long been employed in the challenge protocol to predispose the NE together with the inoculation of bacteria *C. perfringens*. These three factors have been considered to alter the performance of the birds but extensive investigation on the microbiota in the hindgut has been minimal. In the present study, we examined the roles of dietary fishmeal inclusion, *Eimeria* inoculation and *C. perfringens* challenge on the growth performance and NE infections in a 2 × 2 × 2 factorial design.

Materials and methods

Birds and diets

A total of 1344 day-old Ross 308 male broiler chickens (Baiada Country Road Hatchery, Tamworth, NSW, Australia) were divided into groups of 28 birds and placed into 48 floor pens on softwood shavings within 8 hours of hatch (day zero). Pen groups were culled to 25 birds on day seven. Thereafter, the birds (1200) were raised for 35 days in a temperature-controlled room (33–34^{o}C during days zero and seven, decreased by 3^{o}C each week to 21–23^{o}C by day 24) at Kirby Research Station of the University of New England. The birds were subjected to 23 h/d for the first 7 days from placement and then 16 h/d thereafter from fluorescent bulbs at 20 lux. Each pen was assigned to one of eight treatment groups with six replicates per treatment (25 birds/cage) in a 2 × 2 × 2 factorial design with or without: 250g/kg fishmeal (F±) feeding from day 8 to 14, *Eimeria* inoculation on day 9 (E±), and oral gavage of approximately 10^{8} CFU *C. perfringens* type A on days 14 and 15 (C±). The live phase of the study was approved by and conducted according to the conditions set by the Animal Ethics Committee of the University of New England.

Nutrient and dietary composition of the starter and finisher diets are detailed in Table 7.1. Birds were given a common starter diet devoid of fishmeal from zero to seven days of age. Fishmeal was added at a level of 250g/kg from 7 to 14 d to half of the pens as specified. Fishmeal was removed on day 14, with the starter diet alone fed from 15 to 21 d, and finisher fed from 22 and 35 d. Birds had *ad libitum* access to feed and water throughout the experiment. Live weights of the birds and feed intake were determined on days 0, 7, 14, 21, 28, and 35 of the experiment. Feed conversion ratio (FCR) was calculated accordingly: FCR = feed intake (g)/body weight gain (g), and corrected for mortality.

NE Challenge

The NE challenge was performed based on previous experiments conducted at the University of New England, Australia (Kocher *et al*. 2004; Mikkelsen *et al.* 2009; Wu *et al*. 2010) with modifications. Birds were vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease at the hatchery. The research facility was thoroughly cleaned and disinfected prior to bird placement on fresh pine shavings. On day 9, birds to be inoculated with *Eimeria* in appropriate groups were given *per os* a suspension of 5000 sporulated oocysts of vaccine strains of *E. acervulina*, and *E. maxima*, and 2500 sporulated oocycts of a field strain of *E. brunetti* (Bioproperties Pty., Glenorie, NSW, Australia) in 1 mL sterile phosphate-buffered saline (PBS). Birds in other groups were given sterile PBS in place of the *Eimeria* suspension. On days 14 and 15, birds to be challenged with *C. perfringens* were inoculated *per os* with 1 mL of *C. perfringens* suspension at a concentration of 10^8–10^9 CFU/mL. A primary poultry isolate of *C. perfringens* type A strain EHE-NE18 (Keyburn *et al.* 2006) was incubated overnight at 39^{o}C in 100 mL of thioglycollate broth (USP alternative; Oxoid) followed by subsequent overnight incubations of ca. 1 mL of the previous culture in 200 mL of cooked meat medium (Oxoid). The bacteria were then cultured in 1000 mL of thioglycollate broth (USP alternative; Oxoid) containing starch (10 g/L) and casitone (5 g/L) to obtain the challenge inoculum. Birds in unchallenged groups received 1 mL of sterile thioglycollate broth (USP alternative; Oxoid). The pathologic diagnosis of NE was performed using the standards reported previously (Broussard *et al*. 1986; Prescott *et al.* 1978). The NE lesions of duodenum, jejunum, and ileum of all sampled birds were assessed according to Prescott, et al. (1978), and the severity of the lesions scored as 0, 1, 2, 3, or 4 when present in the tissues (where; 0 = healthy tissue, 4 = severe and extensive focal mucosal necrosis).

Table 7.1. Composition of experimental diets (in g/kg)

Ingredients	Starter	Finisher
Wheat	247.6	270.0
Barley	0.0	20.5
Sorghum	372.0	371.5
Tallow	9.8	18.6
Sunflower oil	9.3	0.9
Canola meal (solvent)	23.3	0.0
Soybean meal (solvent)	302.5	282.0
Limestone	13.5	14.0
MDCP	9.8	10.2
Salt	1.4	1.4
Sodium bicarbonate	2.3	2.3
Choline chloride 75%	0.3	0.6
DL-methionine	2.4	2.2
L-lysine	3.2	3.1
L-threonine	0.6	0.5
Vit and mineral premix †	1.9	1.9
Phyzyme XP5000 broiler	0.1	0.1
Phyzyme XP5000 broiler	0.1	0.1
Ronozyme WX CT	0.3	0.3

† Premix provided (per kg finished feed): molybdenum, 1.0 mg; selenium, 0.2 mg; zinc; 120 mg; manganese, 100 mg; iron, 50 mg; copper, 10.0 mg; cobalt, 5.0 mg; iodine, 1.5 mg; retinyl acetate, 4.1 mg; cholecalciferol, 100 µg; DL-tocopheryl acetate, 100.0 mg; menadione, 2.0 mg; pantothenate, 25 mg; thiamine, 3.0 mg; riboflavin, 8.0 mg; niacin, 60.0 mg; pyridoxine, 6.0 mg; cyanocobalamin, 20.0 µg; biotin, 250.0 µg; folate, 2.0 mg.

Calculated nutrient composition with and without 25% fishmeal

Nutrients as fed	Starter	Starter + 25% fishmeal
Metabolisable energy (MJ/kg)	12.24	12.41
Crude fat (g/kg)	40.9	52.1
Crude fibre (g/kg)	29.6	24.7
Crude protein (N × 6.25) (g/kg)	215.0	332.8
Amino acids (g/kg)		
Lysine	13.0	22.8
Methionine	5.7	8.3
Cystine	3.7	4.6
Threonine	8.5	13.0
Leucine	17.8	25.2
Isoleucine	9.5	14.1
Tryptophan	2.6	3.8
Arginine	13.2	20.8
Calcium (g/kg)	9.51	9.11
Phosphorus, available (g/kg)	4.57	4.53
Sodium (g/kg)	1.59	2.32
Choline (mg/kg)	1389	2337

Statistical Analyses

Performance data were analysed using the statistical package IBM® SPSS® Statistics package version 19 (IBM Corporation). The main effects of fishmeal addition, *Eimeria* inoculation and *C. perfringens* challenge, and their interactions on bird performance were examined by analysis of variance using the General Linear Model. As mortality and lesion score data were not normally distributed, these were analysed by the nonparametric Kruskal-Wallis test.

Results

Performance

Overall, the performance of the birds was significantly different among the 8 treatments, and the performance implications of each of the experimental factors was most apparent when the entire duration of the experiment was considered (Tables 7.2 and 7.3).

Body weight. On d 14 (the day of first *C. perfringens* challenge and 5 days following *Eimeria* inoculation, and 7 days following fishmeal feeding) bird body weight was significantly increased by fishmeal addition ($P < 0.001$), but reduced by *Eimeria* inoculation ($P < 0.001$). The highest body weight was attained by birds fed fishmeal, without *Eimeria* inoculation and challenged with *C. perfringens* (F+E-C+), whereas the birds fed basal diet, with *Eimeria* inoculation and without *C. perfringens* challenge (F-E+C-) had the lowest body weight.

At 35 d, all three factors significantly affected the body weight of the birds. Fishmeal addition increased it ($P < 0.05$), and *Eimeria* and *C. perfringens* infections decreased ($P < 0.001$) it. The birds fed fishmeal without *Eimeria* and *C. perfringens* inoculations (F+E-C-) achieved the highest body weight. Interactions among these three treatment factors were not detected for body weight of the birds at d14 and d35.

Mortality adjusted body weight gain. Mortality-adjusted body weight gain (adjusted gain) during the first 14 d was increased ($P < 0.001$) by fishmeal addition and decreased ($P < 0.001$) by *Eimeria* inoculation. The highest adjusted gain was attained by the F+E-C- birds, and the lowest by the F-E+C- birds. Fishmeal addition during the second week did not affect adjusted gain of the birds during 15-35 d. The *Eimeria* and *C. perfringens* infections, however, decreased adjusted gain of the birds during this period ($P < 0.001$, and $P < 0.05$, respectively). As expected, the birds in the F+E-C- group had the highest adjusted gain during the 15 and 35 d period. As for body weight, the overall adjusted gain during the 35 d study was significantly altered by all the three factors, where fishmeal consumption

increased ($P < 0.05$) body weight and challenge with *Eimeria* and *C. perfringens* decreased ($P < 0.001$ and $P < 0.05$, respectively) body weight. The highest adjusted gain appeared in the F+E-C- group, and the lowest was observed in birds fed fishmeal and challenged by both *Emeria* and *C. perfringens* (F+E+C+). No interactions between the treatment factors were observed on adjusted gain measured during both growth periods.

Feed Intake. Fishmeal decreased ($P < 0.001$) feed intake while the *Eimeria* inoculation reduced it during 0 d to 14 d ($P < 0.01$) (Table 7.3). A 7.8% decrease in feed intake was observed due to the addition of 250 g/kg fishmeal during this period. The highest feed intake occurred in the birds without any of the three treatments (F-E-C-), and the lowest feed intake in the birds fed fishmeal, with *Eimeria* inoculation but without *C. perfringens* challenge (F+E+C-). During 15 d to 35 d, fishmeal addition in the earlier diet had no effect on feed intake ($P > 0.05$), but both *Eimeria* and *C. perfringens* reduced ($P < 0.05$) it. This led to the highest feed intake in the F+E+C- group, and the lowest in the F+E+C+ group. An interaction between fishmeal addition and *C. perfringens* challenge was detected for feed intake for 15 d to 35 d ($P < 0.05$). This was evident when birds receiving the fishmeal diet up to 14 d (which coincided with a depression in feed intake) then experienced an increase in feed intake during 15 d to 35 d, but only when the birds were not challenged with *C. perfringens* (data not shown). The three-way interaction between fishmeal, *Eimeria* and *C. perfringens* on feed intake during 15-35 d was also significant ($P < 0.05$). The pattern of feed intake and interactions between the three treatment factors during the entire trial (0-35 d) were similar to those observed during 15-35 d, with the highest feed intake in F+E+C- and the lowest feed intake in F+E+C+.

Feed Conversion Ratio. While fishmeal had a positive effect on FCR during earlier and later periods of trial, *Eimeria* and *C. perfringens* had unfavorable effects on FCR (Table 7.3). Fishmeal addition between d7-14 improved FCR by an average of 0.18 from d -14. However, the initial improvement in feed efficiency observed when birds received fishmeal was reversed during d15-35 ($P < 0.05$). Both *Eimeria* and *C. perfringens* infection led to poorer FCR during the same period ($P < 0.001$ and 0.01, respectively). Fishmeal and *C. perfringens* did not show any influence on FCR overall, however, *Eimeria* challenge decreased ($P < 0.001$) mortality-adjusted FCR by 80 g of feed per kg body weight over the entire study.

Table 7.2 Body weight and mortality adjusted body weight gain of birds relative to *C. perfringens* challenge, *Eimeria* inoculation and fishmeal feeding

	Body weight				Mortality adjusted body weight gain					
	d14		d35		d0-14		d15-35		d0-35	
Treatment	Mean†	SD	Mean†	SD	Mean†	SD	Mean†	SD	Mean†	SD
F-E-C-	424.8^{c}	6.9	2132.8^{b}	47.5	379.1^{bc}	3.5	1695.9^{c}	25.1	2075.0^{c}	25.6
F-E-C+	421.1^{bc}	3.3	2023.0^{ab}	50.4	376.0^{abc}	1.5	1591.8^{bc}	28.9	1967.7^{bc}	28.5
F-E+C-	400.0^{a}	3.7	1918.4^{a}	28.0	358.5^{a}	3.6	1502.6^{abc}	16.1	1861.2^{ab}	16.1
F-E+C+	404.1^{ab}	2.3	1931.5^{a}	31.1	361.7^{ab}	3.2	1440.3^{ab}	27.7	1802.0^{ab}	26.6
F+E-C-	443.8^{d}	4.2	2147.5^{b}	16.7	401.7^{e}	2.8	1708.3^{c}	23.2	2110.1^{c}	25.0
F+E-C+	445.6^{d}	2.1	2106.2^{b}	29.2	400.9^{de}	1.9	1576.2^{bc}	37.4	1977.1^{bc}	37.3
F+E+C-	425.5^{c}	4.7	2032.8^{ab}	21.2	382.2^{cd}	8.7	1587.7^{bc}	48.8	1969.9^{bc}	46.0
F+E+C+	430.7^{cd}	1.1	1955.9^{a}	26.3	388.8^{cde}	3.5	1329.5^{a}	100.2	1718.3^{a}	97.7
One way	***		***		***		***		***	
Main effect‡										
F -	412.5	2.7	2001.4	21.1	368.8	2.3	1557.6	23.2	1926.5	24.6
+	436.4	2.7	2060.6	24.3	393.4	2.9	1550.4	40.1	1943.8	40.2
E -	433.8	2.7	2102.4	16.1	389.4	2.8	1643.1	18.4	2032.5	18.8
+	415.1	3.5	1959.6	20.2	372.8	3.7	1465.0	33.4	1837.8	32.4
C -	423.5	4.0	2057.9	23.9	380.4	4.0	1623.6	22.7	2004.0	24.7
+	425.4	3.3	2004.2	21.8	381.8	3.3	1484.4	34.8	1866.3	34.7
GLM ANOVA‡										
F	***		*		***		NS		*	
E	***		***		***		***		***	
C	NS		*		NS		*		*	
F * E	NS		NS		NS		NS		NS	
F * C	NS		NS		NS		NS		NS	
E * C	NS		NS		NS		NS		NS	
F * E * C	NS		NS		NS		NS		NS	

†Values within a column with unlike superscripts are significantly different.

‡NS: $P \geq 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Bold: variables analysed in the study. *Italic*: statistical analysis applied to the respective data.

Table 7.3. Feed intake and feed conversion ratio (FCR) of birds relative to *C. perfringens* challenge, *Eimeria* inoculation and fishmeal feeding

Treatment	Mortality adjusted feed intake						Mortality adjusted FCR					
	d0-14		d15-35		d0-35		d0-14		d15-35		d0-35	
	Mean† †	SD	Mean†	SD	Mean†	SD	Mean†	SD	Mean†	SD	Mean†	SD
F-E-C-	493.1^{c}	2.9	2890.2^{ab}	32.2	3383.3^{ab}	34.5	1.30^{b}	0.01	1.70^{a}	0.01	1.59^{a}	0.01
F-E-C+	490.4^{c}	4.7	2766.5^{ab}	33.6	3256.8^{ab}	37.2	1.30^{b}	0.01	1.74^{a}	0.02	1.61^{ab}	0.01
F-E+C-	479.9^{bc}	5.4	2677.9^{ab}	16.1	3157.8^{ab}	18.4	1.34^{b}	0.01	1.79^{a}	0.03	1.63^{ab}	0.02
F-E+C+	479.8^{bc}	4.7	2757.4^{ab}	63.2	3237.1^{ab}	66.0	1.32^{b}	0.01	1.95^{ab}	0.04	1.63^{abc}	0.02
F+E-C-	459.9^{ab}	5.1	2952.8^{b}	37.8	3412.6^{b}	40.3	1.15^{a}	0.02	1.73^{a}	0.01	1.66^{abc}	0.01
F+E-C+	449.8^{a}	3.7	2856.5^{ab}	31.3	3306.3^{ab}	33.0	1.13^{a}	0.01	1.81^{a}	0.03	1.68^{abc}	0.02
F+E+C-	436.3^{a}	14.5	2980.8^{b}	118.9	3417.1^{b}	126.7	1.14^{a}	0.02	1.88^{ab}	0.04	1.69^{bc}	0.03
F+E+C+	444.8^{a}	3.1	2621.8^{a}	120.6	3066.6^{a}	120.4	1.15^{a}	0.01	2.1^{b}	0.14	1.73^{b}	0.04
One way ANOVA‡	***		**		*		***		***		***	
Main effect												
F -	485.8	2.5	2773.0	24.5	3258.8	26.1	1.32	0.01	1.79	0.02	1.66	0.01
+	447.7	4.2	2853.0	50.6	3300.7	51.8	1.14	0.01	1.88	0.05	1.64	0.02
E -	473.3	4.4	2866.5	21.1	3339.8	21.3	1.22	0.02	1.75	0.01	1.61	0.01
+	460.2	5.6	2759.5	51.0	3219.7	51.4	1.24	0.02	1.93	0.04	1.69	0.01
C -	467.3	5.9	2875.4	39.1	3342.7	39.3	1.23	0.02	1.77	0.02	1.64	0.01
+	466.2	4.5	2750.6	37.8	3216.7	38.9	1.23	0.02	1.90	0.05	1.66	0.01
GLM ANOVA‡												
F	***		NS		NS		***		*		NS	
E	**		*		*		NS		***		***	
C	NS		*		*		NS		**		NS	
F * E	NS		NS		NS		NS		NS		NS	
F * C	NS		*		*		NS		NS		NS	
E * C	NS		NS		NS		NS		NS		NS	
F * E * C	NS		*		*		NS		NS		NS	

† Values within a column with unlike superscripts are significantly different.

‡ NS: $P \geq 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Bold: variables analysed in the study. *Italic*: statistical analysis applied to the respective data.

Necrotic Enteritis

Bird Mortality. Bird mortality and NE lesion scores in the small intestine were affected largely by *Eimeria* and *C. perfringens* challenge. Treatments receiving *Eimeria* and *C. perfringens* inoculations experienced higher NE-associated moralities than treatments receiving either or neither of the two inoculums, regardless of fishmeal feeding (Figure 7.1). The *P* values for the paired comparisons of the two groups challenged with *Eimeria* and *C. perfringens* with the other 6 groups were all less than 0.05. Respectively, the NE-associated mortalities of the birds were 24% in the group infected with *Eimeria* and *C. perfringens* and fed basal diet (F-E+C+), and 27% in the F+E+C+ group. Although a 3 percentage point higher mortality rate was observed in the F+E+C+ group than in the F-E+C+ group, the difference was not statistically significant ($P = 0.94$).

Intestinal Lesion Scores. The cause of mortalities in the treatment groups was also reflected in the results produced from the analysis of NE lesion scores in the small intestine of birds as shown in Figure 7.2. In the duodenum, the highest NE lesion score was recorded in F+E+C+ birds. The lesion score of this group was significantly higher than those in the groups F-E-C-, F-E-C-, F-E+C+, and F+E-C-. The F-E+C+ birds had significantly higher ($P < 0.05$) NE lesion scores than the birds in F-E-C- group and the birds fed basal diet, challenged by C. perfringens but not with Eimeria (F-E-C+). It is also worth note that the duodenal lesion score was significantly higher in F+E-C+ than that in F-E-C+ and F-E-C- indicating some fishmeal role in the NE occurrence when Eimeria infection was not applied (Figure 7.2a). In the jejunum, the NE lesion scores were higher ($P < 0.05$) in the birds from the F+E+C+ and F-E+C+ groups than in the birds of all groups except F+E-C+ (Figure 7.2b). A similar NE lesion score pattern was identified in the ileum, i.e., the combination of Eimeria and C. perfringens effectively induced NE lesions in this section of small intestine (lesion scores of F+E+C+ = 0.96, and F-E+C+ = 0.83). Conversely, fishmeal addition alone or in combination with either C. perfringens or Eimeria treatment did not significantly contribute to the occurrence of NE lesions in the ileum (Figure 7.2c). When the NE lesion scores from three sections of small intestine were averaged, the two groups with both Eimeria and C. perfringens treatments also had high lesion scores (lesion scores of F+E+C+ = 1.16, and F-E+C+ = 0.95, Figure 7.2d).

Overall, the results of lesion score and mortality analysis indicated that the interaction between *Eimeria* inoculation and *C. perfringens* challenge were significant and both treatments were required to cause the NE lesions in the small intestine, and hence NE-associated mortality.

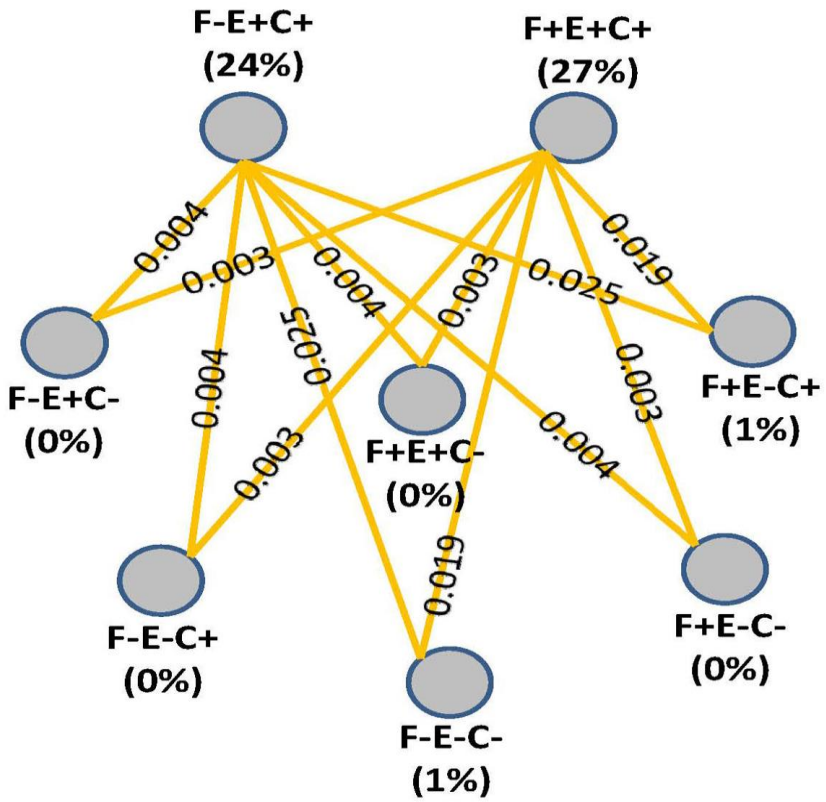


Figure 7.1. The NE-caused mortality of the birds in groups with or without 25% fishmeal addition in the starter diet during days 8 to 14, Eimeria inoculation on day 9, and C. perfringens challenge on days 14 and 15 of the trial. The levels of mortalities (%) are shown in the brackets underneath the names of treatments. The mortalities are significantly different between two treatments if the pair is linked by a line on which *P* value is shown. The data were analysed using nonparametric Kruskal-Wallis test. The abbreviations are: F, fishmeal addition; E, Eimeria inoculation; C, C. perfringens challenge; +, the treatment applied; -, no such treatment.

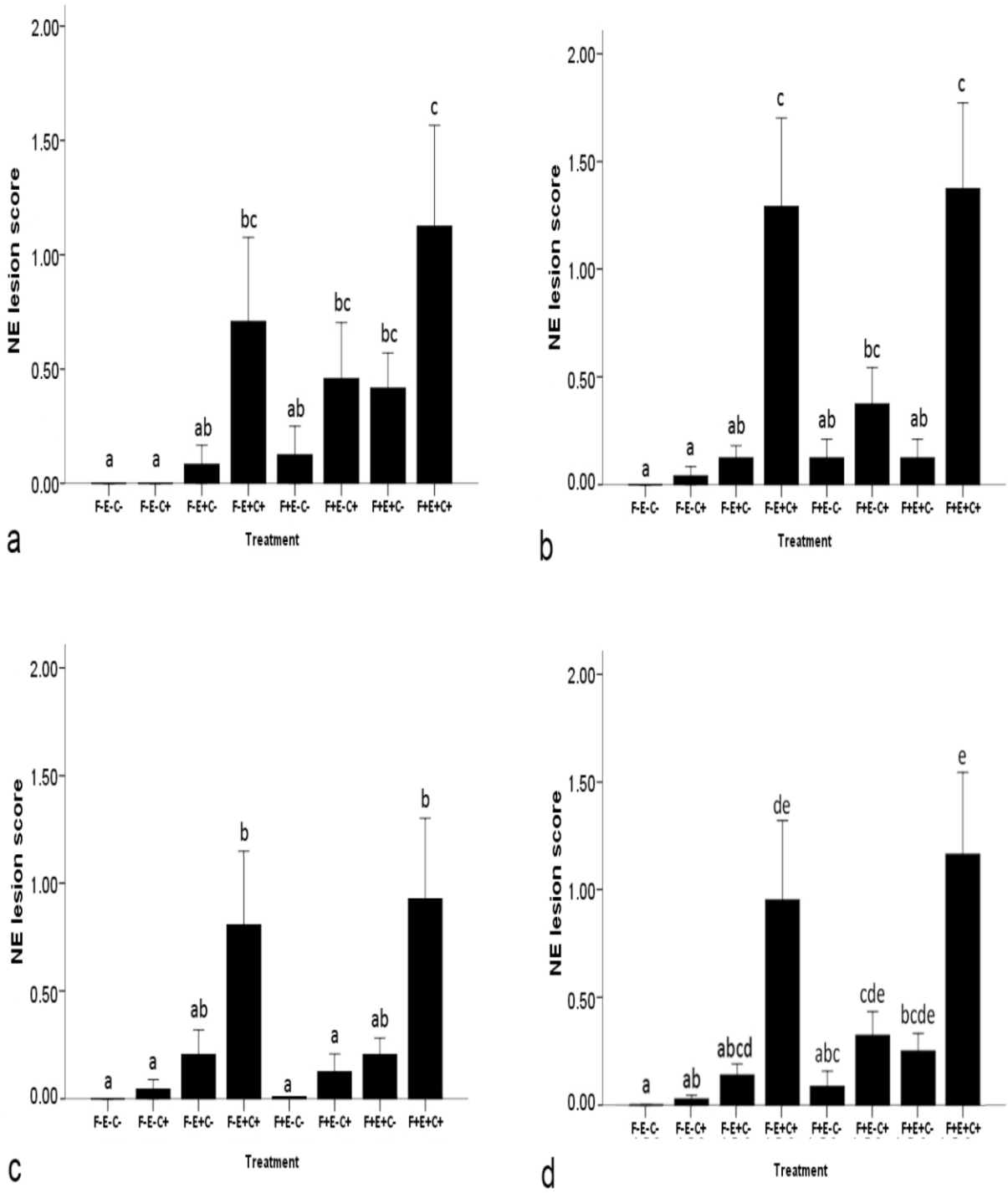


Figure 7.2. Intestinal lesion scores in different sections of the birds with different treatments on day 16. a. Duodenum; b. Jejunum; c. Ileum; d. Mean of three sections. The means not having the same letter above the error bars are significantly different (*P* < 0.05). The error bar is the standard error of the treatment (n = 6). The abbreviations are: F, fishmeal addition; E, Eimeria inoculation; C, C. perfringens challenge; +, the treatment applied; -, no such treatment.

Discussion

In the present study, three factors, i.e. fishmeal addition, *Eimeria* inoculation and *C. perfringens* challenge, which have been previously considered important to the NE challenge model in meat chickens, were examined to assess their roles individually and their interactions on the induction of NE in meat chickens. The *Eimeria* inoculation together with *C. perfringens* challenge proved to be important to induce the disease, whereas fishmeal was not beneficial when *Eimeria* was used.

Fishmeal has been widely used in the NE challenge models, and considered important to the reliable reproduction of NE in the chicken challenge models (Brennan et al. 2003; Cowen *et al.* 1987; Hofacre *et al.* 2003; Prescott 1979; Truscott and Al-Sheikhly 1977; Wu *et al*. 2010). It is thought to promote rapid growth of *C. perfringens* in the gut, leading to localised populations to exceed 10^8 CFU/g of digesta (McDevitt *et al.* 2006). This is believed to be due to the inability of *C. perfringens* to produce most of the essential amino acids it needs for proliferation. So it thrives when these conditions are met, i.e., when given excess protein. It has also been suggested that fishmeal may cause the onset of NE through the production of biogenic amines in the gut. Therefore, it has been considered that challenge models without the inclusion of fishmeal are less successful in reproducing the disease (Kocher and Choct 2008). In the present study, however, the addition of fishmeal did not significantly increase the overall severity of NE lesion scores or NE-associated mortality, although it resulted more severe duodenal lesion in the groups without *Eimeria* treatment, suggesting that fishmeal can be omitted from the NE challenge protocol. This seems not in line with the findings from at least some of the studies (Brennan *et al*. 2003; Cowen *et al.* 1987; Hofacre *et al.* 2003; Prescott 1979; Truscott and Al-Sheikhly 1977; Wu *et al.* 2010). However, the result presented here needs to be repeated in more trials to ensure removal of fishmeal from the model can reliably reproduce NE. It is also worth to investigate the importance of fishmeal inclusion in the previous models. As indicated in this study and some challenge models used in other groups, fishmeal may be important to introduce NE when *Eimeria* is not used in the challenge protocol.

An important application of the NE challenge model is to test potential alternatives to antibiotics under NE challenge by observing not only the frequency and severity of clinical symptoms but also bird performance pre and post challenge to market weight. It is often required that bird performance be at a commercial standard if the efficacy of an additive has any credibility in practice. The feeding of a high fishmeal diet, albeit for a short period of time during the challenge, has always attracted some criticism for its commercial irrelevance. Thus, the removal of fishmeal from the model will remove nutritional bias from future studies and allow the measured performance parameters to be more relevant to industry. Interestingly, 25% dietary fishmeal significantly reduced feed intake, increased body weight gain and improved the FCR of the birds

during 0 d to 14 d. However, this effect did not continue during the rest period of the trial (15-35 d). Rather, the FCR was significantly higher for the birds fed fishmeal than those fed only control diet during 15d to 35 d. Although challenged and unchallenged birds received fishmeal enriched diets in previous studies at our laboratory, feeding such a diet does not allow normal performance pre-challenge (as shown here), and may not therefore allow the most accurate representation of birds performance in treatment groups post-challenge.

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Coccidiosis has been considered an important factor to predispose meat chickens to NE in the challenge models as well as in the field (Al-Sheikhly and Al-Saieg 1980; Bradley and Radhakri 1973; Collier *et al*. 2008). The co-infection of *Eimeria* with *C. perfringens* has been used widely in NE challenge models to induce the disease (Forder *et al.* 2012; Hong *et al.* 2012; Park *et al.* 2008), however, some models can produce clinical symptoms without the need of *Eimeria* inoculation (Sarson *et al.* 2009; Truscott and Al-Sheikhly 1977). In our NE challenge model, it appears that *Eimeria* infection is necessary to assist *C. perfringens* colonisation and thus to produce consistent infection of the birds. Significantly higher intestinal lesion scores and mortality rates in the birds co-infected with *Eimeria* and *C. perfringens* than those in other groups indicate the critical role of *Eimeria* in the NE challenge model.

Conclusion

The present study elucidated the role of predisposing factors, i.e., *Eimeria* infection and fishmeal, together with *C. perfringens* in the introduction of NE disease in an experimental challenge model. The results indicated that fishmeal was not a prerequisite for the successful induction of NE into meat chickens fed an industry standard diet. However, it appears that *Eimeria* infection is important for a challenge model to produce clinical NE especially NE caused mortality in meat chickens.

Chapter 8. Dynamics of intestinal metabolites and morphology in response to necrotic enteritis challenge in meat chickens

Extracted from an original article by Wu et al. (2016)

Introduction

Necrotic enteritis (NE) has been considered an economically important disease for the poultry industry worldwide and the annual costs of up to 6 billion US dollars have been estimated recently for losses incurred in meat chicken production globally (Wade and Keyburn 2015). Both clinical and subclinical NE can be responsible for such losses, but subclinical cases likely account for the majority of financial losses as such cases may only affect growth performance and efficiency of production and in the absence of outwardly visible clinical symptoms. Since the ban on the use of antibiotics in animal feed was implemented in the European Union, the re-emergence of NE has been reported in Europe (Kaldhusdal 2015; Tsiouris 2015). Similarly, the disease has also been considered a concern in North America in recent years due to voluntary removal of antibiotics from chicken rations (Mozisek *et al.* 2015; Smyth 2015). As consumer pressure from across the globe to remove in-feed antibiotics has been increasing, more attention has been paid to NE outbreaks.

Clostridium perfringens has been proved to be responsible for NE in chickens with NetB toxin being essential for its onset (Keyburn *et al.* 2008). It has also been recognised that predisposing factors, such as high dietary protein and non-starch polysaccharides (NSPs), coccidiosis and environmental stressors, are important considerations for managing meat chicken production without the use of in-feed antibiotics (M'Sadeq *et al.* 2015; Timbermont *et al.* 2011). Among those, high protein diets and *Eimeria* infection have been found to be highly associated with NE outbreaks in the field (Annett *et al.* 2002; Broussard *et al.* 1986; Kaldhusdal and Skjerve 1996; Williams 2005), or at least to promote the growth of *C. perfringens* in the intestine of chickens (Bradley and Radhakri 1973; Drew *et al.* 2004). With the application of these predisposing factors, experimental challenge models have been established to elucidate the aetiology and to investigate possible ways to control or reduce the disease (Al-Sheikhly and Al-Saieg 1980; Gholamiandehkordi *et al.* 2007; Keyburn *et al.* 2008; Truscott and Al-Sheikhly 1977; Williams *et al.* 2003; Wu *et al.* 2010).

In chickens, the major energy sources come from glucose through the digestion of dietary starch (Jozefiak *et al.* 2004; Weurding *et al.* 2001) and long chain fatty acids. Although VFAs produced from fermentation of carbohydrates and proteins in the caeca do not contribute a significant portion of the dietary energy to poultry (Choct and Annison 1992; Svihus *et al.* 2013), together with other

acids, such as branded chain fatty acids (BCFA), and lactic acid, they have important roles to play in the GIT of birds, for example, inhibition of the growth of some pathogenic bacteria. This can be achieved by the penetration of these fatty acids through bacterial cell membrane leading to the dissociation of charged anions and protons that decreases bacterial intracellular pH and inhibits essential metabolic reactions, and thus reducing bacterial growth (Cherrington *et al.* 1991; Davidson and Taylor 2007). It has been demonstrated that organic acids show bactericidal effects on *C. perfringens* although this does not necessarily translate into improvement of health and performance of birds with NE. Nevertheless, reduction of NE symptoms by the application of butyric acid and sodium lauroyl lactylate in the diets of NE challenged birds has been reported (Biggs and Parsons, 2008; Huyghebaert *et al.* 2011; Timbermont 2009). Despite a suggested beneficial role of VFAs in the intestine of animals, information on their dynamics in response to the health status of chickens, especially those with NE, is still scarce and the exact role and underlying mechanisms are not yet fully understood. Therefore, more information on the dynamics of VFAs and other fatty acids in the GIT of NE infected chickens is needed especially when different predisposing factors are involved.

We have previously reported the responses in performance, disease occurrence, gut microflora and mucin gene expression of meat chickens to predisposing factors, i.e., fishmeal feeding and *Eimeria* inoculation, as well as the causative agent *C. perfringens* (Kitessa *et al.* 2014; Rodgers *et al.* 2015; Stanley *et al.* 2014; Wu *et al.* 2014). The performance and disease data demonstrate that the *Eimeria* is a more important predisposing factor than fishmeal feeding, and it has been suggested to remove fishmeal from the NE challenge model (Rodgers *et al.* 2015). Metagenomics analyses of caecal microflora have revealed that both fishmeal feeding and *Eimeria* infection induce significant changes in the gut microbiota which may play an important role in predisposing birds to NE prior to the *C. perfringens* challenge at 13 d (Wu *et al.* 2014). Following the *C. perfringens* challenge of the birds, similar effects of fishmeal and *Eimeria* on microbiota at 16 d have been observed in both alpha and beta diversity. These two predisposing factors also enable *C. perfringens* to establish itself after challenge. The study also showed that there was little overlap in the changes caused following *Eimeria* and fishmeal treatments, possibly indicating multiple routes to progress towards clinical symptoms of NE (Stanley *et al.*, 2014). Furthermore, gene expression study shows that mRNA levels of mucin synthesis genes are modulated by fishmeal supplementation in the diet, and *Eimeria* challenge reduced Muc2 gene mRNA levels about 6 d post-inoculation independent of *C. perfringens* challenge. Both *Eimeria* and *C. perfringens* challenges significantly reduced the mRNA levels of CD36 gene, but no additive effect of *Eimeria* and *C. perfringens* on the mRNA levels of this gene can be concluded (Kitessa *et al.* 2014).

In the present study, we examined the roles of dietary fishmeal inclusion, *Eimeria* inoculation, and *C. perfringens* challenge on metabolites and histomorphometry dynamics in the GIT in a 2 × 2 × 2 factorial arrangement of treatments for birds. We hypothesised that *Eimeria* and fishmeal would substantially alter the intestinal metabolites and morphology possibly in favour of the growth of pathogenic *C. perfringens*.

Materials and methods

Birds and diets

The experimental design, diets and animal management was the same as reported previously (Rodgers *et al.*, 2015). Briefly, day-old Ross 308 male meat chickens (Baiada Country Road Hatchery, Tamworth, NSW, Australia) were raised under same conditions of Rodgers *et al.* (2015) in 48 floor pens, 6 pens in each group, on softwood shavings within 8 hours of hatch (d 0) in a temperature-controlled room at Kirby Research Station, University of New England, Australia. The birds were subjected to fluorescent illumination at 20 Lux for 23 h/d for the first 7 d from placement and then 16 h/d thereafter. A 2 × 2 × 2 factorial design, i.e., with 8 treatment groups and a total of 192 birds, was applied with or without: 250g/kg fishmeal (F±) feeding from d 8 to d 14, *Eimeria* inoculation on d 9 (E±), and oral inoculation of approximately 10^8 CFU *C. perfringens* type A on d 14 and d 15 (C±). The experiment was approved by the Animal Ethics Committee of the University of New England. Birds were given a common starter diet from 0 to 7 d. Fishmeal was added at 250 g/kg from 7 d to 14 d to half of the pens (F+) as specified. The other half of pens (F-) received the basal starter diet over the same period. Fishmeal was removed from the F+ treatment group pens on d 14. Birds had *ad libitum* access to feed and water throughout the experiment.

NE challenge

The NE challenge was performed same as in the previous experiment reported by Rodgers *et al.* (2015). In general, on 9 d, birds were inoculated with *Eimeria* (E+), *per os*, a suspension of 5000 sporulated oocysts of vaccine strains of *E. acervulina*, and *E. maxima*, and 2500 sporulated oocycts of a field strain of *E. brunetti* (Bioproperties Pty., Glenorie, NSW, Australia) in 1 mL sterile phosphate-buffered saline (PBS). Birds in non-*Eimeria* (E-) groups were given sterile PBS in place of the *Eimeria* suspension. On d 14 and d 15, birds to be challenged with *C. perfringens* (C+) were inoculated *per os* with 1 mL of *C. perfringens* suspension at a concentration of 10^8–10^9 CFU/mL. A primary poultry isolate of *C. perfringens* type A strain EHE-NE18 (Keyburn *et al.*, 2006) was used for the inoculations. Birds in unchallenged groups received 1 mL of sterile thioglycollate broth (USP alternative; Oxoid). Twelve birds from each experimental group or 2 birds from each pen were sampled for VFA analysis with ileal and caecal digesta pooled from each pen respectively and for gut histomorphometric measurements at d 13 and d 16. Altogether, 96 birds were sacrificed for each sampling day.

Analysis of volatile fatty acids and lactic acid, and histomorphometric measurements

The analysis of VFA and enumeration of bacteria followed the methods used previously (Stanley *et al.* 2014; Wu *et al.* 2010; Wu *et al.* 2014). Briefly, VFA and lactate concentrations were measured in a scale of 2.0 g thawed caecal sample by gas chromatography using 2-ethylbutyric acid as an internal standard. Total VFA concentration was calculated by the sum of all the VFA and lactate measured in a sample, expressed as mmol/g digesta, and individual organic concentration was expressed as the level relative to the total VFA (%). Ileal and caecal pH was measured using a hand-held series pH-meter (Eutech Instruments, Singapore) immediately after the intestinal contents were collected and pooled from two birds sampled in each pen.

For gut histomorphometric measurement, one cm of the duodenal mid-section was collected and fixed in 10% buffered formalin (3.7 % formaldehyde, pH 7.5). Formalin-fixed duodenum samples were processed in consecutive steps of dehydration by serial ethanol solutions (30% to 100%), clearing by xylene, and infiltration by paraffin. The tissue was embedded in paraffin and subsequently sectioned at a thickness of 8 □m with a Reichert-Jung 820 Histocut Microtome (Cambridge Instruments GmbH, Germany). The tissue sections on the slides were stained using Harris's hematoxylin (George Gurr Ltd., London, UK), and eosin (Gur Certistain, VWR International Ltd., Poole, UK), and mounted with DPX (distrene polystyrene xylene) mountant. The sections were viewed under an Olympus Vanox microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and the images captured with a colour video camera LY-MN-HP SUPER CCD (Chengdu Liyang Precision Machinery Co. Ltd, Chengdu, China). Morphometric indices were determined using computer-aided light microscope image processing analysis software VideoPro 32 package (Leading. Edge Pty Ltd, Adelaide, Australia). Villus height and crypt depth were measured in 20 vertically, well-oriented, intact villi and crypts. Villus height to crypt depth ratio was calculated by averaged villus height:averaged crypt depth (VH:CD) for each pen. All measurements were calibrated with a micrometer.

Statistical analyses

The data were analysed using the statistical package IBM® SPSS® Statistics package version 19 (IBM Corporation). At d 13, the analysis of data followed a 2 × 2 factorial design as *C. perfringens* challenge was not yet conducted. At d 16, the analysis of data followed a 2 × 2 × 2 factorial design following the *C. perfringens* challenge performed on d 14 and 15. The main effects of fishmeal addition, *Eimeria* inoculation and *C. perfringens* challenge, and their interactions on measured parameters were examined by analysis of variance using the General Linear Model. One-way ANOVA analysis was performed when the interactions were present and Tukey's paired

comparison procedure was applied. When the data were not normally distributed such as in the cases of some VFAs, nonparametric Kruskal-Wallis analysis was applied.

Results

VFA, pH and histomorphometry at 13 d

At 13 d, fishmeal did not change total VFA level in ileum ($P > 0.05$), but increased both formic ($P < 0.001$) and acetic ($P < 0.01$) acids relative to total VFA level while reduced lactic acid ($P < 0.001$). *Eimeria*, in contrast, significantly increased total VFA level ($P < 0.05$) and lactic acid ($P < 0.001$), but reduced formic ($P < 0.01$) and acetic ($P < 0.001$) acids (Table 8.1). The major VFA in ileum was lactic acid which accounted for an average of 77.7% of total. No interactions between these two treatments were observed in the ileum at d 13 ($P > 0.05$).

Table 8.1. Ileal VFA responses to the addition of 250 g/kg fishmeal in diet during days 8–14 and *Eimeria* inoculation on d 9 in broiler chickens at the age of 13 d.

Treatment		Total VFA (mmol/g)	Formic acid (%)	Acetic acid (%)	Lactic acid (%)
Fishmeal	+	12.4	6.97	19.3	71.9
	-	16.0	2.69	11.8	83.8
Eimeria	+	18.0	3.35	10.8	84.3
	-	10.0	6.51	20.8	70.8
Pooled SE		3.7	0.95	2.7	3.6
GLM *P* values ^					
Fishmeal		NS	***	**	***
Eimeria		*	***	***	***
Fishmeal**Eimeria*		NS	NS	NS	NS

^ The asterisks ***, ** and * represent the *P* values being < 0.001, 0.01 and 0.05 respectively, and NS means the *P* value being > 0.05.

In the caeca, fishmeal did not alter total VFA level, but increased formic ($P < 0.001$), propionic ($P < 0.05$), isobutyric ($P < 0.001$), and isovaleric ($P < 0.01$) acids, while decreased butyric acid ($P < 0.05$) (Table 8.2). The major VFA in the caeca was acetic acid which accounted for an average of 72.0% of the total, and was not affected by either fishmeal or Eimeria application at 13 d ($P < 0.05$). Eimeria did not show any effect on total and individual VFA contents in the caeca ($P < 0.05$). Interactions between fishmeal and Eimeria were observed on formic acid ($P < 0.05$) in ileum. Eimeria reduced only the caecal formic acid of birds fed control diet but not of those fed fishmeal (Figure 8.1).

Table 8.2. Caecal VFA responses to the addition of 250 g/kg fishmeal in diet during days 8–14 and Eimeria inoculation on d 9 in broiler chickens at the age of 13 d.

Treatment		Total VFA (mmol/g)	Acetic acid (%)	Formic acid (%)	Propionic acid (%)	Isobutyric acid (%)	Butyric acid (%)	Isovaleric acid (%)
Fishmeal	+	75.4	72.7	0.70	1.77	0.61	8.86	0.15
	-	73.8	71.2	0.18	1.38	0.28	10.49	0.06
Eimeria	+	75.1	72.0	0.46	1.49	0.46	9.33	0.11
	-	74.0	72.0	0.43	1.84	0.44	9.99	0.10
Pooled SE		5.0	2.4	0.07	0.15	0.07	0.74	0.03
GLM *P* values ^								
Fishmeal		NS	NS	***	*	***	*	**
Eimeria		NS	NS	NS	NS	NS	NS	NS
Fishmeal**Eimeria*		NS	NS	*	NS	NS	NS	NS

^ The asterisks ***, ** and * represent the *P* values being < 0.001, 0.01 and 0.05 respectively, and NS means the *P* value being > 0.05.

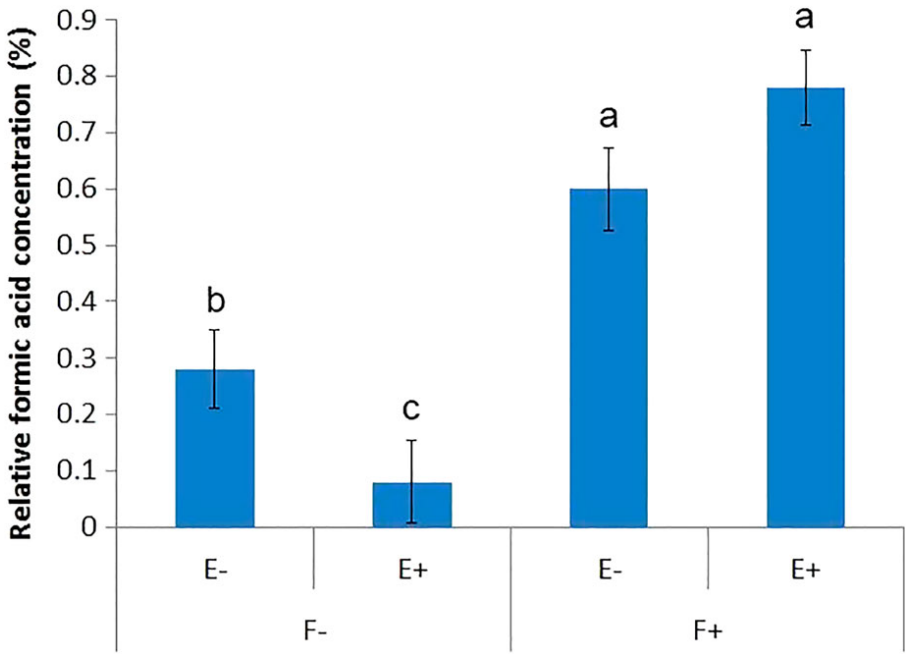

Figure 8.1. Interaction between fishmeal and *Eimeria* on caecal formic acid of chickens at 13 d of age. Error bars show standard error of means. Means not having same letters indicate that the difference is significant ($P < 0.05$).

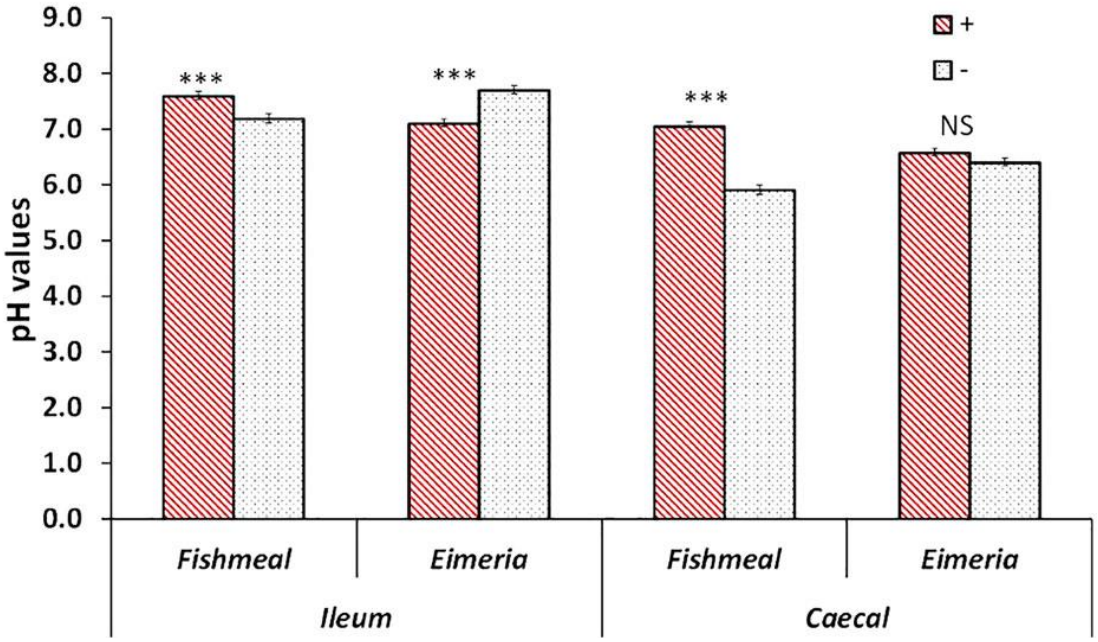

Figure 8.2. Ileal and caecal pH responses at 13 d to the addition of 250 g/kg fishmeal in diet during days 8–14 and Eimeria inoculation on d 9 of broiler chickens. Fishmeal+, or – represents pH values with or without fishmeal supplementation regardless of Eimeria inoculation, and Eimeria+, or – represents pH values with or without Eimeria inoculation regardless of fishmeal supplementation. The asterisks * represent the P values being < 0.001, and NS means the P value being > 0.05.**

At the same time, fishmeal elevated pH values in both the ileum ($P < 0.001$) and caeca ($P < 0.001$), while *Eimeria* decreased ileal pH value ($P < 0.001$) and did not affect caecal pH value ($P > 0.05$) (Figure 8.2). There was no effect of fishmeal or Eimeria at 13 d ($P > 0.05$) on duodenal histomorphometry.

Ileal VFA, lactic acid and pH at 16 d

At 16 d, the main effects of treatments were detected on the VFA and pH in the ileum (Table 8.3). Total ileal VFA concentration was increased by the inoculation of *Eimeria* ($P < 0.001$) and *C. perfringens* ($P < 0.001$), but not by dietary fishmeal addition ($P > 0.05$). The relative formic acid to total VFA concentration was reduced by *Eimeria* ($P < 0.001$) but not affected by *C. perfringens* and dietary fishmeal addition ($P > 0.05$). Acetic acid was not affected by all the treatments as main effects ($P > 0.05$). The relative propionic acid concentration was increased by both *C. perfringens* ($P < 0.05$) and *Eimeria* ($P < 0.01$) but not affected by fishmeal ($P > 0.05$). Similarly, butyric acid was increased by both *Eimeria* ($P < 0.05$) and *C. perfringens* ($P < 0.05$) but not by fishmeal ($P > 0.05$). Lactic acid was the major VFA components in ileum with an average of 76.4% relative to the total, however was not affected by the treatments as main effects. Furthermore, pH in ileum was reduced by both *Eimeria* ($P < 0.001$) and *C. perfringens* ($P < 0.001$).

Two-way interactions among these factors were observed on the VFAs and pH in the ileum at 16 d (Table 8.3). No fishmeal × *Eimeria* interactions were observed but fishmeal × *C. perfringens* interactions were present on butyric acid ($P < 0.05$) and lactic acid, and *Eimeria* × *C. perfringens* interactions were present in propionic acid ($P < 0.05$), lactic acid ($P < 0.05$) and pH ($P < 0.01$). Relative butyric acid was increased by *C. perfringens* challenge in the birds only when fishmeal was fed (7.92% vs 0.04%). Noticeably, the butyric acid relative concentration was 11.64% in the birds challenged with *Eimeria* and *C. perfringens* and fed with fishmeal while there was no detectable level of the acid observed in the group without any of experimental treatments applied. On the other hand, relative lactic acid concentration was reduced by *C. perfringens* challenge only when the birds were fed additional fishmeal. Relative propionic acid was increased by *Eimeria* inoculation only when *C. perfringens* challenge was applied, and it was the case *vice versa*. In contrast, lactic acid was reduced by *C. perfringens* challenge only when *Eimeria* was applied, whereas no change was made by *C. perfringens* otherwise. In addition, ileal pH was lowered by *C. perfringens* when birds were not inoculated with *Eimeria*, and at the same time *Eimeria* inoculation significantly reduced ileal pH regardless of *C. perfringens* challenge.

Table 8.3. Ileal VFA responses to the addition of 250 g/kg fishmeal in diet during days 8–14, *Eimeria* inoculation on d 9 and *C. perfringens* challenge on days 14 and 15 in broiler chickens at the age of 16 d.

Treatment [$]			Total VFA (mmol/g)	Formic acid (%)	Acetic acid (%)	Propionic acid (%)	Butyric acid (%)	Lactic acid (%)	pH ileum
Fishmeal	*Eimeria*	*C. perfringens*							
-	-	-	6.3^{c}	3.26	19.1^{ab}	0.00^{b}	0.00^{b}	76.4^{ab}	7.85^{ab}
-	-	+	7.6^{c}	3.89	17.7^{ab}	0.00^{b}	0.00^{b}	77.3^{ab}	7.26^{bc}
-	+	-	16.5^{bc}	0.60	16.9^{ab}	0.04^{ab}	4.01^{ab}	77.0^{ab}	6.46^{de}
-	+	+	42.2^{a}	1.25	12.9^{ab}	0.14^{ab}	5.33^{ab}	77.4^{ab}	5.90^{e}
+	-	-	9.6^{bc}	3.81	17.5^{ab}	0.00^{b}	0.00^{b}	78.0^{ab}	8.11^{a}
+	-	+	24.8^{abc}	3.61	13.5^{ab}	0.00^{b}	3.46^{ab}	78.4^{ab}	6.70^{cd}
+	+	-	24.5^{abc}	0.00	6.4^{b}	0.00^{b}	0.16^{b}	92.5^{a}	6.15^{de}
+	+	+	30.1^{ab}	2.64	25.4^{a}	0.35^{a}	11.64^{a}	55.0^{b}	6.10^{de}
Pooled SE			5.0	1.01	4.3	0.07	2.4	6.9	0.16
Main effect									
Fishmeal	-		18.2	2.25	16.6	0.04	2.33	77.0	6.87
	+		22.1	2.47	15.8	0.18	3.81	75.8	6.76
Eimeria	-		11.5	3.65	17.1	0.00	0.75	77.5	7.48
	+		28.3	1.12	15.4	0.13	5.26	75.5	6.15
C. perfringens	-		14.2	1.92	15.0	0.01	1.02	81.0	7.14
	+		26.3	2.81	17.5	0.13	5.18	71.7	6.49
GLM *P* values ^									
Fishmeal			NS	NS	NS	NS	NS	NS	NS
Eimeria			***	***	NS	**	*	NS	***
C. perfringens			***	NS	NS	*	*	NS	***
Fishmeal**Eimeria*			NS	NS	NS	NS	NS	NS	NS
Fishmeal**C. perfringens*			NS	NS	NS	NS	*	*	NS
*Eimeria*C. perfringens*			NS	NS	NS	*	NS	*	**
Fishmeal**Eimeria*C. perfringens*			*	NS	*	NS	NS	NS	**

^ The asterisks ***, ** and * represent the *P* values being < 0.001, 0.01 and 0.05 respectively, and NS means the *P* value being > 0.05. [$] Means not having same letters indicate that the difference is significant (*P* < 0.05.

Three-way interactions were significant on total VFA content ($P > 0.05$), acetic acid level ($P > 0.05$) and pH ($P < 0.01$) in the ileum (Table 8.3). *C. perfringens* challenge significantly increased total VFA content only when *Eimeria* was inoculated in the birds without dietary fishmeal addition. In contrast, no effects of *Eimeria* and *C. perfringens* on total VFA content were significant when fishmeal was used. *C. perfringens* challenge increased acetic acid content only when birds were fed fishmeal and inoculated with *Eimeria* but no effects of the treatments could be seen otherwise. Further, *C. perfringens* challenge significantly reduced pH in ileum only when the birds were fed fishmeal and not inoculated with *Eimeria*.

Caecal VFA, lactic acid and pH at 16 d

Main effects of the treatments were detected on caecal VFAs and pH at 16 d (Table 8.4). The total VFA concentration was increased in the caeca by fishmeal addition ($P < 0.01$) but no change was observed following *C. perfringens* and *Eimeria* inoculation ($P > 0.05$). Acetic acid was the major VFA component in the caeca with an average of 66.9% relative to the total. *Eimeria* and fishmeal reduced the relative acetic acid concentration ($P < 0.05$ and 0.01, respectively), while *C. perfringens* and *Eimeria* increased the relative propionic acid concentration ($P < 0.001$ and 0.001 respectively). The relative isobutyric acid concentration was elevated by both *C. perfringens* ($P < 0.05$) and *Eimeria* ($P < 0.001$). On the other hand, the relative butyric acid concentration was increased by *Eimeria* ($P < 0.001$) but reduced by fishmeal ($P < 0.01$). *C. perfringens* ($P < 0.001$) and *Eimeria* ($P < 0.001$) increased the level of isovaleric acid, whereas fishmeal increased relative valeric acid level ($P < 0.05$). The relative succinic acid level was also increased by fishmeal ($P < 0.001$).

Two-way interactions among these factors were observed on VFAs and pH in the caeca at 16 d (Table 8.4). No fishmeal × *Eimeria* interactions on VFAs or pH were present. However, a fishmeal × *C. perfringens* interaction on pH ($P < 0.01$), and *Eimeria* × *C. perfringens* interactions on pH ($P < 0.001$), propionic ($P < 0.01$), isobutyric ($P < 0.05$), isovaleric ($P < 0.01$) and succinic acids ($P < 0.05$) were shown. The pH in the caeca was reduced by *C. perfringens* only in the birds without *Eimeria* inoculation, and by *Eimeria* inoculation only in the birds without *C. perfringens* challenge. Propionic acid was reduced by *C. perfringens* only when birds were inoculated with *Eimeria*, but increased by *Eimeria* only when birds were challenged by *C. perfringens*. Isobutyric and isovaleric acids were altered by the treatments in the same way. Succinic acid, however, was increased by *Eimeria* inoculation only when birds were challenged by *C. perfringens*.

No three-way interaction was observed among all the measurements in caeca ($P > 0.05$).

Table 8.4. Caecal VFA responses to the addition of 250 g/kg fishmeal in diet during days 8–14, *Eimeria* inoculation on d 9 and C. perfringens challenge on days 14 and 15 in broiler chickens at the age of 16 d.

Treatment[$]			Total VFA (mmol/g)	Formic (%)	Acetic (%)	Propionic (%)	Isobutyric (%)	Butyric (%)	Isovaleric (%)	Valeric (%)	Succinic (%)	pH caeca
Fishmeal	*Eimeria*	*C. perfringens*										
-	-	-	84.0	0.10	74.3	1.60^{c}	0.33^{c}	13.6	0.06^{c}	0.16	9.7^{b}	7.05^{a}
-	-	+	87.9	0.14	73.5	1.72^{c}	0.42^{bc}	13.4	0.10^{c}	0.22	10.4^{b}	6.24^{ab}
-	+	-	80.8	0.83	65.3	1.60^{c}	0.65^{abc}	18.8	0.32^{bc}	0.19	11.9^{ab}	5.95^{b}
-	+	+	69.3	0.00	67.5	3.57^{a}	1.26^{a}	17.3	0.89^{a}	0.31	9.0^{b}	6.54^{ab}
+	-	-	93.1	0.47	67.0	1.53^{c}	0.37^{c}	11.6	0.09^{c}	0.41	18.2^{ab}	8.22^{a}
+	-	+	89.3	0.07	63.2	1.56^{c}	0.33^{c}	9.9	0.06^{c}	0.22	23.7^{a}	6.24^{ab}
+	+	-	105.5	0.71	62.8	1.93^{bc}	0.78^{abc}	16.0	0.26^{bc}	0.41	17.6^{ab}	6.57^{ab}
+	+	+	89.9	0.07	62.1	3.27^{ab}	1.05^{ab}	15.0	0.70^{ab}	0.43	11.0^{b}	6.56^{ab}
Pooled SE			8.0	0.27	2.8	0.35	0.14	1.3	0.11	0.10	2.8	0.19
Main effect												
Fishmeal	-		81.0	0.28	70.3	2.06	0.64	15.7	0.32	0.21	10.3	6.44
	+		94.5	0.21	63.5	2.07	0.63	13.1	0.28	0.37	17.6	6.90
Eimeria	-		88.6	0.25	69.5	1.60	0.36	12.1	0.08	0.25	15.5	6.94
	+		87.1	0.23	64.0	2.55	0.92	16.8	0.53	0.34	12.5	6.40
C. perfringens	-		90.8	0.42	67.3	1.66	0.53	15.0	0.18	0.29	14.4	6.95
	+		84.7	0.05	66.3	2.48	0.74	13.7	0.42	0.29	13.7	6.39
GLM *P* values [$]												
Fishmeal			*	0.750	**	0.841	0.757	**	0.405	*	***	***
Eimeria			0.708	0.855	*	***	***	***	***	0.212	0.111	***
C. perfringens			0.252	*	0.609	***	*	0.220	***	0.963	0.671	***
Fishmeal**Eimeria*			0.141	0.079	0.276	0.794	0.920	0.92	0.419	0.709	0.069	0.317
Fishmeal**C. perfringens*			0.610	0.888	0.388	0.455	0.247	0.81	0.531	0.211	0.878	**
*Eimeria***C. perfringens*			0.247	0.710	0.515	**	*	0.864	***	0.332	*	***
Fishmeal**Eimeria***C. perfringens*			0.879	0.055	0.920	0.571	0.599	0.591	0.848	0.584	0.273	0.282

^ The asterisks ***, ** and * represent the *P* values being < 0.001, 0.01 and 0.05 respectively, and NS means the *P* value being > 0.05. [$] Means not having same letters indicate that the difference is significant.

Duodenal histomorphometry at 16 d

Eimeria inoculation reduced villus height ($P < 0.001$), fishmeal feeding increased crypt depth ($P < 0.05$), and *Eimeria* inoculation reduced VH:CD ($P < 0.05$) (Table 8.5). No significant effect of *C. perfringens* challenge was observed on villus-crypt architecture although there was a tendency ($P = 0.077$) to reduce villus height. Furthermore, no significant interactions between the treatments were detected on histomorphometric measurements.

Table 8.5. Duodenal histomorphometric responses to the addition of 250 g/kg fishmeal in diet during days 8–14, *Eimeria* inoculation on d 9 and *C. perfringens* challenge on days 14 and 15 in broiler chickens at the age of 16 d.

Treatment [$]			Villus height	Crypt depth	Villus height : crypt depth
Fishmeal	*Eimeria*	*C. perfringens*			
-	-	-	1510[a]	186	8.12[ab]
-	-	+	1454[ab]	171	8.75[b]
-	+	-	1313[ab]	194	6.78[ab]
-	+	+	1083[b]	188	5.83[a]
+	-	-	1590[a]	207	7.94[ab]
+	-	+	1525[a]	207	7.45[ab]
+	+	-	1350[ab]	185	7.37[ab]
+	+	+	1234[ab]	215	5.71[a]
Pooled SE			94	11	0.61
Main effect					
Fishmeal	-		1340	185	7.37
	+		1425	204	7.12
Eimeria	-		1520	193	8.07
	+		1245	196	6.43
C. perfringens	-		1441	193	7.56
	+		1324	195	6.94
GLM *P* values ^					
Fishmeal			NS	*	NS
Eimeria			***	NS	***
C. perfringens			0.077	NS	NS
Fishmeal**Eimeria*			NS	NS	NS
Fishmeal**C. perfringens*			NS	NS	NS
*Eimeria*C. perfringens*			NS	NS	NS
Fishmeal**Eimeria*C. perfringens*			NS	NS	NS

^ The asterisks ***, ** and * represent the *P* values being < 0.001, 0.01 and 0.05 respectively, and NS means the *P* value being > 0.05. [$] Means not having same letters indicate that the difference is significant.

Discussion

In the present study, we examined how fishmeal addition, *Eimeria* inoculation and *C. perfringens* challenge altered VFAs, lactic acid, pH and histomorphometry in the GIT during the challenge procedure. As stated previously, we hypothesise that *Eimeria* and fishmeal substantially alter the intestinal metabolites and morphology possibly in favour of the growth of pathogenic *C. perfringens*. It has been demonstrated the dynamics of VFAs, lactic acid, pH and histomorphometry in the GIT is rather complex, so it is not conclusive to state that our hypothesis has been accepted or rejected. Nevertheless, these factors significantly influenced VFAs and other fatty acids including lactic acid, pH and histomorphometry in one way or another, and it appears that at least for some measurements the changes are related to NE infections in the birds. As has been reported previously on the same experiment (Rodgers *et al.* 2015), average lesion scores in the small intestine were: 0, 0.03, 0.14, 0.94, 0.08, 0.32, 0.25, and 1.15 for the groups of F-E-C-, F-E-C+, F-E+C-, F-E+C+, F+E-C-, F+E-C+, F+E+C-, F+E+C+, respectively. The lesion data showed that challenged groups with both *C. perfringens* and *Eimeria* had significantly higher lesion scores than the groups without such challenge. Similarly, mortalities in these two groups were higher than other groups, i.e., 24% and 27% mortality in F-E+C+ and F+E+C+, compared to only 0-0.67% mortality in other groups. This indicates the successful challenge of the birds with NE in the experiment under study.

Addition of 25% fishmeal in the starter diet meant that there was a massive excess in dietary protein for the birds, leading to some protein ending up in the hindgut for fermentation. Such fermentation is expected to change the fatty acid profile in the GIT compared with a diet balanced in carbohydrates and protein to more accurately meet but not exceed the dietary requirements of birds at this age. In the present study, fishmeal did not alter the concentrations of the total VFA content both in the ileum and caeca but increased pH in both sections of the gut at 13 d of the experiment, i.e., the day prior to the application of *C. perfringens* challenge and the day before removal of fishmeal from starter diet. Whereas, at 16 d, i.e., two days following *C. perfringens* challenge of birds and the removal of fishmeal from the starter diet, the residual effect of the fishmeal inclusion was no longer apparent on ileal VFAs, lactic acid and pH. As expected, the relative increase in the availability of carbohydrates, as opposed to protein, as a result of the withdrawal of fishmeal, increased total caecal VFAs and lactic acid contents, in the meantime, lower caecal pH. It appears that excessive fishmeal effect lasted until two days after its removal from starter diet possibly by its action on the change of microbiota in the caeca and the stress posed to the birds by the feed changeover. It is interesting that dietary fishmeal treatment during 8-14 d increased the total measured fatty acids in the caeca of birds aged at 16 d, and at the same time elevated rather than lowered the pH. This was thought to be contradictory. However, this phenomenon has been reported previously at least in pigs (Nyachoti *et al.* 2006; Pluske *et al.*

2003). Different molar amounts of the acids increased but not acidity likely due to the profile change of produced acids. Indeed, different acid composition can give rise to different pH values which are dependent on the respective p*Ka* and proportion of VFAs contained in the GIT and even the buffering capacity of dietary nutrient (Pluske *et al.* 1998).

High VFA level in the gut has been generally regarded as a healthier gut. However, NE is not a disease usually associated with the caeca *per se* but with the small intestine (Shojadoost *et al.* 2012; Timbermont *et al.* 2011). Therefore, caeca containing higher VFA concentration may not protect the small intestine from NE infection. On the other hand, excessive dietary fishmeal feeding resulted in deeper jejunal crypts as shown in the current study, a sign of inflammation in the gut (Yason *et al.* 1987), and reduced the relative levels of lactic acid in the ileum of the birds prior to the *C. perfringens* challenge. This suggests that fishmeal addition to the starter diet, and indeed, the changeover at d 14 by removal of fishmeal, have affected the intestinal environment possibly through a shift in the gut microflora (Wu *et al.* 2014). Henceforth, excessive dietary fishmeal as an NE predisposing factor has been frequently used in challenge models (Engberg *et al.* 2012; Keyburn *et al.* 2013), although we consider it unnecessary in our NE challenge model where *Eimeria* is used (Rodgers *et al.* 2015).

Eimeria spp, recognised as an important predisposing factor for NE (Al-Sheikhly and Al-Saieg 1980), has been used in various NE experimental challenge models (Park *et al.* 2008; Rodgers *et al.* 2015; Williams *et al.* 2003). We have identified that *Eimeria* inoculation at d 9 led to marked changes in measured fatty acid contents in the ileum and caeca. Interestingly, while the *Eimeria* inoculation evidently altered VFAs and lactic acid in the ileum it did not affect them in the caeca at 13 d. As the *Eimeria* species we used, i.e., *E. acervulina*, *maxima*, and *brunetti*, target only the small intestine, it is expected microbial metabolism in the small intestine is affected. Three days later, i.e., at 16 d, *Eimeria* inoculation not only affected VFA contents in the ileum but also in the caeca, in contrast with the data at 13 d. Three possible reasons can be proposed: a. *Eimeria* infection may have become more severe in the small intestine at 16 d, which may affect the microbial flow from the small intestine to the caeca such that caecal VFAs were affected; b. the greater metabolic change in the ileum may directly alter the contents in the caeca through peristaltic movements, c. it is possible that a massive damage of the gut affected the entry of fermentable substrates into the caeca and thus VFA contents.

Individually, *Eimeria* reduced the relative levels of acetic and formic acids but increased the relative amount of lactic acid in the ileum at 13 d. At 16 d, it reduced the relative concentration of formic acid but had no effect on the relative levels of acetic and lactic acids in the ileum as a main effect. Moreover, it significantly increased the relative level of ileal and caecal butyric acid at 16 d. It is

intriguing to observe that *Eimeria* infection elevated lactic or butyric acids which are thought to benefit GIT by their antimicrobial nature to inhibit pathogenic microbes (Huyghebaert *et al.* 2011; Naidu *et al.* 1999; Nava *et al.* 2005). For example, butyric acid has been found to decrease the incidence of subclinical NE (Huyghebaert *et al.* 2011; Timbermont 2009). Also, infected chickens with *E. tenella* led to reduced caecal *Lactobacillus* – lactic acid producing bacteria (Bradley and Radhakri 1973). In our study, *Eimeria* was used to predispose chickens to NE, thus its application to increase antimicrobial lactic and butyric acids was totally unexpected. It is likely that the beneficial effect of lactic and butyric acids in the ileum caused by *Eimeria* is not large enough to overcome the damage *Eimeria* inflicted on the intestinal epithelium (Dahiya *et al.* 2006; Shane *et al.* 1985). It was observed in the current study that *Eimeria* significantly reduced jejunal villus height and VH:CD, a sign of gut damage. A caecal microbiota analysis indicated that *Eimeria* inoculation strongly influenced the microbial population while fishmeal is present (Stanley *et al.* 2014), implying its important role in predisposing birds to NE. Further, a three-way interaction analysis indicated that *Eimeria* increased the total VFA content only when birds were challenged with *C. perfringens* and fed a diet without fishmeal. This, too, may be an ileal microbial response to the *Eimeria* challenge as a protective measure. More investigations to explore reasons underlying such an interesting phenomenon are warranted.

C. perfringens is the causative agent for NE outbreaks in poultry (Parish 1961; van Immerseel *et al.* 2009). Its role in producing NE has been extensively studied (Keyburn *et al.* 2008; Lee *et al.* 2011; M'Sadeq *et al.* 2015). However, how the infection of *C. perfringens* changes levels and profiles of intestinal VFAs and other fatty acids has not been systematically investigated. In the present study, we found that *C. perfringens* infection significantly increased total VFA contents as well as lactic acid concentration in the ileum but not in the caeca. It also altered the relative amounts of some individual fatty acids in the ileum and caeca. Particularly, *C. perfringens* increased the relative level of ileal butyric acid of the birds fed additional fishmeal during 7-14 d of age. Similar to *Eimeria* inoculation as stated previously, it was expected *C. perfringens* infection would lead to a gut environment more suitable for the growth of pathogenic microbes, for example, less butyrate acid, as increased butyric acid in the gut makes the intestine healthier (Huyghebaert *et al.* 2011; Naidu *et al.* 1999; Nava *et al.* 2005). However, this was not the case in our experiment. As butyrate has been used in different ways to control *C. perfringens* in birds to reduce the occurrence of NE (Timbermont *et al.* 2010), it is interesting that more butyric acid was present in the ileum under *C. perfringens* challenge. We speculate that butyrate producers yield more butyric acid most likely in response to greater presence of *C. perfringens* possibly to compete against it through modulation of mucin gene expression in the gut (Gaudier *et al.* 2004) or influence of the immune system (Meijer *et al.* 2010). These may include the close relatives of *C. perfringens* as it has been shown that an unknown *Clostridium* was positively correlated with butyric acid content

(Stanley *et al.* 2014). However, it should be noted that caecal butyric acid was not affected by *C. perfringens* in the present study. Furthermore, butyrate producing bacteria have been reported to be reduced in the caeca by NE infection (Stanley *et al.* 2012). Yet, whether this is due to *C. perfringens* or other predisposing factors are not known. Hence, the dynamics of intestinal butyric acid content in response to *C. perfringens* challenge in chickens is complex and worth further investigation.

A three-way interaction analysis has revealed that *C. perfringens* challenge increased acetic acid and reduced lactic acid when the birds were exposed to both predisposing factors, i.e., fishmeal feeding and *Eimeria* inoculation. We have reported previously that birds challenged with *C. perfringens*, fed fishmeal and inoculated with *Eimeria* had high mortality rates and severe NE lesions (Rodgers *et al.* 2015). Therefore, the relatively high contents of acetic acid and the low relative content of lactic acid in the ileum at 16 d may be related to the NE onset either as a cause or result, or otherwise synergetic correlations may be involved.

The observation on the effects of fishmeal and *Eimeria* on VFAs at 13 d is interesting as fishmeal and *Eimeria* appeared to have opposite effects on relative formic, acetic and lactic acids to the total VFA although both of them have been considered as predisposing factors for NE infection in poultry. As has been discussed previously, excessive fishmeal in the diet may lead to some protein ending up in the hindgut for fermentation which may then lead to change of VFA profile there. In terms of its role in the challenge model, fishmeal is thought to promote rapid growth of *C. perfringens* in the gut, leading localised populations to exceed 10^8 CFU/g of digesta (McDevitt *et al.* 2006), and to cause the onset of NE through the increasing presence of biogenic amines in the gut (Ratcliff 2001). The reduction of lactic acid by fishmeal supplementation at 13 d may indicate its action on inhibition of lactic acid bacteria which has been considered as probiotics. *Eimeria*, on the other hand, causes intestinal damage that predisposes the intestinal epithelium to *C. perfringens* colonisation by increased available nutrients for fermentation and more favourable environment for *C. perfringens* to proliferate (Timbermont *et al.* 2011). It appears that the predisposing effects of fishmeal and *Eimeria* on birds to infect NE are implemented through different mechanisms. Thus, the changes of intestinal environment may be somewhat divergent which may result in different microbial and thus fatty acid changes in the GIT. Indeed, while *Eimeria* played a major role in predisposing birds to NE in the current experiment, fishmeal showed only limited effect (Rodgers *et al.* 2015), which may have reflected different intestinal environments for VFA production and intestinal histomorphometry as shown in the current study.

Conclusions

The factors predisposing birds to NE including fishmeal and *Eimeria*, and the causative agent *C. perfringens* all play significant roles in the dynamics of VFAs and lactic acid, and intestinal histomorphometry in different ways. These changes may be important for the onset of NE but may be only the synergetic responses to the micro environmental stress. Based on the intestinal VFA responses to the treatments, *Eimeria* appears to be more important than fishmeal in predisposing birds to NE, thus the application of *Eimeria* in NE challenge model provides more consistency in inducing the disease under experimental conditions. However, the metabolite responses to various adverse environmental factors such as those used in disease challenge experiments are very complex, intensive efforts towards more understanding of the disease are required so as to achieve the control of NE especially upon removal of antibiotics from poultry diets worldwide.

Chapter 9. The roles of feed additives on performance and disease control in the birds challenged with NE

Introduction

Necrotic enteritis (NE) is a major disease threatening poultry production. It is estimated to cost the world's broiler industries over $6 billion annually (Wade and Keyburn 2015). The disease had been controlled by antibiotics until the EU enforced a ban on the prophylactic use of antibiotics in feed (Casewell *et al*. 2003). Apart from EU countries, consumer pressure to ban the use of antibiotics in animal diets is also increasing (Dahiya *et al.* 2006). Therefore, antibiotic alternatives to control diseases and to improve performance have been sought worldwide in food-producing animals (Allen *et al*. 2013).

The objective of the present study was to investigate whether some alternatives to dietary antibiotics had the ability to control necrotic enteritis in a challenge model.

Materials and methods

In the present study, we tested several potential dietary antibiotic alternatives in an NE challenge model for the ability to control NE in broiler chickens. Eight experimental treatments were administered: four control treatments with or without NE challenge, and with or without antibiotics (zinc bacitracin) added to the feed, and four treatments challenged with NE without antibiotics, and each with the addition of one of four feed additives to the diets. The additives and corresponding doses were: product A, a mixture of xylanase (40,000 U/g) and amylase at 350 g/t, product B, containing subtilisin (12,000 U/g) supplemented with xylanase and amylase (300 g/t), product C, a mixture of different organic acids at 1500, 1000 and 500 g/t in starter, grower and finisher respectively, and product D, protease (75,000 U/g, 200 g/t).

On day 0 of the study, 1344 day-old male broilers were randomly allocated to 48 floor pens, and fed the same formulations of starter, grower and finisher diets at 0-10, 11-24, and 25-35 days of age, respectively, except for the additives and Zn bacitracin that were supplemented according to the experimental design. The necrotic enteritis challenge followed the procedure described in Chapter 6 with a major modification. Fishmeal was not added in the starter diet according to the findings in Chapter 6 that 25% fishmeal addition did not increase the necrotic enteritis occurrence when birds were inoculated with *Eimeria* and challenged by *C. perfringens*. On day 9, all of the birds to be challenged were given *per os* a suspension of 2,500-5,000 oocysts of *E. acervulina*, *E. maxima*, and *E. brunetti* (Bioproperties Pty., Glenorie, NSW, Australia) in 1 mL sterile PBS. On days 14 and 15 the challenge groups were inoculated *per os* with 1 mL of *C. perfringens*

suspension at a concentration of 10^8–10^9 CFU/mL. Birds in unchallenged groups received 1 mL of sterile thioglycollate broth (USP alternative; Oxoid).

The NE lesions of duodenum, jejunum, and ileum of all sampled birds were assessed on the day before and day after *C. perfringens* challenge (d 13 and d 16) following the standard of (Prescott 1979). The lesions were scored from 0 to 4 in the tissues, where a score of 0 indicated healthy epithelium and a score of 4 indicated widespread and severe necrosis. Performance data were collected on days 10, 24 and 35.

Statistical Analyses

Performance data were analysed using the statistical package IBM® SPSS® Statistics package version 19 (IBM Corporation). The effects of combinations of feed additives and challenge on bird performance were examined by one-way analysis of variance using the General Linear Model. As livability and lesion score data were not normally distributed, these were analysed by the nonparametric Kruskal-Wallis test.

Results

From days 0- 10, no differences in feed intake, body weight gain and feed conversion ratio (FCR) were observed among the treatments. During days 11-24 the feed intakes of the birds supplemented with all four additives were lower than the feed intake of the birds without challenge and fed Zn bacitracin ($P < 0.05$). At the same time, weight gains of the birds supplemented with the four additives were lower than the controls (Table 9.1). Similarly, the FCR values of the birds fed the four additives were higher than the controls during this period of time (Table 9.2). During the last period of the trial (days 25-35) feed intakes of the birds fed products A and D were higher compared to the controls and products A and C. These two products also showed higher body weight gain than the unchallenged birds without the addition of Zn bacitracin in the diet (Table 9.1). However, no improvement of FCR was observed by addition of the four additives to the diets of the challenged birds (Table 9.2).

Similar responses were seen in the whole period of the trial: higher feed intakes for the A (3439.5 g) and D (3335.0 g) products than that of challenged control without antibiotics addition (3023.7 g); A and D gave higher body weight gains (2296.7 g and 2282.5 g) compared to non-challenge control without antibiotics addition (2075.0 g) (Table 9.1).No FCR benefits of these four additives were observed (Table 9.2).

Table 9.1. Feed intake and body weight gain of the birds in response to different treatments

Additive & challenge	Feed Intake (g/bird)				Body weight gain (g/bird)			
	0-10 d	11-24 d	25-35 d	0-35 d	0-10 d	11-24 d	25-35 d	0-35 d
AGP* & -	238.8	1072.9^{a}	1650.2^{b}	2961.9^{b}	221.8	778.0^{a}	1095.9^{b}	2095.7^{bc}
None & -	240.5	1066.9^{ab}	1576.2^{b}	2883.5^{b}	224.9	775.6^{ab}	1074.6^{b}	2075.0^{c}
AGP & +	237.9	1051.7^{abc}	1728.9^{b}	3018.5^{b}	221.3	756.1^{ab}	1141.6^{b}	2119.1^{abc}
None & +	248.1	1046.5^{abc}	1729.1^{b}	3023.7^{b}	230.6	735.3^{bc}	1126.5^{b}	2092.3^{bc}
A & +	247.5	974.3^{c}	2217.7^{a}	3439.5^{a}	229.1	671.0^{d}	1396.6^{a}	2296.7^{a}
B & +	258.3	1008.0^{c}	1702.1^{b}	2968.4^{b}	240.2	690.7^{d}	1083.1^{b}	2014.0^{c}
C & +	244.8	1014.3^{bc}	1688.7^{b}	2947.8^{b}	227.5	703.9^{cd}	1089.3^{b}	2020.6^{c}
D & +	254.3	1004.9^{c}	2075.8^{a}	3335.0^{a}	231.7	689.1^{d}	1361.7^{a}	2282.5^{ab}
Pooled SEM	2.1	8.0	41.1	40.1	1.8	7.4	26.3	26.6
P value	0.148	0.010	0.000	0.000	0.146	0.000	0.000	0.028

***** Zinc bacitracin

Table 9.2. FCR, lesion scores in duodenum (Duo), jejunum (Jej) and ileum (Ile) and livability of the birds in response to different treatments

Additive & challenge	Feed conversion ratio				NE score				Livability
	0-10d	11-24d	25-35d	0-35d	Duo	Jej	Ile	mean	35 d
AGP* & -	1.08	1.38^{c}	1.51	1.41^{de}	0.00^{d}	0.00^{c}	0.00	0.00^{c}	97.4
None & -	1.07	1.38^{c}	1.47	1.39^{e}	0.00^{d}	0.00^{c}	0.00	0.00^{c}	93.9
AGP & +	1.08	1.39^{c}	1.52	1.43^{cde}	0.08^{cd}	0.00^{c}	0.25	0.11^{c}	94.7
None & +	1.08	1.42^{b}	1.54	1.45^{bcd}	0.88^{b}	2.17^{a}	0.33	1.13^{a}	96.5
A & +	1.08	1.45^{a}	1.59	1.50^{a}	0.54^{bc}	0.88^{b}	0.17	0.53^{b}	92.1
B & +	1.08	1.46^{a}	1.58	1.48^{ab}	1.63^{a}	1.88^{a}	0.25	1.25^{a}	93.9
C & +	1.08	1.44^{ab}	1.55	1.46^{abc}	0.67^{b}	1.17^{b}	0.00	0.61^{b}	96.5
D & +	1.10	1.46^{a}	1.54	1.47^{abc}	0.67^{b}	0.92^{b}	0.00	0.53^{b}	91.2
Pooled SEM	0.00	0.01	0.01	0.01	0.10	0.27	0.04	0.16	1.96
P value	0.600	0.000	0.135	0.000	0.000	0.000	0.154	0.000	0.364

* Zinc bacitracin

Although the mortalities in the challenged birds were low, severe lesions in the small intestine of the challenged birds were observed. The mean lesion score of the group challenged and without antibiotic treatment was significantly higher than the other three control groups ($P < 0.001$). Lesion scores as high as 3 were recorded in the jejunum of birds from this group. Birds fed products A (mixture of xylanase 40,000 U/g and amylase at 350 g/t), C (mixture of different organic acids at 1500, 1000 and 500 g/t in starter, grower and finisher respectively) and D (protease (75,000 U/g, 200 g/t) demonstrated lower mean lesion scores in the jejunum and overall small intestine compared to that of the group with NE challenge and without antibiotic treatment ($P < 0.001$) (Table 9.2).

Conclusion

Although the additives did not improve performance overall, some of them reduced the impact of necrotic enteritis when birds were challenged. This positive outcome of the additives to control NE warrants further investigation under different growth conditions. Understanding of the mechanisms underlying the improvement will be beneficial to the application of these additives in the poultry industry.

Chapter 10. Development and application of a poultry intestinal tract chip (PITChip2), a phylogenetic microarray, for analysis of Australian poultry intestinal microbiomes

Introduction

The gut microbiota in chickens is complex in both composition (thousands of species) and structure (large variation in population sizes). Although a few species of pathogens are known to cause mortality and reduced performance, it is the interactions among the host, microbiota as a whole, and the diet that determine the health status and growth performance of the birds. This hypothesis is supported by the fact that although various dietary interventions have been tested to improve bird health and productivity, the efficacy observed varied considerably in terms of magnitude and repeatability. The lack of adequate understanding of the microbiota and, to a lesser degree, the host makes it extremely difficult to pinpoint the reasons and devise dietary means in a rational manner. This knowledge gap is attributed to the inability to comprehensively analyse and assess the complex gut microbiota and gene expressions that underpin the metabolism and growth of the host.

To address this challenge, our research team members at The Ohio State University have developed microarrays for both the gut microbiota and the host genome. The first generation gut microarray (referred to Poultry Intestinal Tract Chip 1, or PITChip 1) was developed based on signature genes of bacteria found in chickens and turkeys in North America and other countries. Some bacterial species found in chickens in Australia were also represented on this microarray. Geographic differences in gut microbiota composition are expected, but while most of the bacteria found in the chickens in Australia are likely represented on the microarray, it was our intention to further expand this chicken gut microarray by including more signature genes from bacteria that are present in chickens raised in Australia for the purpose of making the PITChip2 technology more appropriate for usage in Australia. The chicken genome array is developed based on the chicken genome, and the expression and regulation of all the possible genes can be analysed simultaneously. We believe combined analysis of the gut microbiota using the gut microarray and the gene expression using the chicken genome microarray will provide unprecedented opportunities to identify key factors governing chicken health, nutrition, and growth, which eventually may lead to development of rational dietary and managerial strategies

to effectively enhance poultry health, welfare and productivity with reduced reliance on antibiotics.

Materials and methods

Sample selection and preparation

Microbial DNA from caecal samples was isolated as described in earlier chapters and transported in 96-well plates from the CSIRO Australian Animal Health Laboratory to the Ohio State University.

Chickens in each experiment were ranked based on their feed conversion ratio (FCR). For each experiment in Phase 1 trials (P1E1, P1E2 and P1E3; described in Chapter 1), metagenomic DNA samples from 9 birds each at the high and the low ends of the FCR spectrum were used for microbial analysis using the PITChip2. All birds received the same commercial starter crumble. For each experiment in Phase 2 trials (P2E1, P2E2 and P2E3; described in Chapter 2), metagenomic DNA samples from 5 birds each in one of the two dietary treatments at the high and the low ends of the FCR spectrum were selected for downstream analysis. The experimental diets for P2E1 were (1) Control and (2) Control with zinc bacitracin (50 ppm active ingredient, for P2E2 were (1) Control and (2) Control with avilamycin (15 ppm active ingredient), and for P2E3 were (1) wheat-based diet and (2) sorghum-based diet with the same nutrient specifications as the wheat-based diet. For Experiment 1 in Phase 3 (described in Chapter 3), 12 samples were randomly selected from each of the three treatments applied to incubating eggs 24 hours from hatch. These were were (1) control PBS solution, (2) caecal inoculum #1, and (3) caecal inoculum #2. The inocula were derived from high performing chickens in Phase 1 trials described in Chapter 1. In total, 150 samples were analysed by PITChip2.

From each metagenomic DNA sample 16S rRNA gene was amplified by PCR using universal bacterial primer set T7/27F and 1525R. PCR was performed with 33 cycles (denaturation, 95℃ for 30 s; annealing, 55℃ for 45 s; and extension, 72℃ for 90 s) using a PTC-100 thermocycler (MJ Research, Waltham, MA). Amplicons were confirmed by agarose gel electrophoresis, purified using QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The same amount of each purified PCR product (100 ng) was then added as template for in vitro transcription using the MEGAScript T7 in vitro transcription kit (Ambion, Austin, TX). Synthesised cRNA was again confirmed by agarose gel electrophoresis, purified using the MEGAClear kit (Ambion,

Foster City, CA) and quantified using a NanoDrop ND-1000 spectrophotometer. Equal amount (500 ng) of the purified cRNA was then labelled with Cy5 fluorescent dye at 37℃ for 1 hour using the Label IT μArray Cy5 reagent (Mirus, Madison, WI). The labelled cRNA was purified to remove the free Cy5 dye using the MEGAClear kit and stored at -80℃ until microarray hybridisation. All subsequent microarray experiment procedures (e.g. hybridisation and washing) after the Cy5 labelling step were performed in dark to avoid light bleaching of the light-sensitive Cy5 dye.

Microarray hybridisation and washing of microarray slides

The hybridisation was performed following manufacturer's protocol provided by MYcroarray. Briefly, for each array 60 μL hybridisation solution was prepared with 18 μL 20×SSPE buffer (3M NaCl, 20mM EDTA, 118.2mM NaH2PO4, and 81.8mM Na2HPO4), 6 μL formamide, 0.6 μL 1% bovine serum albumin, 0.6 μL 1% tween-20, 0.6 μL Control-oligos provided by the manufacturer, 0.6 μL Cy5-labeled internal controls, and 26 μL Cy5-labeled cRNA. The hybridisation solution was incubated at 65℃ for 5 minutes, then immediately cooled on ice for another 5 minutes. Each array was applied with 54 μL hybridisation solution. Microarray slide was placed in an Agilent Hybridisation Chamber (Agilent, Santa Clara, CA) and the hybridisation was carried out in an HB-1000 hybridisation oven (UVP, Upland, CA) at predetermined optimal hybridisation temperature for 20 h with rotation set at 10 rpm.

The washing of microarray following hybridisation was performed per manufacturer’s protocol. Immediately before the end of the hybridisation, 1×SSPE buffer and 0.25×SSPE buffer were placed in the hybridisation oven along with the microarray slide so that they remained at hybridisation temperature in the subsequent washing steps. The microarray slide was first washed in 1×SSPE buffer for 5 minutes, followed by washing in 0.25×SSPE buffer for 30 seconds. The microarray slide was then dried for 10 min using a high speed microarray centrifuge (Arrayit, Sunnyvale, CA).

Signal detection and data analysis

Microarray slides were scanned using a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA) with oversaturation tolerance set to 0.01%, photomultiplier sensitivity set at auto-PMT gain, and resolution set to 5 μm. The information of each probe spot on the scanned images was extracted by fitting the gene allocation list (GAL) file provided by the manufacturer which carries the annotation information of each spot on the microarray. The

scanned image was then quantified using GenePix Pro 6.0 software (Axon Instruments, Union City, CA). Probe spots of negative detection were flagged out by the auto-alignment function of the GenePix software. Probe spots with oversaturation, bad shape, or suspected contamination were flagged manually.

The GenePix extracted results of each array were exported as a GenePix Results Format (GPR) file. ExpressConverter version 2.1 of the TM4 Microarray Suite was used to convert each GPR file to an annotation (ANN) file and a multiexperiment viewer (MEV) file. From the MEV file, the median signal intensity of each spot after subtracting the background noise was used for downstream analysis. The converted data was entered into Ginkgo version 1.01 (J. Craig Venter Institute, Rockville, MD) and the replicated spots in the array were consolidated using the standard “In-Slide Replicates Analysis” option of the Ginkgo software.

The microarray data were normalised based on the signal intensities of internal control probes. Normalised data were imported into MeV program (version 4.8.1) within the TM4 Microarray Suite for statistical analysis. Heat maps were generated based on the log2 values of the median intensities. Clustering of samples was performed using hierarchical clustering (HCL) in the MeV program with a Manhattan distance metric. Average linkage was used to determine cluster-to-cluster distances when constructing the hierarchical tree. Principal component analysis (PCA) was conducted using the MeV program to identify clusters of samples with similar caecal microbiome structure. The standard “TTEST” and “ANOVA” functions of the MeV program were used to identify the hybridised probes that were significantly ($P \leq 0.05$) correlated with FCR.

Results

Phase 1 trials

The hierarchical tree generated from the HCL analysis (Figure 10.1) showed that the samples selected from P1E1 and P1E2 were generally clustered based on FCR but not the samples selected from P1E3, suggesting that the overall caecal microbiome structure of chickens differed between the low- and high-FCR birds. The PCA plots also showed separation of caecal microbiome along PC1 between the low and high-FCR birds even though the separation was not clear or complete (Figures 10.2 and 10.3). Consistent with the hierarchical tree, no separation of caecal microbiome between the low- and high-FCR birds was visible for the samples collected from P1E3 (Figure 10.4). These observations suggested variations among experiments in association between caecal microbiome and FCR in the chickens.

Individual genus- and species-level OTUs were examined for association with FCR. Based on t-test implemented in MeV, 10, 46, and 5 phylotypes associated with host FCR were identified in experiments P1E1, P1E2, and P1E3, respectively (Figures 10.5 – 10.7). Across P1E1 and P1E2, one genus-level phylotype (*Faecalibacterium*) and 3 species-level phylotypes (unclassified *Bacteria*_39, unclassified *Clostridiales*_23, and unclassified *Oscillospira*_6) were more predominant in chickens with low FCR than in chickens with high FCR. However, these trends were not found in in P1E3. One species-level phylotype (unclassified *Eubacterium*_4) was more predominant in the high FCR chickens in P1E2 but was less predominant in the high FCR chickens in P1E3. We did not find phylotypes that are consistently associated with host FCR across all the three experiments.

All the samples in Phase 1 trial, regardless of different experiments, were ranked based on their FCR values. 10 samples with lowest FCR and 10 with highest FCR were selected and compared. HCL and PCA analysis revealed no clear separation of caecal microbiome based on FCR (Figure 10.8 and 9). However, T-test suggested that 15 phylotypes were more predominant in chickens with low FCR whereas 3 phylotypes were more predominant in chickens with high FCR (Figure 10.10).

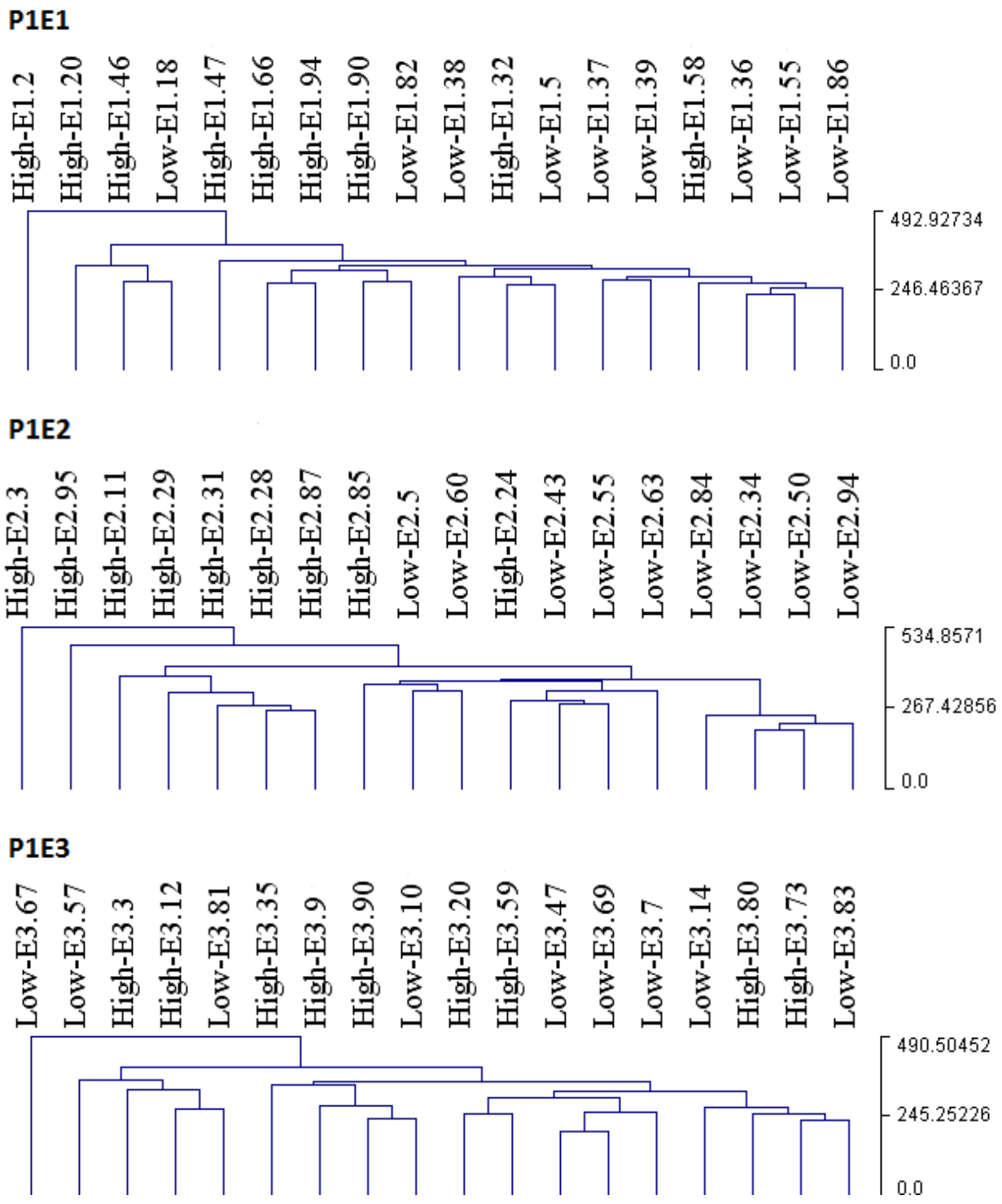

Figure 10.1. Hierarchical trees generated from HCL analysis of entire caecal microbiome. Low, low FCR good-performance chicken; High, high-FCR poor performance chicken.

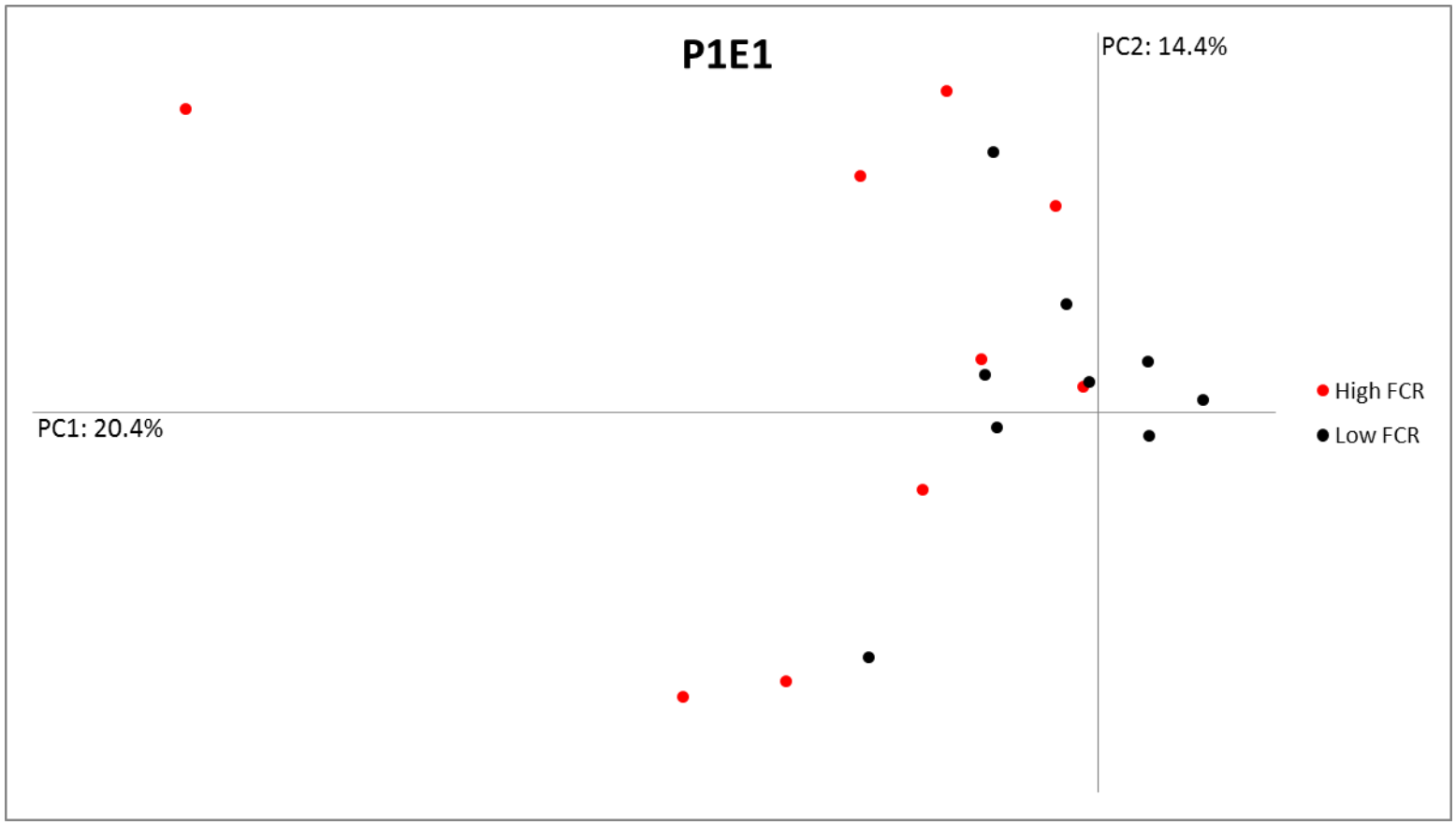

Figure 10.2. PCA plot showing the grouping of chickens from P1E1 based on the overall structure of their caecal microbiome.

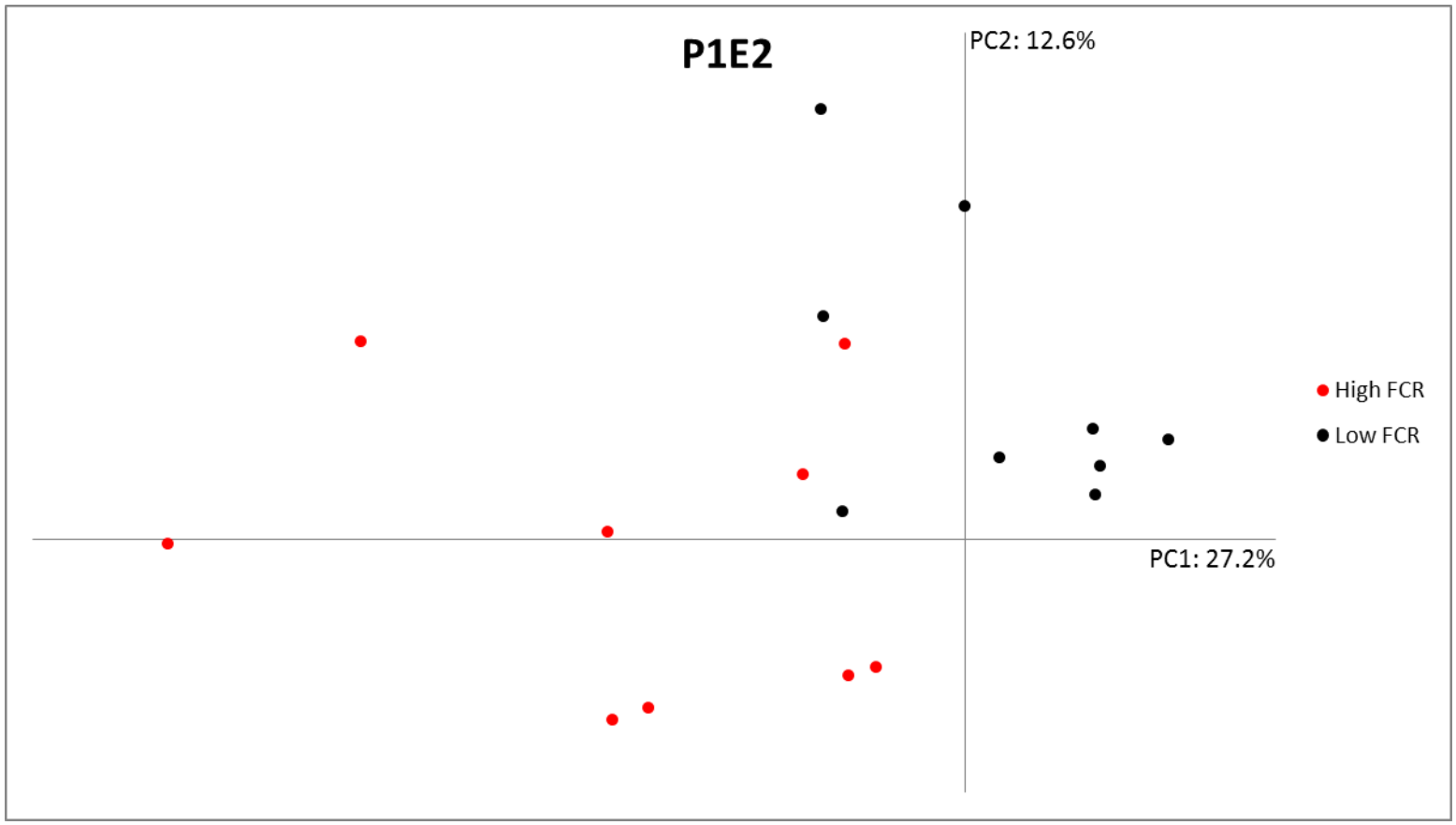

Figure 10.3. PCA plot showing the grouping of chickens from P1E2 based on the overall structure of their caecal microbiome.

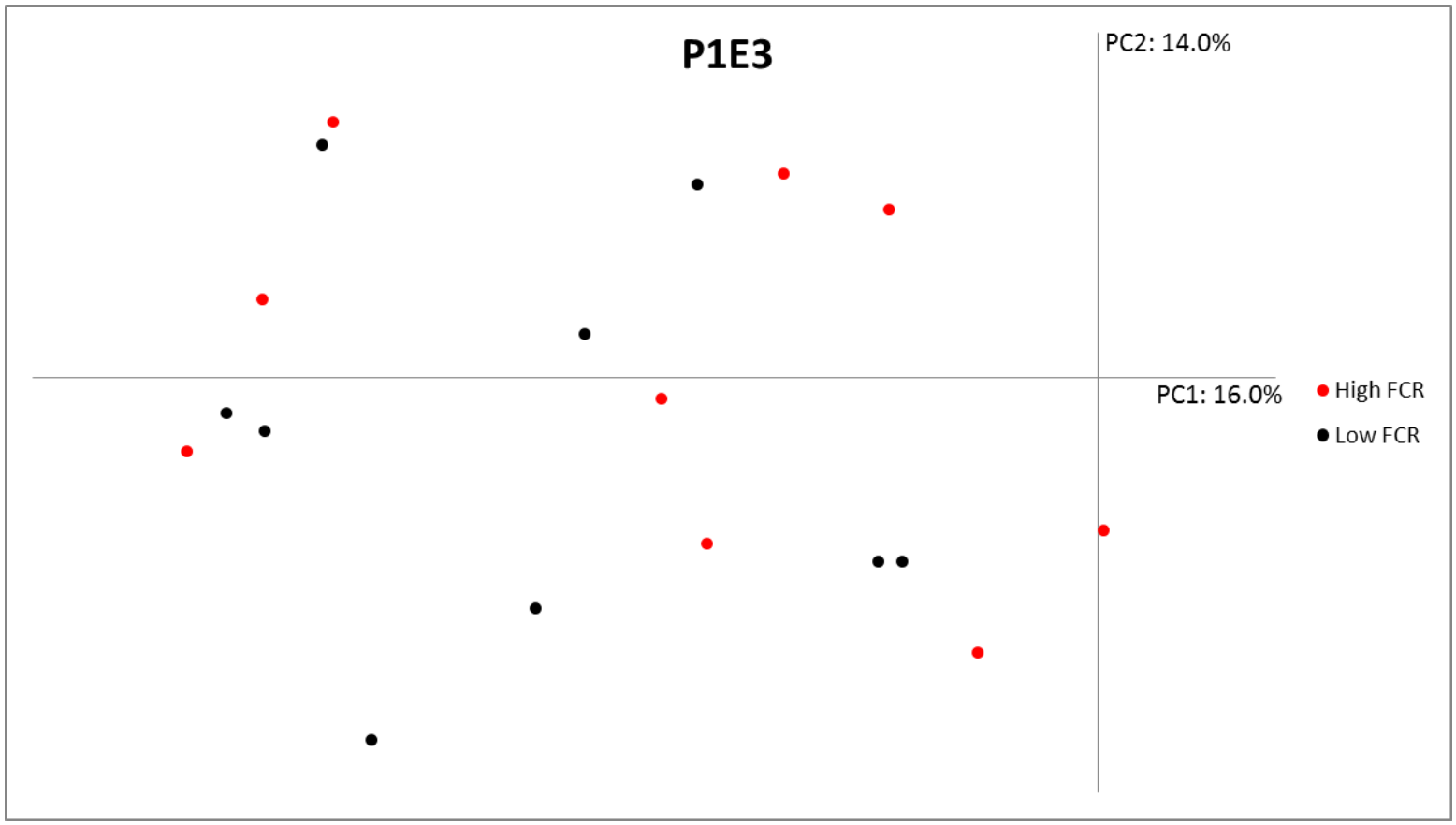

Figure 10.4: PCA plot showing the grouping of chickens from P1E3 based on the overall structure of their gut microbiome.

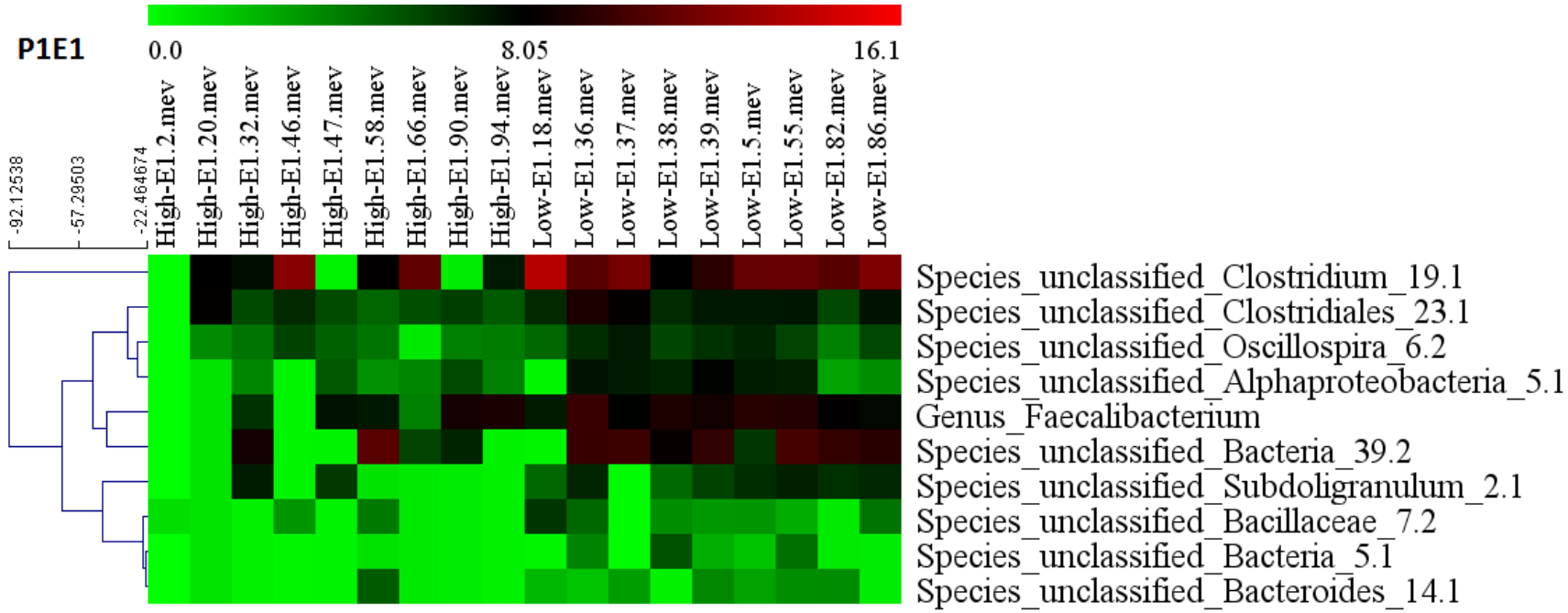

Figure 10.5. 10 phylotypes differing in abundance (p≤0.05) between birds with low or high FCR in experiment P1E1. Heat map was generated based on the log2 value of the ratio between the mean fluorescence intensities of each group of birds and the internal standard, and displayed using the standard Green-Black-Red scheme with red indicates high relative abundance and green indicates low relative abundance. Low, low FCR good-performance chicken; High, high FCR poor-performance chicken.

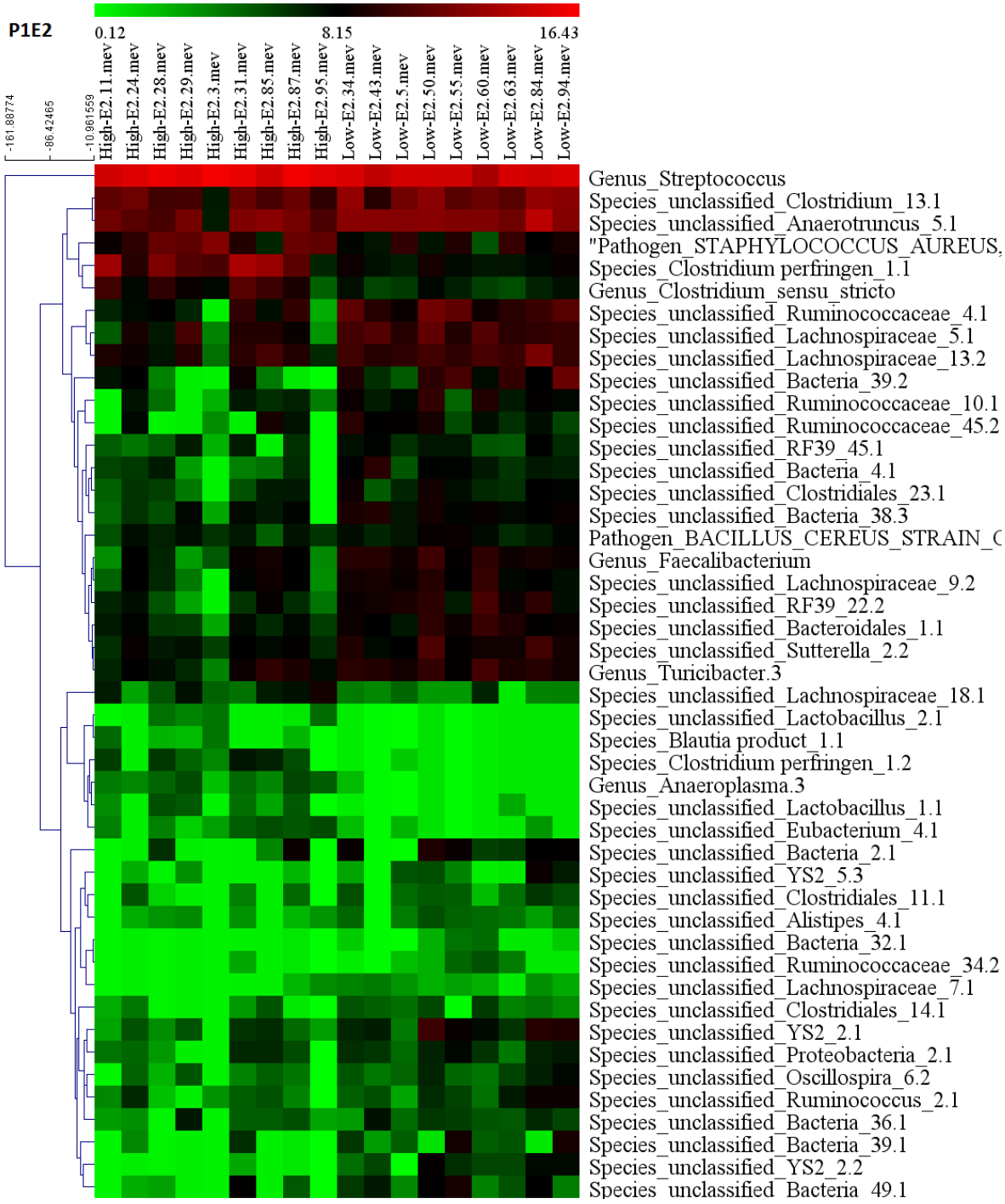

Figure 10.6. 46 phylotypes differing in abundance (*P* ≤ 0.05) between birds with low or high FCR in experiment P1E2. Heat map was generated based on the log2 value of the ratio between the mean fluorescence intensities of each group of birds and the internal standard, and displayed using the standard Green-Black-Red scheme with red indicates high relative abundance and green indicates low relative abundance. Low, low FCR good-performance chicken; High, high FCR poor-performance chicken.

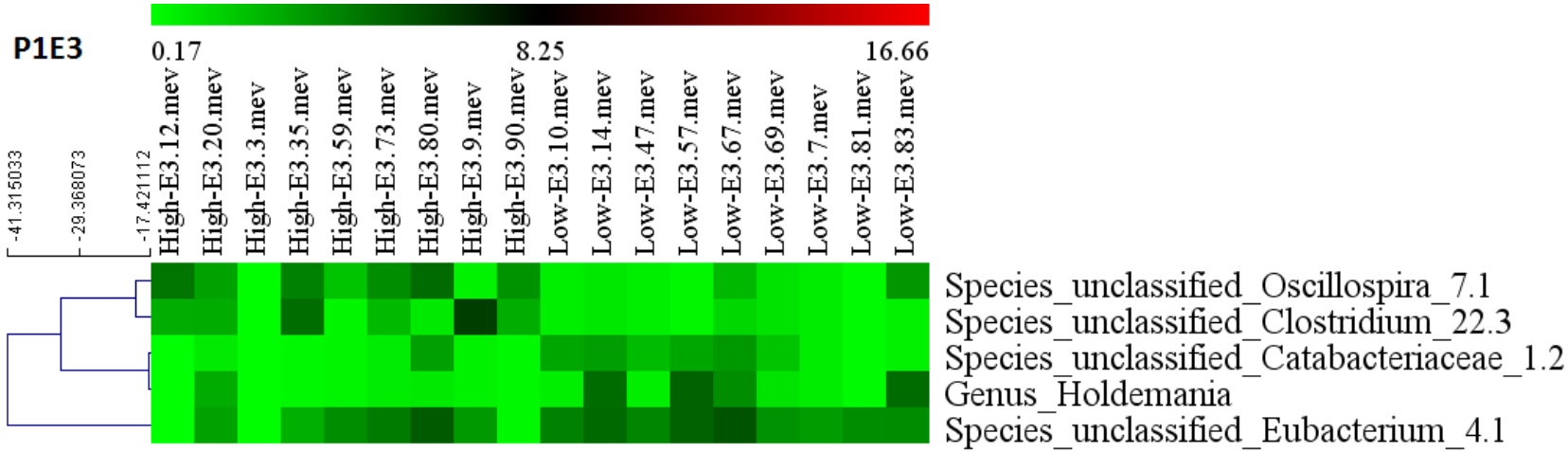

Figure 10.7. 5 phylotypes differing in abundance (p≤0.05) between birds with low or high FCR in experiment P1E3. Heat map was generated based on the log_2 value of the ratio between the mean fluorescence intensities of each group of birds and the internal standard, and displayed using the standard Green-Black-Red scheme with red indicates high relative abundance and green indicates low relative abundance. Low, low FCR good-performance chicken; High, high FCR poor-performance chicken.

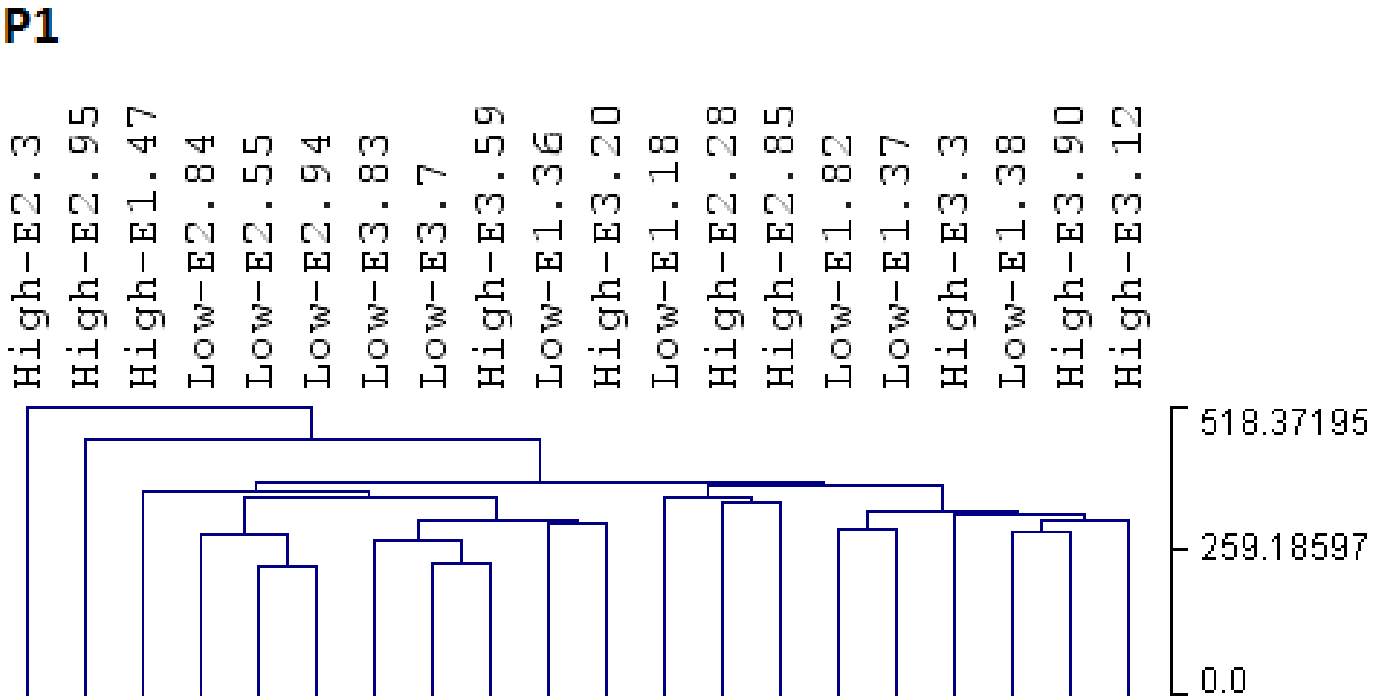

Figure 10.8. Hierarchical trees generated from HCL analysis of caecal microbiome of the three experiments of P1. Low, low FCR good-performance chicken; High, high-FCR poor performance chicken.

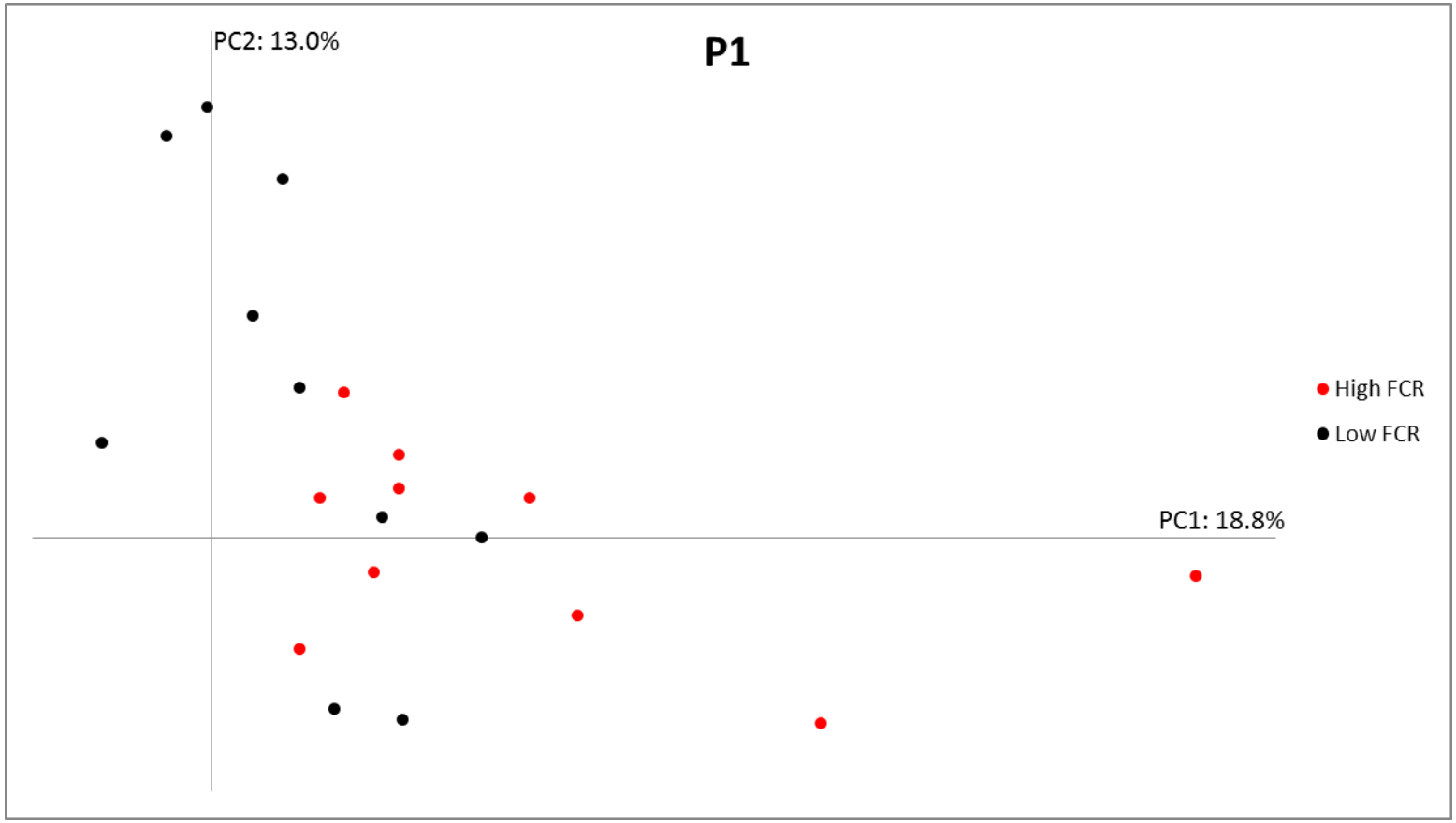

Figure 10.9: PCA plot showing the grouping of 20 chickens from all the three experiments of P1 based on the overall structure of their caecal microbiome.

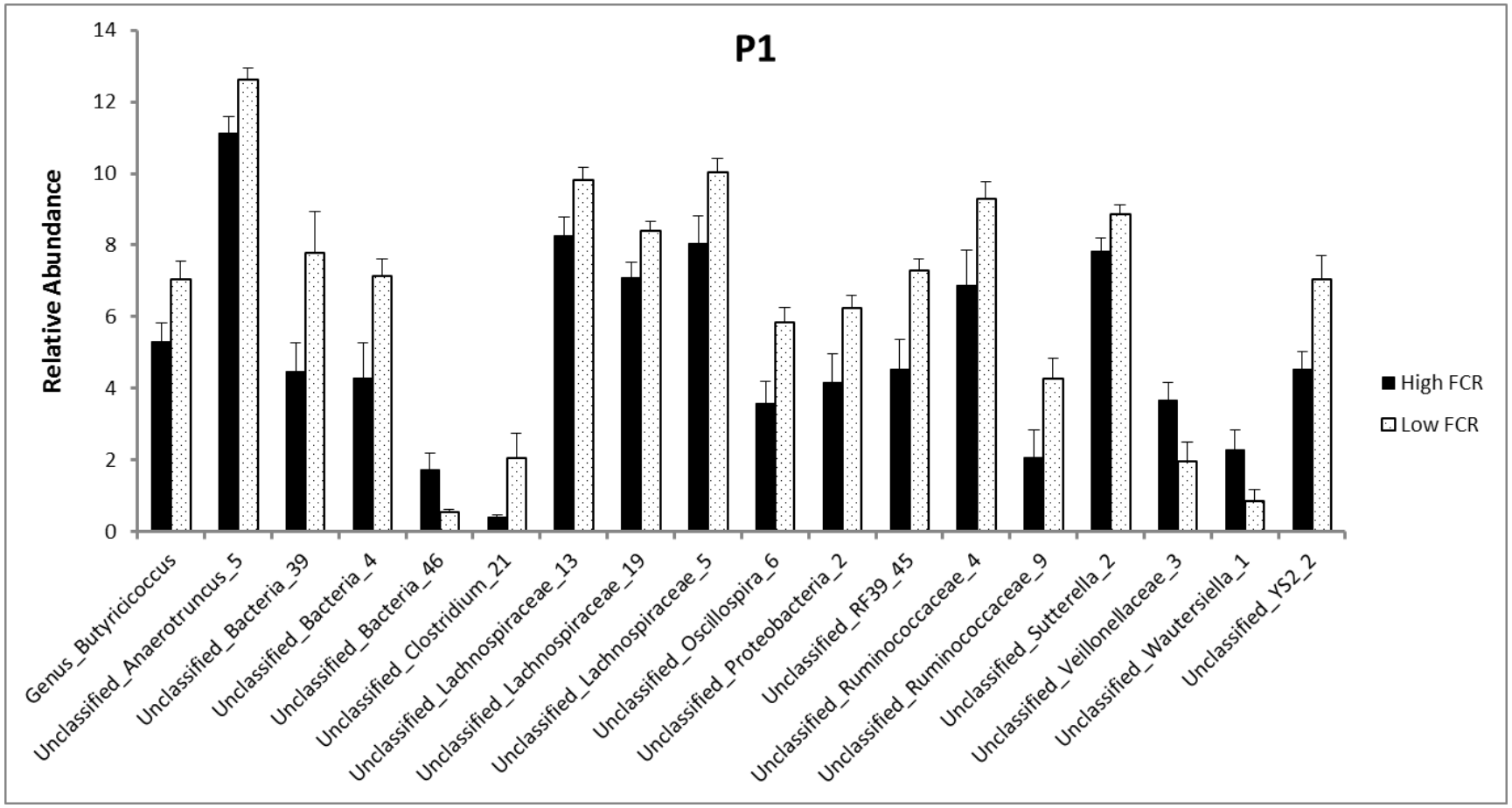

Figure 10.10. 18 phylotypes differing in abundance ($P \leq 0.05$) between birds with low or high FCR in all the experiment of P1. Relative abundance was calculated using the $\log_2$ value of the ratio between the mean fluorescence intensities of each group of birds and the internal standard.

Phase 2 trials

Each experiment in Phase 2 used two different diets. The experimental diets for P2E1 were (1) Control and (2) Control with zinc bacitracin (50 ppm active ingredient, for P2E2 were (1) Control and (2) Control with avilamycin (15 ppm active ingredient), and for P2E3 were (1) wheat-based diet and (2) sorghum-based diet with the same nutrient specifications as the wheat-based diet.Based on PCA analysis of the overall microbiome structure, no clear separation was noted between birds with respect to FCR or diets (Figures 10.11 – 10.13).

By using t-test, we were able to identify a number of phylotypes associated with host FCR in each experiment for the two diets (Figures 10.14 – 10.16). In P2E1, 3 genera and 4 species-level phylotypes and 3 genera and 5 species-level phylotypes differed in predominance between the high- and low-FCR birds for diet 1 and diet 2, respectively. In P2E2, 2 genera and 9 species-level phylotypes and 1 genera and 5 species-level phylotypes differed in predominance between the high- and low-FCR birds for diet 1 and diet 2, respectively. In P2E3, 1 genus and no species-level phylotypes and 1 genus and 7 species-level phylotypes differed in predominance between the high- and low-FCR birds for diet 1 and diet 2, respectively. However, no phylotype was found consistently associated with FCR across all the 3 experiments in Phase 2 trial. In addition, chickens fed with different diets did not share FCR-associated phylotypes.

All the samples in Phase 2 trials, regardless of different experiments and diets, were also ranked based on their FCR values. 10 samples with lowest FCR and 10 with highest FCR were selected and compared. On the PCA plot, chickens with high FCR were grouped in the upper-right corner whereas birds with low FCR were scattered along the first principle component (Figure 10.17). T-test suggested that 9 phylotypes were more predominant in chickens with low FCR whereas 12 phylotypes were more predominant in chickens with high FCR (Figure 10.18).

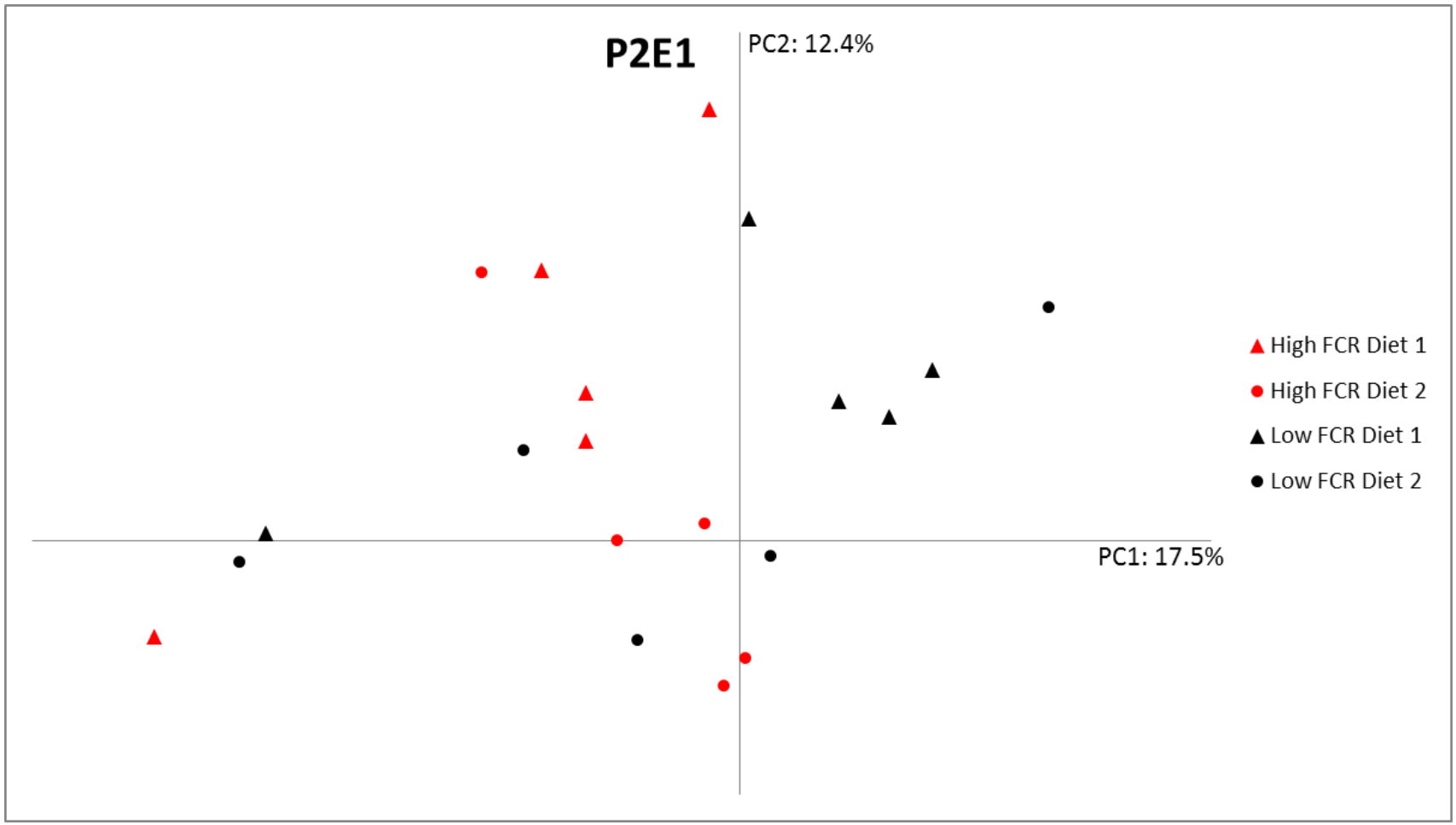

Figure 10.11: PCA plot showing the grouping of chickens from P2E1 based on the overall structure of their caecal microbiome.

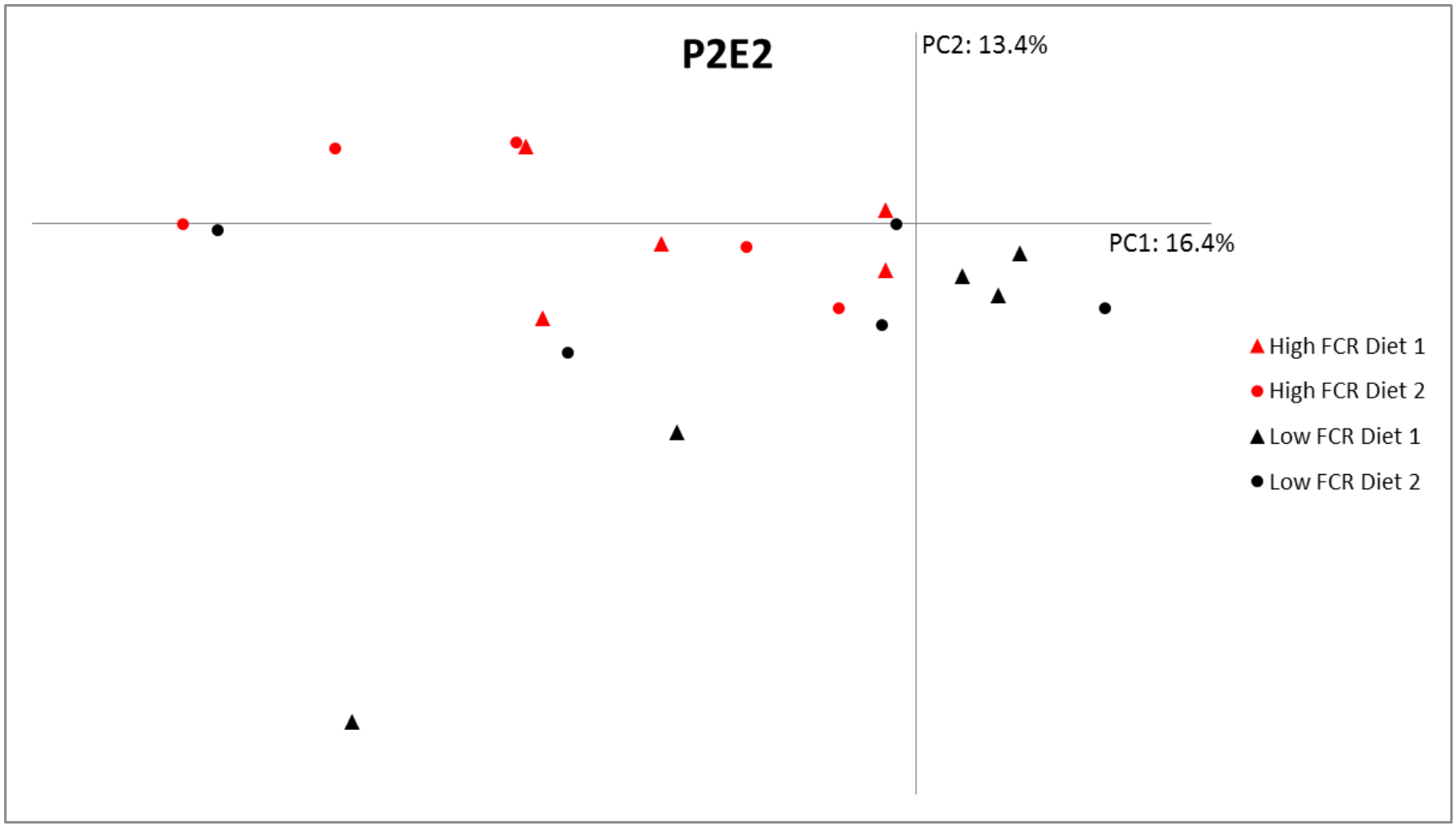

Figure 10.12. PCA plot showing the grouping of chickens from P2E2 based on the overall structure of their caecal microbiome.

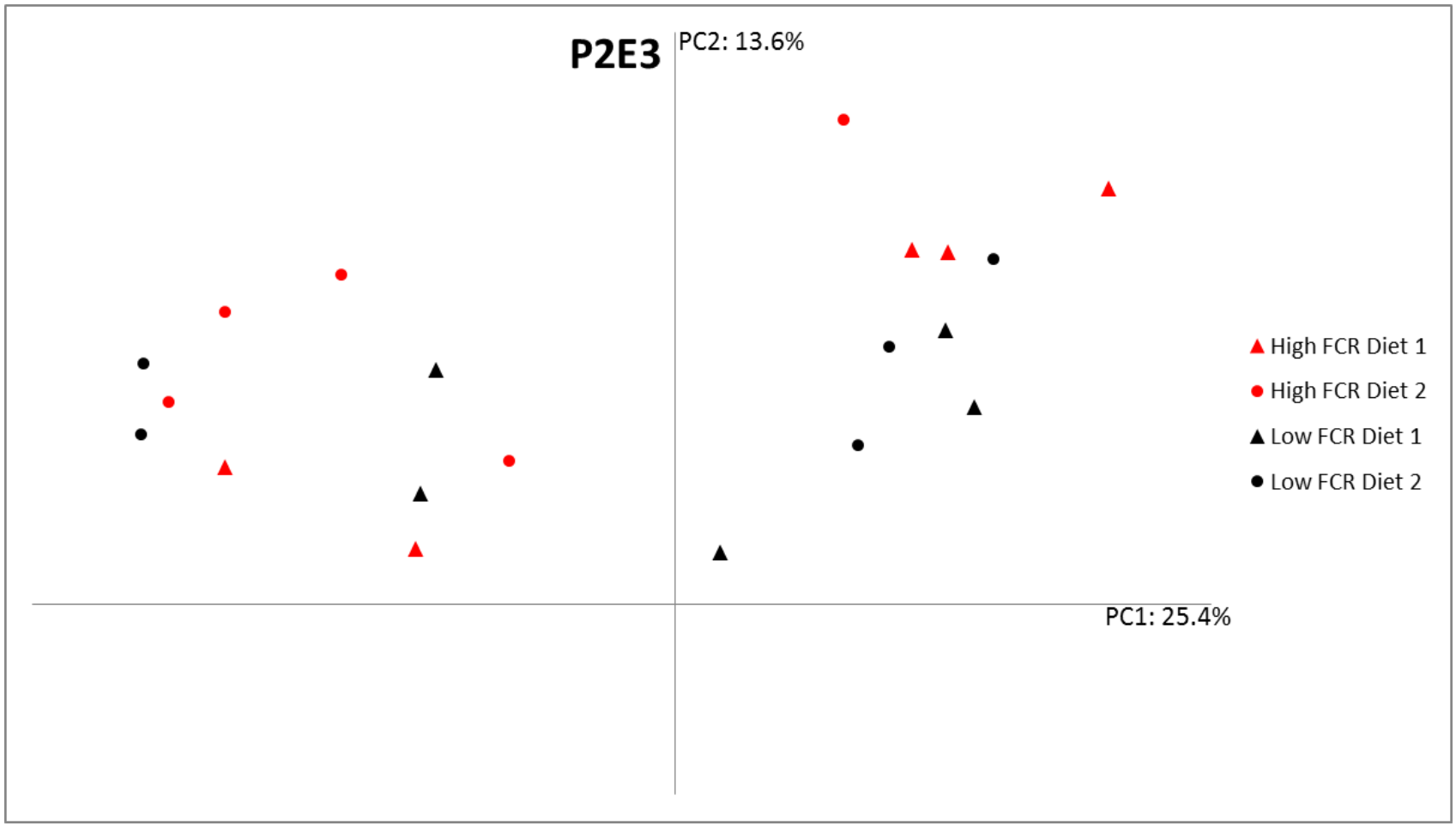

Figure 10.13. PCA plot showing the grouping of chickens from P2E3 based on the overall structure of their caecal microbiome.

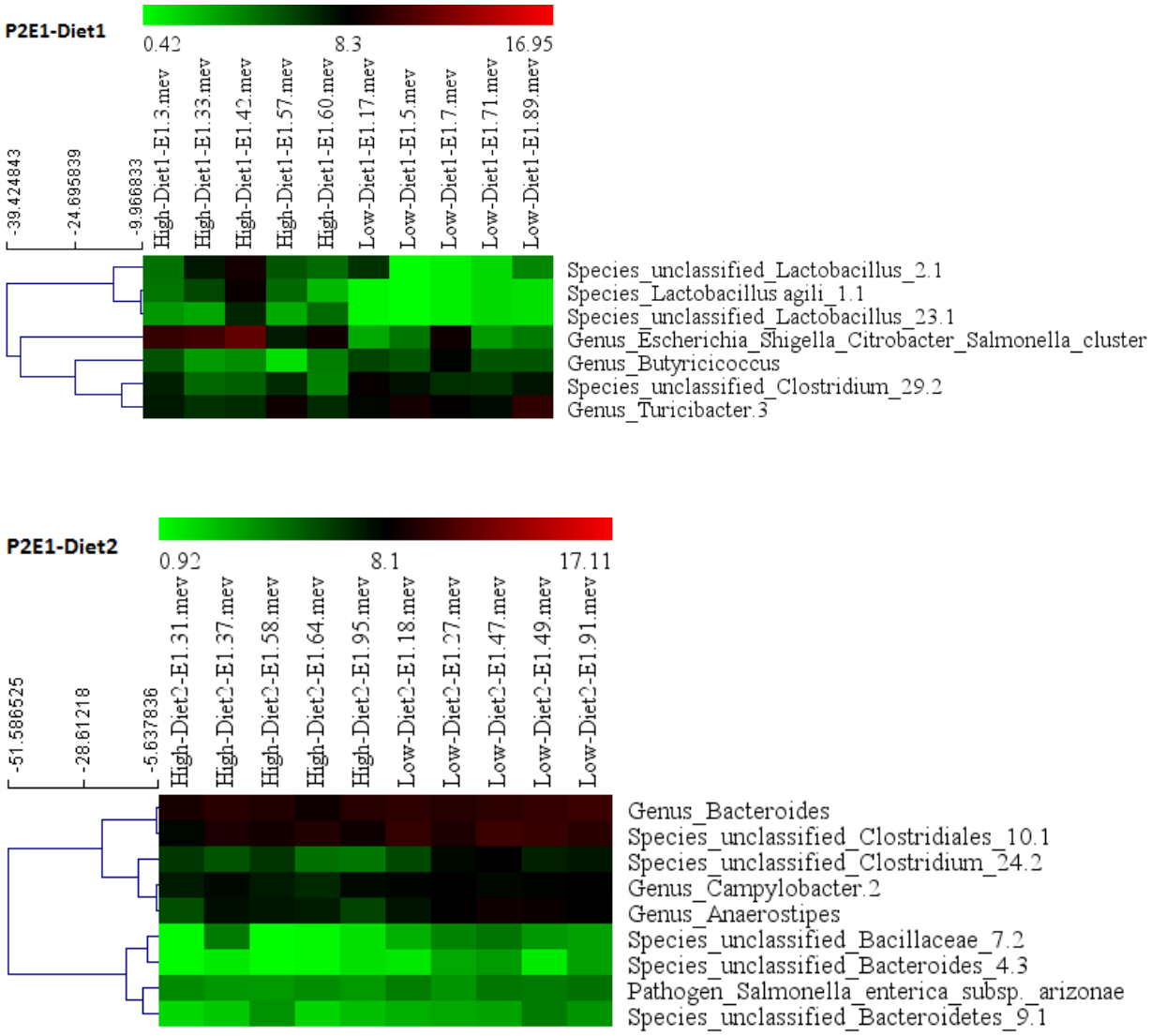

Figure 10.14. Phylotypes differing in abundance (p≤0.05) between birds with low or high FCR in experiment P2E1. Heat map was generated based on the $\log_2$ value of the ratio between the mean fluorescence intensities of each group of birds and the internal standard, and displayed using the standard Green-Black-Red scheme with red indicates high relative abundance and green indicates low relative abundance. Low, low FCR good-performance chicken; High, high FCR poor-performance chicken.

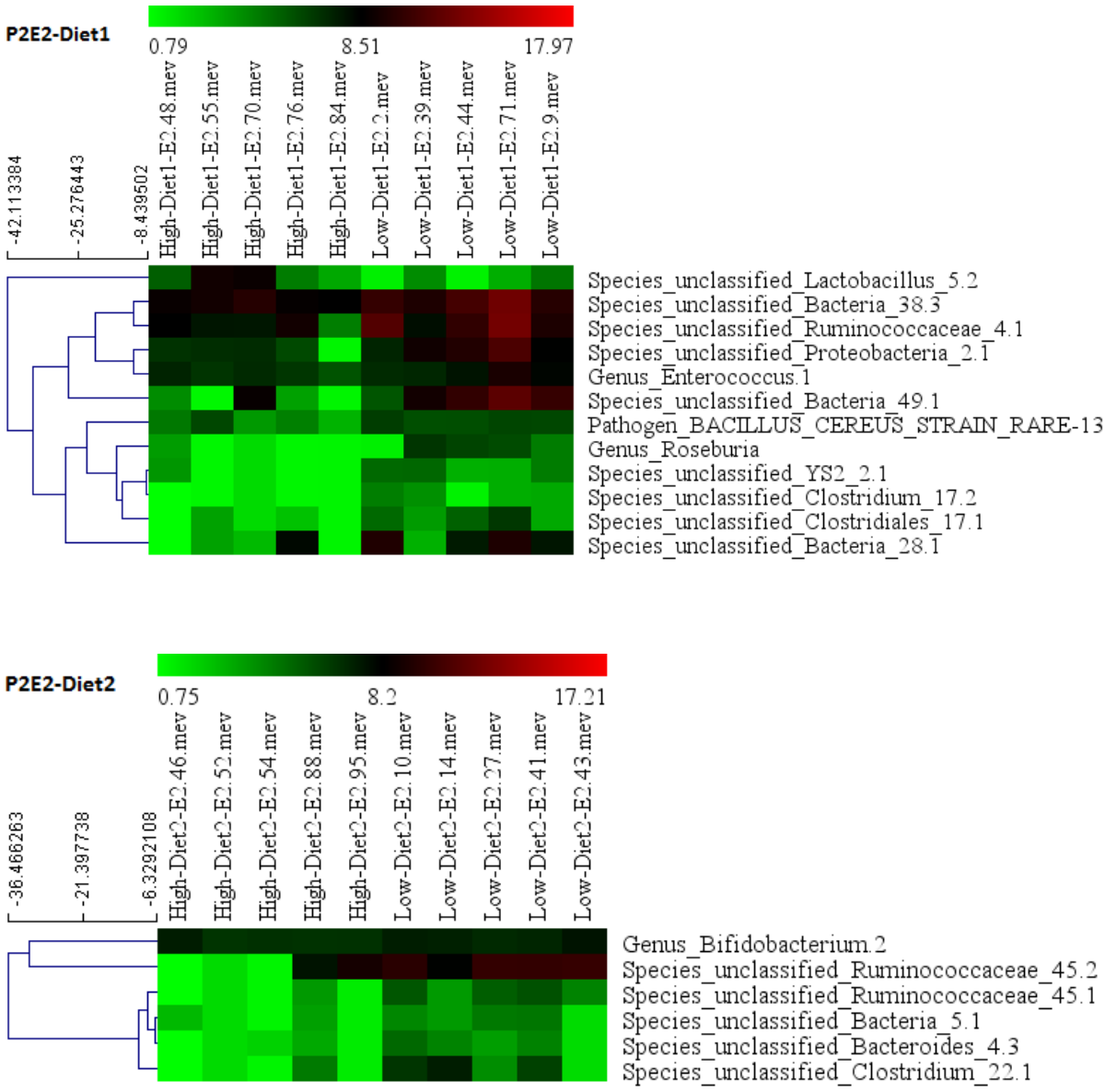

Figure 10.15. Phylotypes differing in abundance (p≤0.05) between birds with low or high FCR in experiment P2E2. Heat map was generated based on the $\log_2$ value of the ratio between the mean fluorescence intensities of each group of birds and the internal standard, and displayed using the standard Green-Black-Red scheme with red indicates high relative abundance and green indicates low relative abundance. Low, low FCR good-performance chicken; High, high FCR poor-performance chicken.

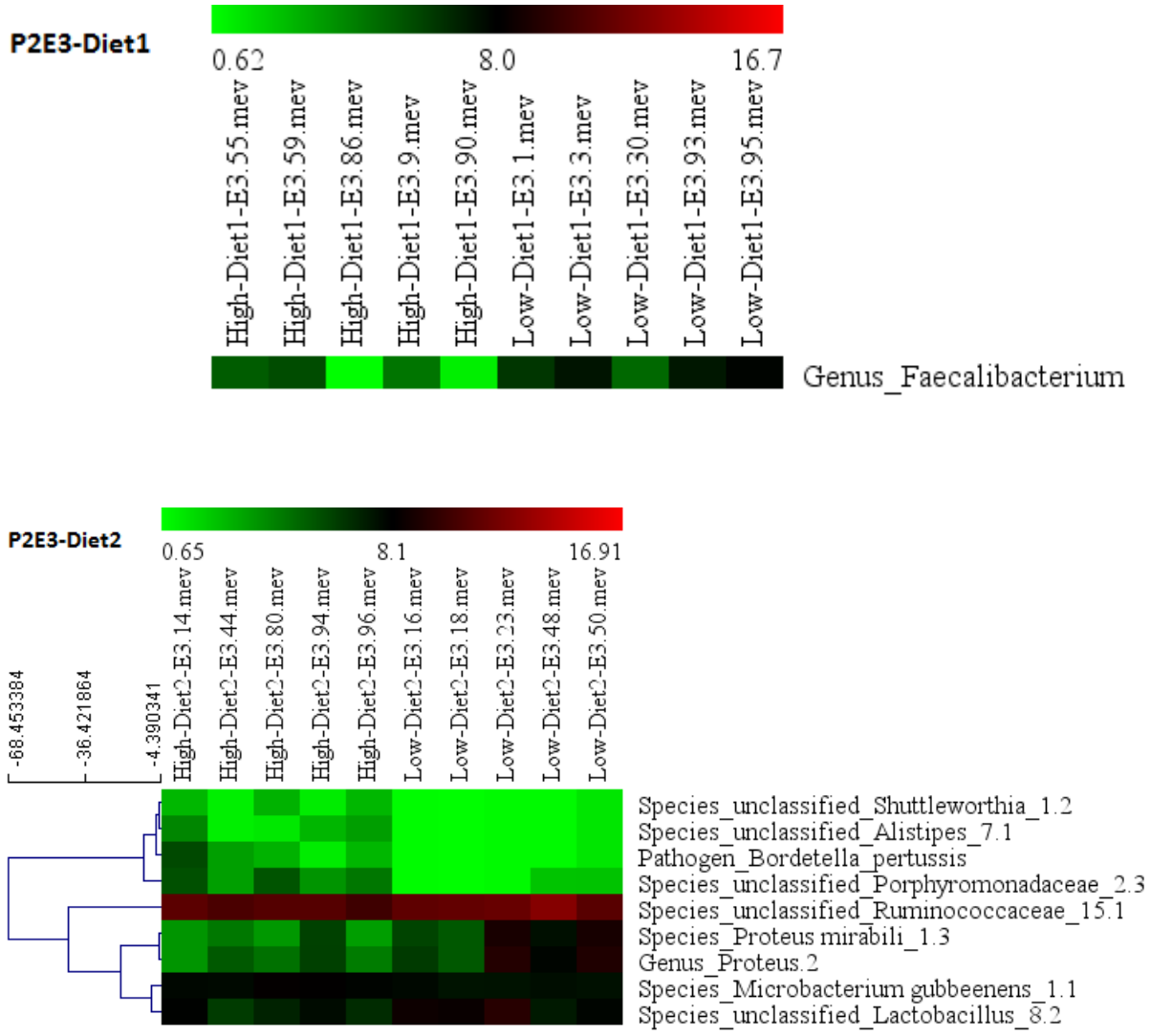

Figure 10.16. Phylotypes differing in abundance (p≤0.05) between birds with low or high FCR in experiment P2E3. Heat map was generated based on the log_2 value of the ratio between the mean fluorescence intensities of each group of birds and the internal standard, and displayed using the standard Green-Black-Red scheme with red indicates high relative abundance and green indicates low relative abundance. Low, low FCR good-performance chicken; High, high FCR poor-performance chicken.

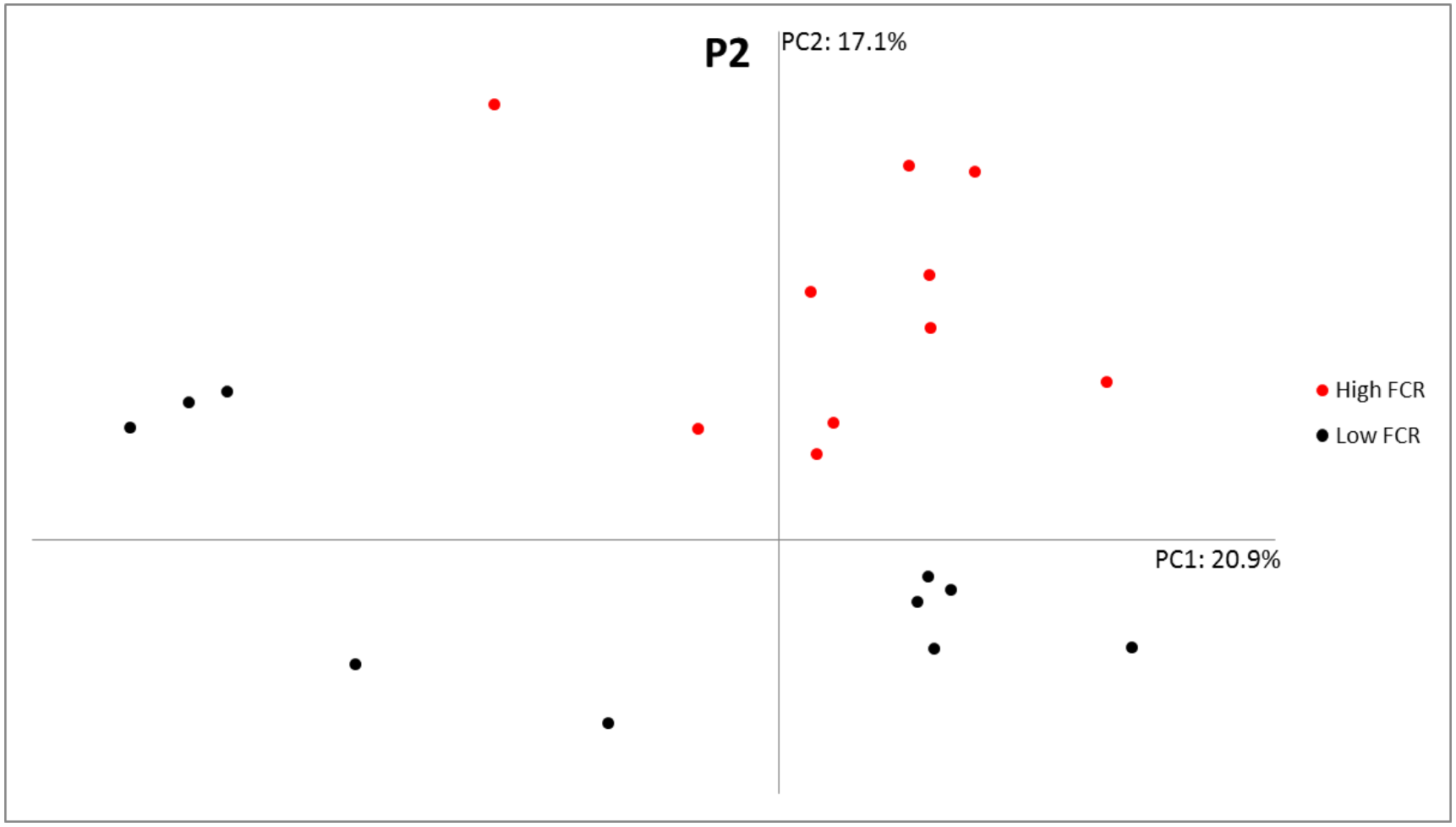

Figure 10.17. PCA plot showing the grouping of 20 chickens from the three experiments of P2 based on the overall structure of their caecal microbiome.

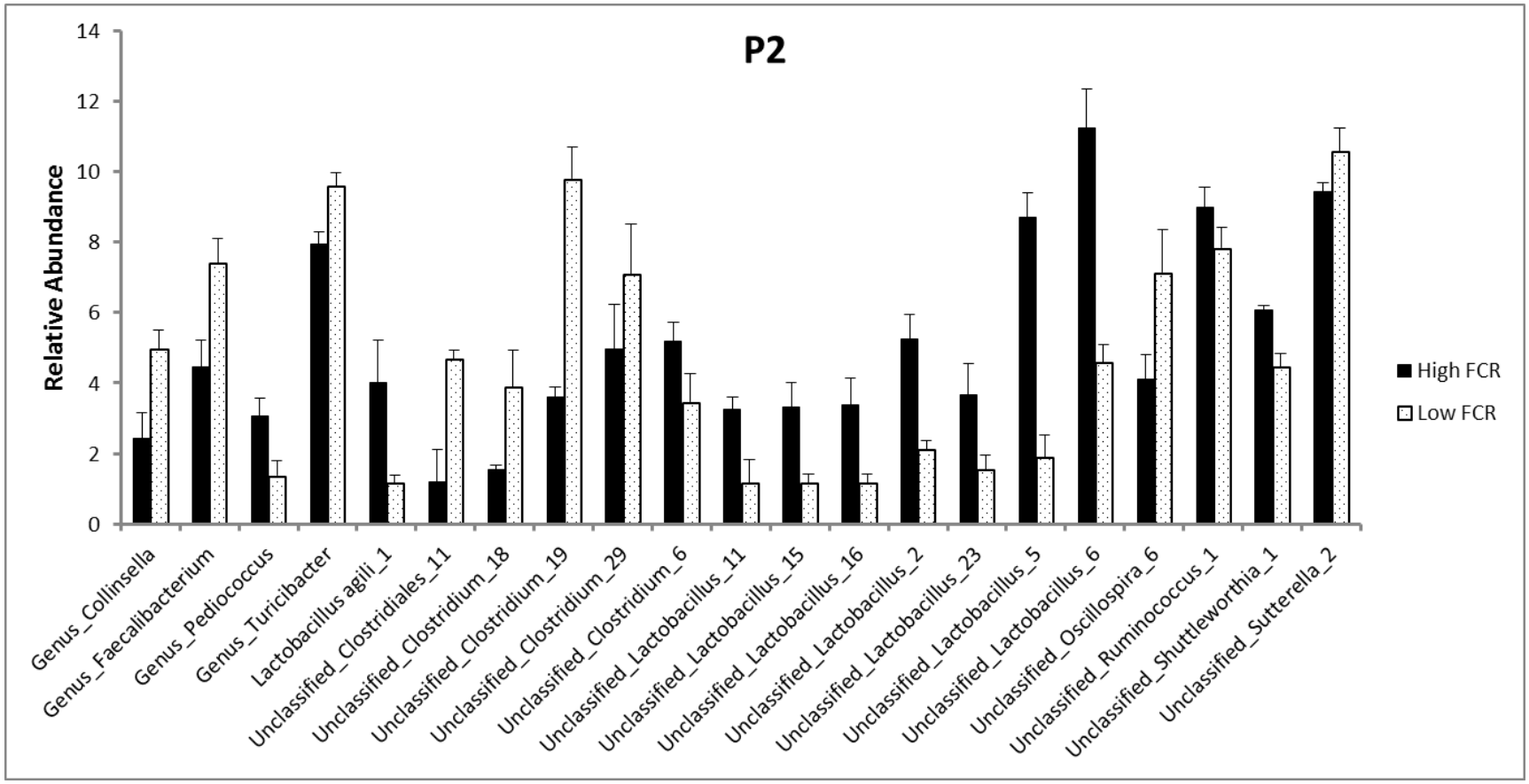

Figure 10.18. 21 phylotypes differing in abundance (P ≤ 0.05) between birds with low or high FCR in the three experiments of P2. Relative abundance was calculated using the log_2 value of the ratio between the mean fluorescence intensities of each group of birds and the internal standard.

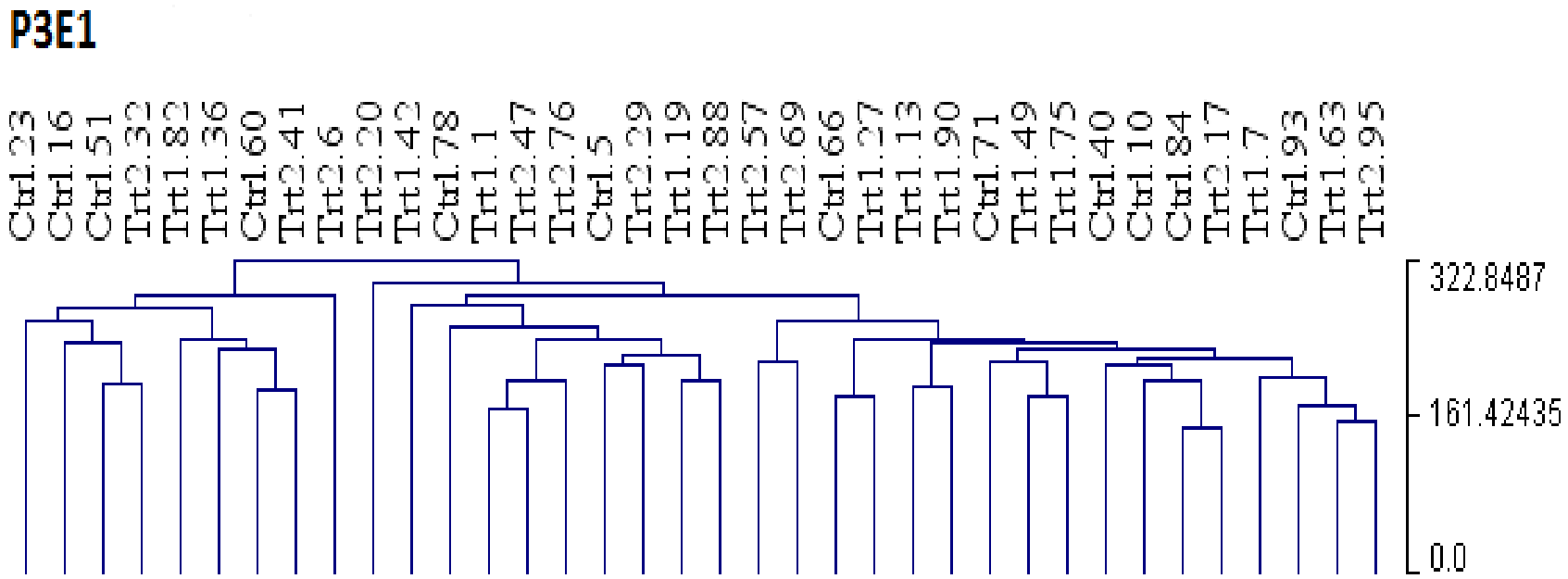

Figure 10.19. Hierarchical trees generated from HCL analysis of the caecal microbiome. Ctrl, control; Trt1, caecal inoculum #1; Trt2, caecal inoculum #2.

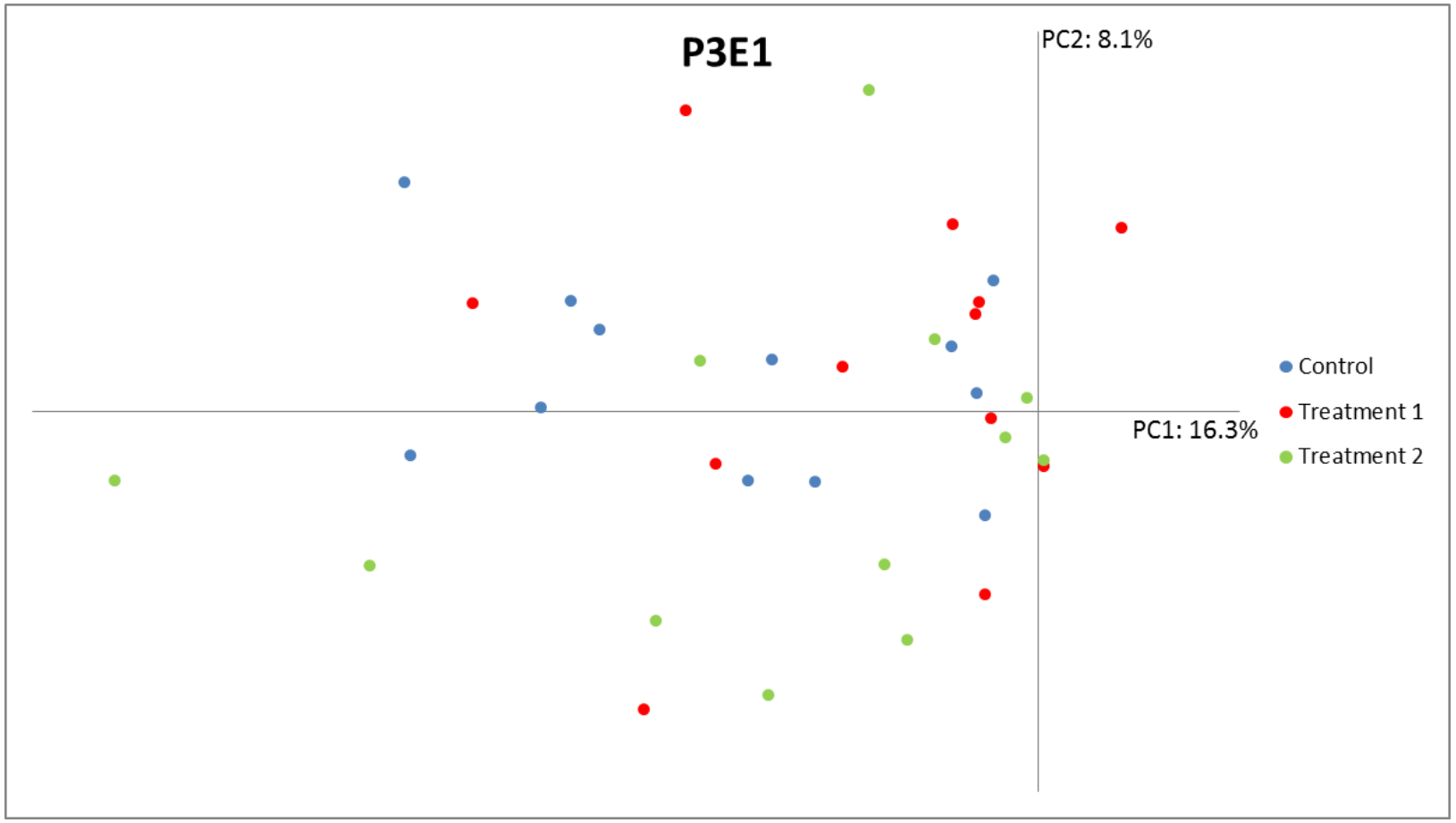

Figure 10.20. PCA plot showing the grouping of chickens from P3E1 based on the structure of their caecal microbiome.

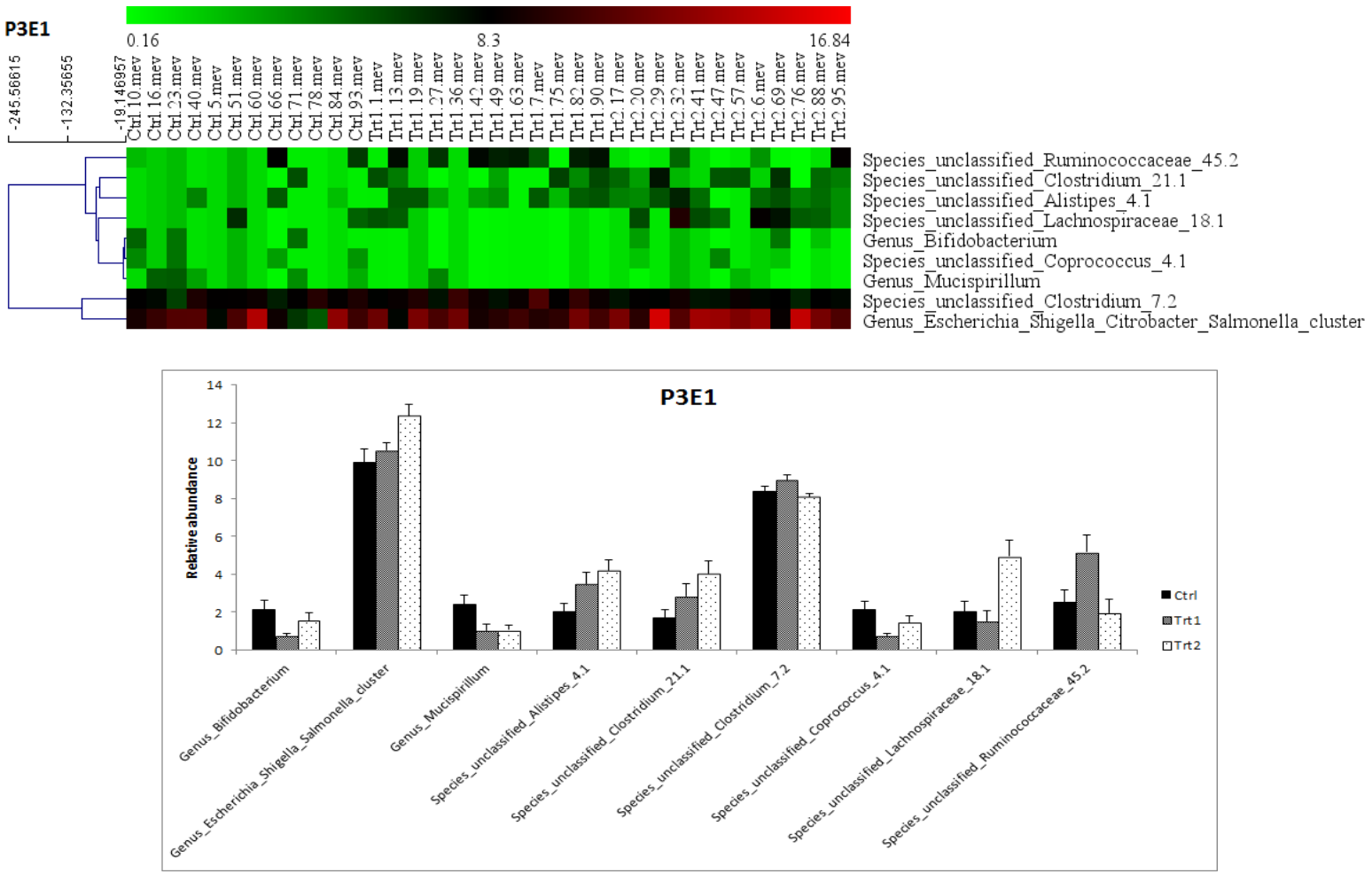

Figure 10.21. Phylotypes differing in abundance ($P \leq 0.05$) between birds with different treatments in experiment P3E1. Heat map was generated based on the $\log_2$ value of the ratio between the mean fluorescence intensities of each group of birds and the internal standard, and displayed using the standard Green-Black-Red scheme with red indicates high relative abundance and green indicates low relative abundance. Ctrl, control; Trt1, caecal inoculum #1; Trt2, caecal inoculum #2.

Phase 3 trial

Both HCL and PCA analysis indicated that there was no clear separation of caecal microbiome between birds with different treatments (Figures 10.19 and 10.20). ANOVA test identified 9 phylotypes associated with treatment, including 3 genera and 6 species-level phylotypes (Figure 10.21).

Discussion

This study investigated the relationship between gut microbiome and host feed utilisation efficiency using PITChip2, a poultry specific phylogenetic microarray. Ninety six male Cobb 500 broiler chickens were used in this study and they were ranked from 1 to 96 based on their feed conversion ratio (FCR). On day 25 post-hatch the birds were euthanised. Metagenomic DNA extracted from caecal content collected from 24 birds on each end of the FCR spectrum was used for microbial analysis using PITChip2. There was no clear separation in the gut microbiome structure between high-performance (low FCR) and low-performance (high FCR) chickens as indicated by the hierarchical clustering (HCL) analysis or principal component analysis (PCA). Sixty-nine phylotypes of bacteria were found to exist universally in all 48 birds, with 16 phylotypes were identified as predominant, including phylotypes in *Ruminococcaceae*, *Faecalibacterium*, *Oscillibacter*, *Bacteroidetes*, and *Lactobacillales*. One hundred phylotypes were found to correlate with bird performance ($p \leq 0.05$), with 48 of them showing a greater than 3-fold difference in relative abundance between birds with high and low FCR. Three phylotypes representing species of *Bacteroides*, *Peptococcus*, and *Ruminococcaceae*, and two unclassified phylotypes were found to be more abundant in high-performance than in 47 low-performance chickens.

In the current study we have identified five phylotypes that were found more abundant in the caeca of birds with higher feed utilisation efficiency as indicated by low FCR. These five phylotypes may serve as bacterial indicators of feed utilisation efficiency and potential probiotic candidates or targets of prebiotics for improvement of growth performance of meat chickens. While association does not mean causality, the current study has suggested the direction of future researches. If any of these identified phylotypes are repeatedly shown to increase with efficient feed utilisation in chickens, intervention strategies (e.g. feeding probiotics or prebiotics) can then be designed to increase the abundance of such phylotypes, which may in turn benefit the growth of the birds. Such intervention strategies may serve as alternatives for currently widely used in-feed antibiotic growth promoters (AGP) and help control the spreading of antibiotic resistance among bacteria residing in food animals.

While next-generation sequencing is becoming more affordable and being widely used in microbiome studies, the current study showed that our phylogenetic microarray, PITChip2, can

serve as an alternative tool that enables high-throughput and comprehensive analysis of the intestinal microbiome in chicken's GI tract. However, probes on PITChip2 were designed from the V3 region of bacterial 16S rRNA gene sequence, limiting its ability to reliably detect and identify bacteria present in chicken's gut. The phylogenetic microarray PITChip3 with probes designed from longer region of bacterial 16S 61 rRNA gene sequences, such as the V1-V3 region, is likely to generate more accurate and reliable results. Experimental work underpinning development of PITChip3 is described in Chapter 11.

PITChip2 and 454 pyrosequencing analyses of extracted bacterial DNA from experiments described in Chapters 1 and 2 are in agreement in the sense that there is no one exclusive handful of micro-organisms associated with high feed efficiency, at least under Australian conditions. Excellent FCR values can be achieved in the presence of many different consortia of bugs. This has the very important implication that more than one probiotic product could support high feed efficiency, and no manufacturer can claim superiority over their opposition without very strong evidence, and good performance from their product does not constitute strong evidence – much like the situation with exogenous enzyme products – all work to an extent so it comes down to competitive pricing, once efficacy is established.

On the other hand, there is mounting evidence from PITChip2 and 454 analyses that certain specific organisms are associated with poor FCR but without causing overt disease or health issues. It will be useful for industry to know what the identity of these undesirable organisms are, and to be provided with practical advice and ways to exclude these organisms from the developing gut microbiota. PITChip3 technology developed by inclusion of the longer V1-V3 region of bacterial 16S rRNA gene sequences, could provide practical outcomes here. Once the commensal population is developed and stabilised, it is nigh on impossible to alter this without on-going dietary treatments involving pre- and pro-biotic products and /or antibiotics at extra cost to production, and criticisms from consumers, potentially.

Conclusions

Five phylotypes were identified that were found more abundant in the caeca of birds with higher feed utilisation efficiency as indicated by low FCR. These five phylotypes may serve as bacterial indicators of feed utilisation efficiency and potential probiotic candidates or targets of prebiotics for improvement of growth performance of meat chickens.

Chapter 11. Development of PITChip3 and comparative analysis of gut microbiome of meat chickens fed corn- or a wheat-based diet

Extracted from an original article by Pan (2014)

Introduction

A phylogenetic microarray on a single chip can have hundreds of thousands of probes, which can provide semi-quantitative information on members of microbial ecosystems with great diversity (DeSantis *et al*. 2007). The microarray can have some advantages over other high-throughput techniques such as 454 DNA pyrosequencing. Firstly, the microarray can provide a uniformed, robust assessment of individual populations of complex microbiomes (Brodie *et al.* 2006). Secondly, nucleic acid samples can be directly hybridised with the microarray chip, avoiding inherent PCR bias, which has been shown to compromise the assessment of microbiomes. Last but not least, microarray chips can be custom fabricated or printed in house at relatively low cost (less than US $250 per microarray chip), updated quickly when new probes need to be added, and analysis of microarray data is much less resource demanding.

To date, phylogenetic microarrays have been developed for the assessment of human gut microbiome as well as complex microbiomes in other environments, and have gained recognition and acceptance as a highly efficient phylogenetic fingerprinting tool (DeSantis *et al*. 2007; Rajilić-Stojanović *et al*. 2009; Kang *et al*. 2010). This chapter describes the development of PITChip3, a poultry specific phylogenetic microarray, which is intended to be used as a comparative analytical tool to comprehensively study bacterial community in the poultry gastrointestinal tract. The design is based on the V1-V3 region of bacterial 16S rRNA gene sequences of poultry origin, and with up to three probes targeting the same target, the probes on PITChip3 are able to simultaneously detect 62 genus-level phylotypes and 662 species-level phylotypes, as well as 34 pathogens. Each PITChip3 slide has six microarrays and every probe has 4 replicates randomly located on each microarray. To test its utility, PITChip3 was used to study metagenomic DNA recovered from caecal content of broiler chickens fed with either wheat-based or corn-based diets.

Materials and methods

Oligonucleotide probe design

Three sources of bacterial 16S rRNA gene sequences were used to construct a sequence dataset for the design of oligonucleotide probes for PITChip3: 1) 3,922 bacterial 16S rRNA gene sequences of poultry origin (chicken and turkey) retrieved from the GenBank and the RDP

database (Release 10, Update 32) using search terms such as ‘poultry’, ‘chicken(s)’, ‘chick(s)’, ‘broiler(s)’, and ‘turkey’; 2) 4,095 bacterial 16S rRNA gene sequences (V1-V3 region) from chicken gut microbiome kindly provided by Prof Moore at CSIRO Livestock Industries, Australia; 3) bacterial 16S rRNA gene sequences (V1-V3 region) from broiler chickens generated using 454 FLX Titanium pyrosequencing (sequences deposited at the MG-RAST server [Meyer *et al*. 2008] with ID:9131 and 9132) from another two projects at Ohio State University (unpublished). The sequences from these sources were combined into a single dataset and subjected to quality screening to eliminate those sequences of low quality. The Mothur program (Schloss *et al*. 2009) was used to de-replicate identical sequences, filter out sequences with ambiguous bases, and remove sequences shorter than 400 bp. Chimeric sequences were detected and removed using UCHIME (Edgar *et al*. 2011) and DECIPHER (Wright *et al*. 2012). The remaining sequences were then aligned against the SILVA alignment reference database that was incorporated into Mothur. Based on the alignment, only sequences that contain the V1-V3 hypervariable region were retained, and the V1-V3 region were delineated and sliced out for subsequent probe design.

Oligonucleotide probes at two taxonomy levels (genus- and species-equivalent) were designed for PITChip3. In order to design probes at genus level, sequences that passed the above mentioned quality check procedures were first classified using RDP Classifier (Wang *et al*. 2007). Sequences that cannot be classified at genus level (with confidence threshold set to 70%) were not used for genus-level probe design. Genera with only one sequence were also excluded from subsequent design of genus-level probes. Sequences used for genus-level probe design were aligned against the SILVA alignment reference database using Mothur. The alignment was imported to Geneious Basic version 5.6.6 (Kearse *et al*. 2012) and one consensus sequence for each genus was generated. The probe design program Picky version 2.20 (Chou *et al*. 2004) was used to design probes from these consensus sequences. Sequences not used for genus-level probe design were imported into Picky as nontarget so that the probes designed will not hybridise to these sequences. Picky was set to design oligonucleotide probes with sizes of 20-30 nt and with the minimum temperature difference (i.e. minimum safe difference between a probe's target melting temperature and its highest nontarget melting temperature) set to 7℃. In addition, Picky was instructed to design three oligonucleotide probes targeting different regions of each target sequence, and only unique probes (probes that can only hybridise to one target) were selected. For genera that Picky failed to design unique probes from consensus sequences, a distance matrix with Jukes-Cantor correction was calculated using ARB (Ludwig *et al*. 2004). The distance matrix was then used to generate a representative sequence for each genus using Mothur. Representative sequences from these genera were then imported into Picky to design unique oligonucleotide probes.

Species-level probes were designed following similar procedures as for genus-level probe design. Briefly, ARB was used to produce a distance matrix, which was then used to cluster sequences into species-equivalent operational taxonomic units (OTUs, defined at 0.04 phylogenetic distance). Unique oligonucleotide probes were first designed based on the consensus sequences of each OTU. For OTUs that had no unique probes designed from consensus sequences, additional probes were designed based on the representative sequences. The parameters in Picky used for species-level probe design were identical to those used for genus-level probe design.

All the pathogen probes on PITChip2 were also included on PITChip3. Designed from the 16S rRNA gene or genes encoding virulence factors using Picky, these probes target poultry pathogens, including pathogenic bacteria, virus and parasite, which have their 16S rRNA or virulence genes archived in GenBank.

In addition to the aforementioned probes, PITChip3 has positive and negative control probes and also internal control probes, the latter of which was used for microarray data normalisation. The probe EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') which targets most bacteria was used as positive control probe on PITChip3 (Banerjee *et al*. 2002). Negative control probe was designed from human mitochondrial 16S rRNA gene sequence using Picky. In order to design internal control probes, 4 randomly generated DNA sequences (1,600 nt) were imported into Picky as targets, while the sequences used for genus- and species-level probe design were imported into Picky as nontarget, thus assuring the designed internal control probes will not hybridise to the targets of genus and species-level probes. Picky designed several oligonucleotide probes from the 4 randomly generated DNA sequences, from which 6 probes were used on PITChip3. These 6 internal control probes were further tested using the probe match tool in RDP to ensure that they do not hybridise to any bacterial 16S rRNA gene sequences archived in RDP.

Fabrication of PITChip3

The PITChip3 slides were custom-fabricated by MYcroarray (Ann Arbor, MI). There are 6 arrays on each slide with each array consists of a grid of 56 columns by 94 rows for a total of 5,264 spots, among which 264 spots are reserved for internal quality control, leaving 5,000 spots available for the customised oligonucleotide probes. Each probe has 4 replicates occupying 4 spots randomly located on each microarray and each spot has approximately 1.5×10^9 probes.

Sample collection and DNA extraction

Intestinal microbial samples from another study (unpublished) in our lab were used to test PITChip3. Briefly, 200 straight run (unsexed) Cobb 500 broiler chicks at age of one day were randomly assigned to two different diets (wheat-based diets vs. corn-based diets). Chickens were

housed in 40 floor pens (n=5 birds per pen) with 20 replicate pens per dietary treatment. One bird per pen was randomly picked for sampling at 14, 20, and 35 days of age. Ileal mucosa and caecal content samples were collected from the birds selected and frozen at -80℃ until DNA extraction. The birds were handled and cared of following a protocol approved by the Institutional Animal Care and Use Committee.

Caecal content samples collected from 4 birds per treatment at 14 and 35 days of age were used for microarray experiments. Metagenomic DNA was extracted from each sample using the repeated bead beating plus column (RBB+C) method developed by Yu and Morrison (2004). Extracted DNA was stored at -20℃ until use.

Sample preparation and labelling

From each metagenomic DNA sample 16S rRNA gene was amplified by PCR using universal bacterial primer set T7/27F (5 - TCTAATACGACTCACTATAGGGAGAGTTTGATCMTGGCTCAG-3', T7 promoter region is the underlined region) and 1525R (5'-AAGGAGGTGWTCCARCC-3') as described previously (Devereux and Willis, 1995; Kang *et al*. 2010). PCR was performed with 33 cycles (denaturation, 95℃ for 30 s; annealing, 55℃ for 45 s; and extension, 72℃ for 90 s) using a PTC-100 thermocycler (MJ Research, Waltham, MA). Amplicons were confirmed by agarose gel electrophoresis, purified using QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

The same amount of each purified PCR product (100 ng) was then added as template for in vitro transcription using the MEGAScript T7 in vitro transcription kit (Ambion, Austin, TX). Synthesised cRNA was again confirmed by agarose gel electrophoresis, purified using the MEGAClear kit (Ambion, Foster City, CA) and quantified using a NanoDrop ND-1000 spectrophotometer. Equal amount (500 ng) of the purified cRNA was then labelled with Cy5 fluorescent dye at 37℃ for 1 hour using the Label IT μArray Cy5 reagent (Mirus, Madison, WI). The labelled cRNA was purified to remove the free Cy5 dye using the MEGAClear kit and stored at -80℃ until microarray hybridisation. All subsequent microarray experiment procedures (e.g. hybridisation and washing) after the Cy5 labelling step were performed in dark to avoid light bleaching of the light-sensitive Cy5 dye.

Microarray hybridisation and washing of microarray slides

The hybridisation was performed following manufacturer's protocol provided by MYcroarray. Briefly, for each array 60 μL hybridisation solution was prepared with 18 μL 20×SSPE buffer (3M NaCl, 20mM EDTA, 118.2mM NaH2PO4, and 81.8mM Na2HPO4), 6 μL formamide, 0.6 μL 1% bovine serum albumin, 0.6 μL 1% tween-20, 0.6 μL Control-oligos provided by the manufacturer,

0.6 µL Cy5-labelled internal controls, and 26 µL Cy5-labelled cRNA. The hybridisation solution was incubated at 65℃ for 5 minutes, then immediately cooled on ice for another 5 minutes. Each array was applied with 54 µL hybridisation solution. Microarray slide was placed in Agilent Hybridisation Chamber (Agilent, Santa Clara, CA) and the hybridisation was carried out in an HB-1000 hybridisation oven (UVP, Upland, CA) at predetermined optimal hybridisation temperature for 20 h with rotation speed set at 10 rpm.

The washing of microarray following hybridisation was performed per manufacturer's protocol. Right before hybridisation, 1×SSPE buffer and 0.25×SSPE buffer were placed in the hybridisation oven along with the microarray slide so that they remained at hybridisation temperature in the subsequent washing steps. The microarray slide was first washed in 1×SSPE buffer for 5 minutes, followed by washing in 0.25×SSPE buffer for 30 seconds. The microarray slide was then dried for 10 min using a high speed microarray centrifuge (Arrayit, Sunnyvale, CA).

Signal detection and data analysis

Microarray slides were scanned using a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA) with oversaturation tolerance set to 0.01%, photomultiplier sensitivity set at auto-PMT gain, and resolution set to 5 µm. The information of each probe spot on the scanned images was extracted by fitting the gene allocation list (GAL) file provided by the manufacturer which carries the annotation information of each spot on the microarray. The scanned image was then quantified using GenePix pro 6.0 software (Axon Instruments, Union City, CA). Probe spots of negative detection were flagged out by the auto-alignment function of the GenePix software. Probe spots with oversaturation, bad shape, or suspected contamination were flagged manually.

The GenePix extracted results of each array were exported as a GenePix Results Format (GPR) file. ExpressConverter version 2.1 of the TM4 Microarray Suite (Saeed *et al*. 2006) was used to convert each GPR file to an annotation (ANN) file and a multiexperiment viewer (MEV) file. From the MEV file, the median signal intensity of each spot after subtracting the background noise was used for downstream analysis. The converted data was entered into Ginkgo version 1.01 (J. Craig Venter Institute, Rockville, MD) and the replicated spots in the array were consolidated using the standard "In-Slide Replicates Analysis" option of the Ginkgo software.

The microarray data were normalised based on the signal intensities of internal control probes. Normalised data were imported into MeV program (version 4.8.1) within the TM4 Microarray Suite for statistical analysis. Heat maps were generated based on the log2 values of the median intensities. Clustering of samples was performed using hierarchical clustering (HCL) in the MeV program with a Manhattan distance metric. Average linkage was used to determine cluster-to-

cluster distances when constructing the hierarchical tree. Principal component analysis (PCA) was conducted using the MeV program to identify clusters of samples with similar caecal microbiome structure. The standard “TTEST” option of the MeV program was used to identify the hybridised probes that were significantly ($p \leq 0.05$) correlated with treatments.

Linear detection range and optimal hybridisation temperature determination

In order to determine the probe specificity, optimal hybridisation temperature, and linear detection range, the reverse complementary oligonucleotides of the 6 internal control probes and 20 other probes (10 genus-level probes, 8 species-level probes, and 2 pathogen probes) were selected and synthesised by Alpha DNA (Montreal, Quebec, Canada). These 26 oligonucleotides were first labelled using the Label IT μArray Cy5 kit. The labelling procedure was modified based on manufacturer's protocol so that most of the oligonucleotides (20-30 nt) can be labelled by Cy5 fluorescent dye. Briefly, 500 ng oligonucleotides were labelled in a 50 μL labelling reaction. The Cy5 dye used in each reaction was increased from the recommended 2 μL to 6 μL. The incubation time was increased from 1 hour to 2 hours. The labelled oligonucleotides were purified using the Oligo Clean-Up and Concentration Kit (Norgen, Thorold, Ontario, Canada). NanoDrop ND-1000 spectrophotometer was then used to quantify both the oligonucleotides and the Cy5 dye. It was estimated that there was one Cy5 label every 20nt. Since the sizes of oligonucleotide targets range from 20-30nt, it is safe to say most of the oligonucleotides were labelled by at least 1 Cy5 fluorescent dye.

The hybridisation was performed following procedures described above. In order to determine linear detection range, Cy5-labelled targets for the 6 internal control probes, namely C1, C2, C3, C4, C5, C6, were serially diluted and applied to each array at 0.5, 5, 50, 500, 5000, and 50000 pg, respectively. Each array was also hybridised with the other 20 synthetic oligonucleotide targets, 109 copies each, to determine the specificity of the probes. Microarray hybridisation was carried out in an HB-1000 hybridisation oven (UVP, Upland, CA) at 42℃ for 20h with rotation speed set at 24 rpm. The experiment was repeated at 45℃, 48℃, and 52℃ to determine optimal hybridisation temperature.

Results and Discussion

PITChip3 design

Bacterial 16S rRNA gene sequences from three sources were combined into a single dataset and subjected to a series quality check and screening processes, leaving 7,492 partial (V1-V3 hypervariable region) 16S rRNA gene sequences for subsequent genus and species-level probe design. From the 7,492 16S rRNA gene sequences, 4,402 sequences could be classified at genus level using RDP Classifier and were used to design genus-level probes based on either the

consensus or the representative sequences of each genus. After removing 50 singletons (genera with only one sequence), 4,352 sequences representing 90 genera were used to design genus-level probes based on consensus sequence from each genus. In total 107 unique probes were designed for 62 genera, with 64 probes designed from consensus sequences targeting 34 genera and 43 probes designed from representative sequences targeting another 28 genera. Picky was not able to design unique probes for 28 genera. To our knowledge, it is almost impossible to design unique probes for every genus of the 90 genera without having cross hybridisation issues as the 16S rRNA gene sequences of some genera (e.g. genera in the same family) are too similar with each other. Therefore, PITChip3 will not be able to analyse those 28 genera. Another approach to design genus-level probes would be to use genus-equivalent OTUs defined at 0.05 phylogenetic distance. The OTU0.05 approach may allow design of probes for more genera than the approach we used here. However, the genus-equivalent OTUs are not the same as the genera classified in the RDP database. As such, the probes designed using the OTU0.05 approach do not target the established genera. The current approach was used because we want PITChip3 to be able to analyse the established genera whose physiology and ecology are known.

For species-level probes design, the 7,492 16S rRNA gene sequences were clustered into 1,739 species-equivalent OTUs at 0.04 phylogenetic distance. The conventional 0.03 phylogenetic distance (97% similarity) was not used as it was suggested by Kim *et al*. (2011) that 0.04 phylogenetic distance will allow more accurate phylogenetic analysis when species-equivalent OTUs clustering was based on V1-V3 hypervariable region. In total, 1,006 unique probes were designed for 662 species-equivalent OTUs. Almost two thirds of the species-equivalent OTUs had no unique probes designed from Picky. As mentioned previously it is not feasible to design unique probes for all of the sequences without having cross-hybridisation issues because the sequences used for probe design are quite similar. Although in the current study the hypervariable regions of the 16S rRNA gene sequences were used for probe design, when comparing to other genes they are still highly conserved, especially those sequences belonging to the same genus or family.

On PITChip3 there are 83 probes targeting the 16S rRNA genes or virulence genes of 34 known poultry pathogens including pathogenic bacteria (e.g. *Clostridium perfringens*), viruses (e.g. avian adenovirus), and parasites (e.g. *Eimeria maxima*). Identification of pathogens using oligonucleotide probes targeting non-16S rRNA gens is applicable only when metagenomic DNA or metatranscriptome are analysed.

Taken together, in total there are 1,204 customised oligonucleotide probes on PITChip3, including 107 genus-level probes targeting 62 genera, 1,006 species-level probes targeting 662 species-equivalent OTUs, and 83 pathogen-specific probes targeting 34 pathogens, plus 8 control probes

(i.e. positive and negative control and internal control probes). The sequence information about the probes and their targets is available upon request.

Optimal hybridisation temperature and linear detection range determination

When performing microarray hybridisation, if hybridisation temperature is too low, cross-hybridisation is likely to occur, resulting in false positives. On the other hand, when hybridisation temperature is too high, hybridisation between target and probe may not occur even with a perfect match and false negatives may result (Kim 2011). The optimal hybridisation temperature would be the one at which least false positives and least false negatives result. In order to determine the optimal hybridisation temperature for PITChip3, synthetic oligonucleotide targets for 20 probes (10 genus-level probes, 8 species-level probes, and 2 pathogen probes) were labelled with Cy5 dye and hybridised to PITChip3 at 42℃, 45℃, 48℃, and 52℃ for 20 hours. When the hybridisation temperature was set to 42℃ and 45℃, there were 4 and 2 false positives detected on the array, respectively. No false negatives were found at those two temperatures. Neither false positives nor false negatives were detected at 48℃. At 52℃, there were no false positives but 3 false negatives. Therefore, based on the 20 synthetic oligonucleotide targets tested, 48℃ was selected as the optimal hybridisation temperature for PITChip3. It is worth noting that the selected 20 probes only make up a very small fraction (<2%) of the probes on PITChip3. Optimal hybridisation temperature determined based on these 20 probes may not necessarily be optimal for all the other probes on PITChip3.

Theoretically we could order synthetic oligonucleotide targets for all 1204 probes and use them to determine optimal hybridisation temperature. However, it is impractical to do so. It should also be noted that although at optimal hybridisation temperature the Picky designed probes do not likely cross-hybridise to non-target within V1-V3 region, some probes may hybridise to non-target 16S rRNA sequence at locations beyond the V1-V3 region (Kim 2011). Such a possibility cannot be excluded based just on the 20-30nt synthetic oligonucleotide targets tested here. All microarrays, especially phylochips, have these potential limitations, but these limitations should not affect comparative analysis of related or similar microbiome samples.

The linear detection range of PITChip3 at 48℃ was determined using serially diluted and Cy5-labelled oligonucleotide targets for the 6 internal control probes. These 6 oligonucleotides, namely C1, C2, C3, C4, C5, C6, were applied to the array at 0.5, 5, 50, 500, 5,000, and 50,000 pg (approximately 3×10^{7} to 3×10^{12} copies), respectively. The linear detection range was determined based on the log10 value of median signal intensity of each probe-target hybrid (Figure 11.1). Signal intensity reached plateau when the copy number of target was 3×10^{11}. When the copy number of targets ranged from 3×10^{7} to 3×10^{10}, it had a linear relationship with log10 signal

intensity, indicating PITChip3 has a linear detection range of three orders of magnitude (10^7-10^{10} copies per array). The linear detection is narrower than that of quantitative real-time PCR, and PITChip3 is not as sensitive as qPCR. However, it allows much more comprehensive analysis of at both genus and species-equivalent levels.

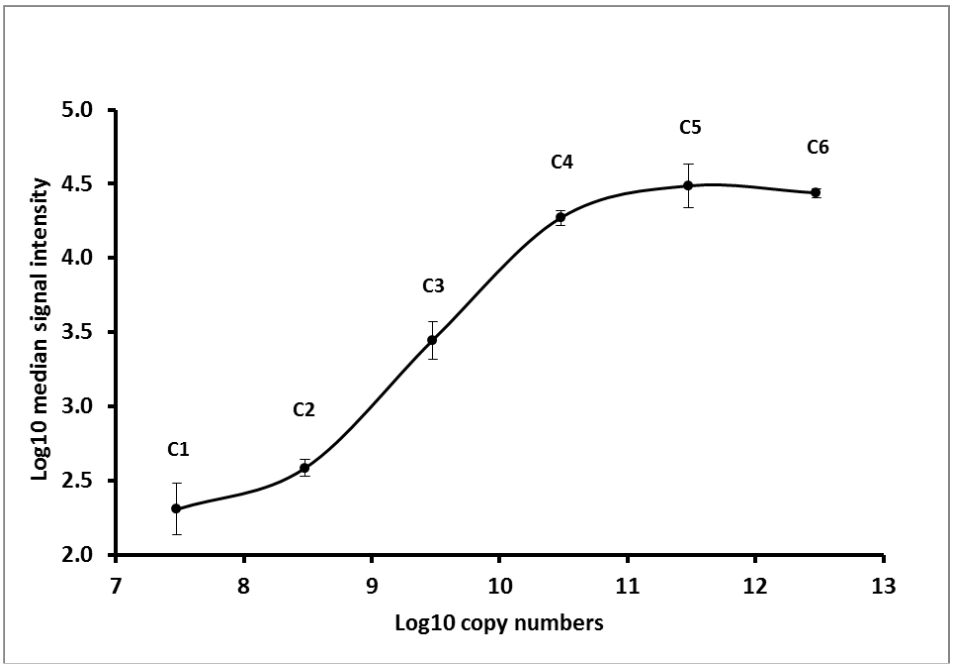

Figure 11.1. Linear detection range of PITChip3. Targets for 6 internal controls, from left to right, had copy numbers ranging from 3×10^{7} to 3×10^{12}. The log10 median signal intensity is shown as mean ± SD. PITChip3 has a linear detection range from 3×10^{7} to 3×10^{10}.

Microarray data summary

Across all treatments, PITChip3 was able to detect 419 targets (or phylotypes), including one target for positive control (i.e. total bacteria), 44 targets for genus-level probes, 345 targets for species-level probes, and 29 pathogens. At 14 days of age, birds fed with corn and wheat-based diets had 188 and 242 targets detected, respectively. At 35 days of age, 230 and 198 targets were detected from chickens fed with corn- and wheat-based diet, respectively. A Venn diagram (Figure 11.2) was constructed to show the distribution of detected targets among different groups of chickens.

Based on PCA analysis of the PITChip3 data, birds fed corn-based diet had distinct caecal microbiome from birds fed wheat-based diet at 14 and 35 days of ages (Figure 11.3). This is not surprising because diet has the greatest potential impact on the intestinal microbiome in poultry as dietary components that escape host digestion and absorption serve as the substrates for the growth of bacteria residing in the gut. Depending on the nature of feed ingredients, a diet may selectively enhance the growth and proliferation of some bacteria while having no effect or even adverse effects on others (Pan and Yu 2013). Corn-based diet may favour the growth and proliferation of a group of bacteria which does not thrive in the GI tract of chickens fed with wheat-based diet, and vice versa.

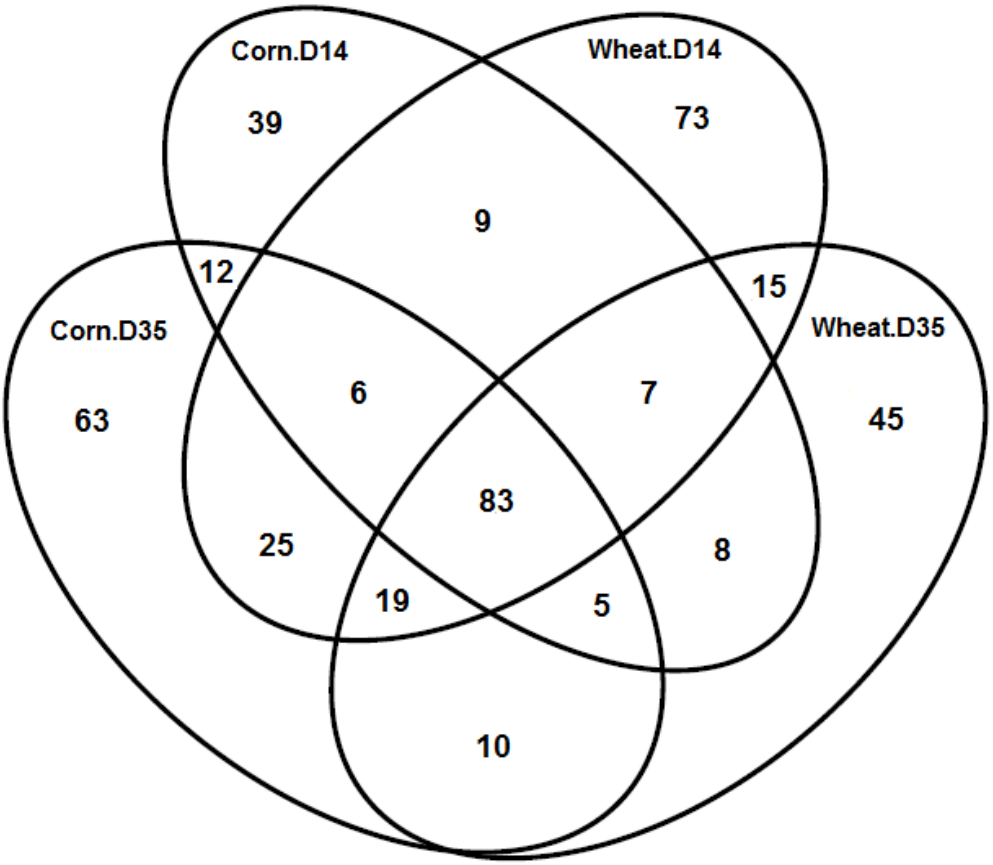

Figure 11.2. A Venn diagram showing the number of phylotypes shared among different groups of chickens. D14 and D35 represent birds at 14 and 35 days of age, respectively.

Impact of dietary grain on caecal microbiome structure of chickens

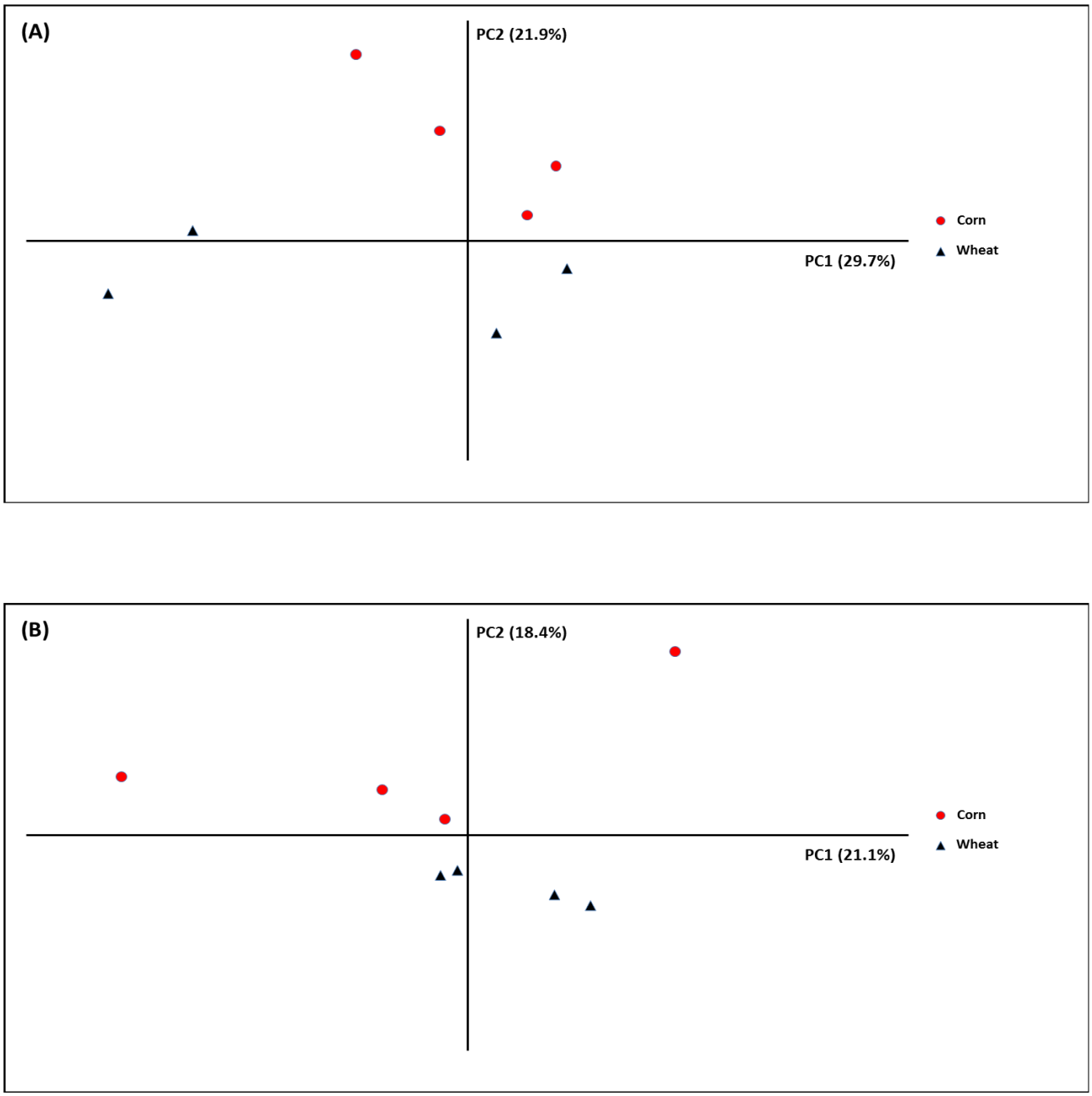

Figure 11.3. PCA plots showing the grouping of chickens based on the structure of their gut microbiome at 14 (A) and 35 (B) days of age.

The PCA analysis revealed difference in the caecal microbiome structure of chickens fed corn- and wheat-based diet, but it did not provide any information on what phylotypes were contributing to such difference. T-test in MeV was therefore used to further identify phylotypes with different abundance in chickens fed different diet. At 14 days of age, eight species-level phylotypes and two genus-level phylotypes were found more abundant in chickens fed wheat-based diet, whereas two species-level phylotypes were more abundant in corn-based diet (Figure 11.4). At 35 days of age, seven species-level and one genus-level phylotypes were more abundant in birds fed wheat-based diet, while two species-level and one genus-level phylotypes were less abundant in wheat-based diet group (Figure 11.5).

Only three species-level phylotypes, namely *Akkermansia muciniphila*, *Clostridium perfringens*, and *Corynebacterium variabile* can be classified to species level. *A. muciniphila* is the type species of the relatively new genus *Akkermansia*, which is proposed a decade ago by Derrien *et al*. (2004). Commonly found in the GI tract of human, this species is known as a mucin degrader and has shown to be inversely associated with body weight in humans and rodents (Derrien *et al*. 2008; Lyra *et al*. 2010; Everard *et al*. 2013).

In the current study we found that, at 14 days of age, *A. muciniphila* was more abundant ($P \leq 0.05$) in chickens fed wheat-based diet. At 35 days of age, however, no statistical difference was found in the abundance of *A. muciniphila* between chickens fed the two different diets. It is worth noting that, at both ages, *A. muciniphila* was present in only one (out of four) bird in the corn-based diet group, while all birds in the wheat-based diet group had this phylotype. Considering that chickens fed with corn-based diet had greater body weight ($P < 0.05$) than those fed with wheat-based diet (460 versus 375 g/bird at 14 days of age, and 2,000 versus 1,1815 g/bird at 35 days of age), it is reasonable to speculate that *A. muciniphila* might have an inverse association with body weight of chickens fed corn- or wheat-based diets. Previous studies on mice suggested that *A. muciniphila* is involved in the control of host gut barrier function and physiological homeostasis during obesity and type 2 diabetes (Everard *et al*. 2013). To explain why in the current study *A. muciniphila* was found more abundant in wheat-fed chicken and to elucidate the potential connection between *A. muciniphila* and chicken body weight, however, will require further studies.

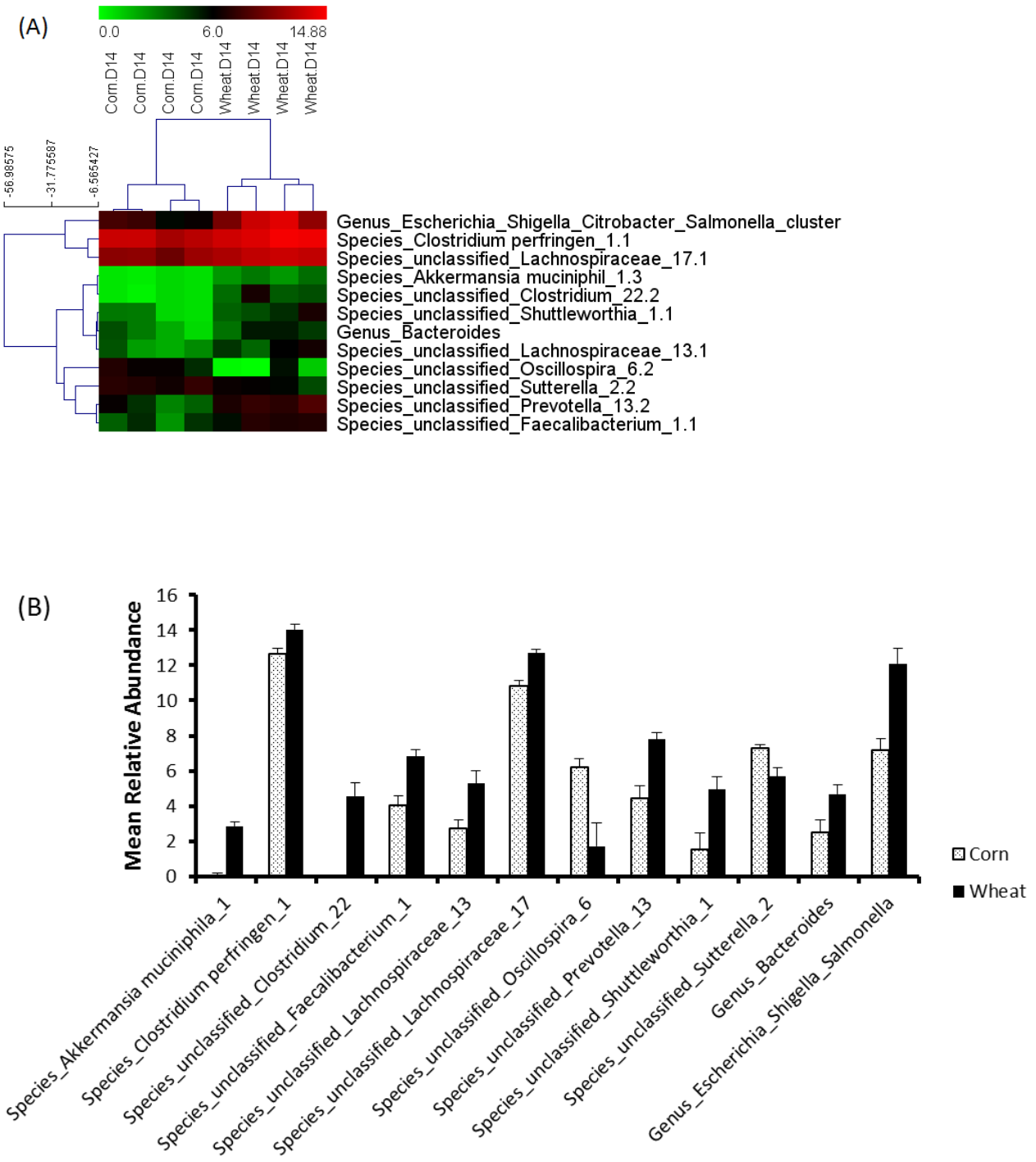

Figure 11.4. Phylotypes with different abundance in 14-day-old chickens. Heat map (A) was generated based on the log2 value of the ratio between the mean fluorescence intensities of each phylotypes and the internal standard and displayed using the standard Green-Black-Red scheme. The mean relative abundance (shown as mean ± SE) of each phylotype for each diet (B) is also represented as the log2 ratio between the mean fluorescence intensities of each phylotypes and the internal standard.

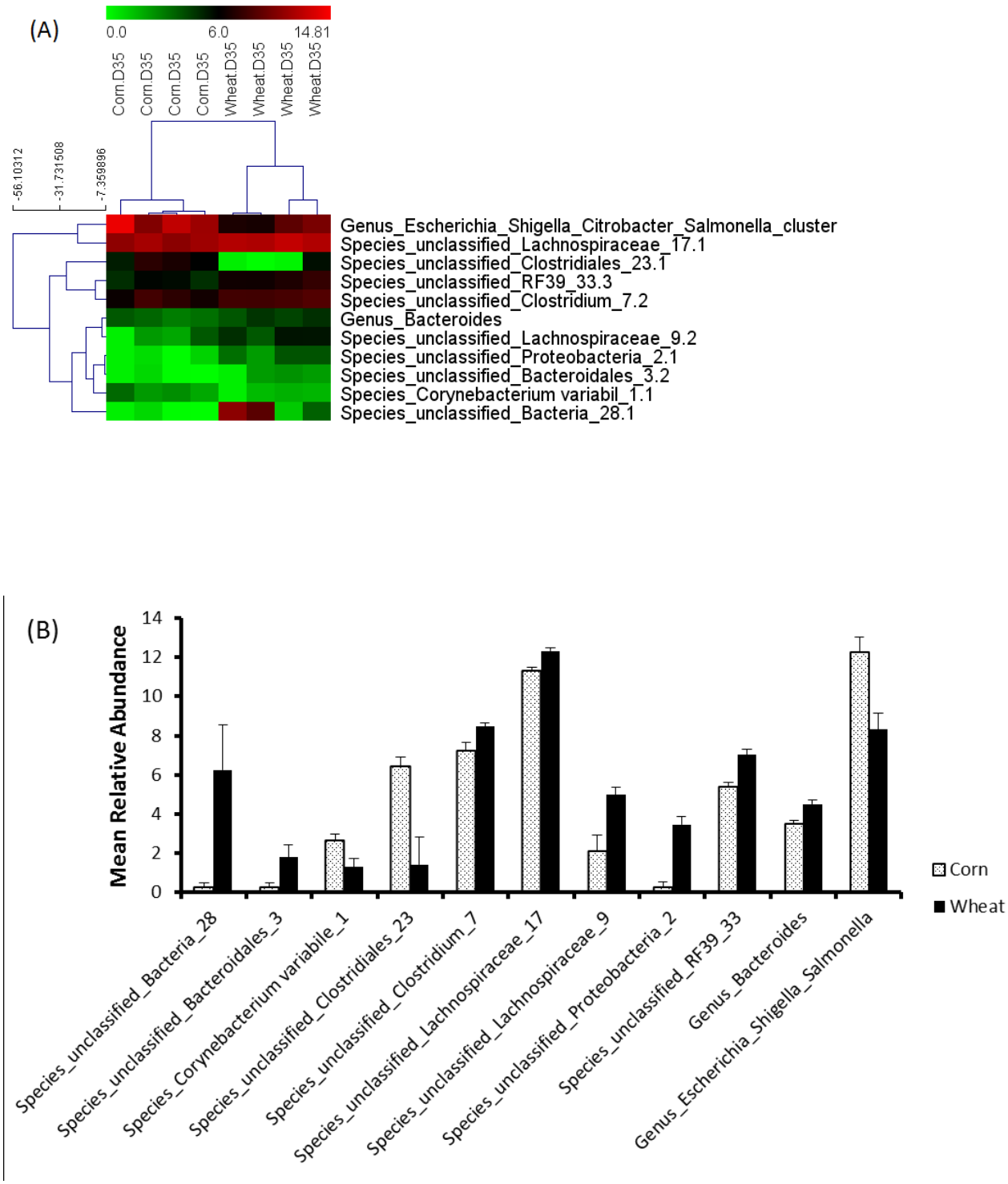

Figure 11.5. Phylotypes with different abundance in 35-day-old chickens. Heat map (A) was generated based on the log2 value of the ratio between the mean fluorescence intensities of each phylotypes and the internal standard and displayed using the standard Green-Black-Red scheme. The mean relative abundance (shown as mean ± SE) of each phylotype for each diet (B) is also represented as the log2 ratio between the mean fluorescence intensities of each phylotypes and the internal standard

C. perfringens is the causative organism of necrotic enteritis (NE), a common and costly enteric disease in young chickens at approximately 4 weeks of age (Van Immerseel *et al*. 2004a; Timbermont *et al*. 2011). This species is ubiquitous in the environment and commonly found in the GI tract of healthy birds (Si *et al*. 2007). In the current study *C. perfringens* was detected in all 16 chickens yet none of them showed symptoms of NE. Indeed, the presence of *C. perfringens* alone does not necessarily lead to the development of NE in chicken. Other predisposing factors are required for the disease to take place. Previous studies indicated that wheat-based diets, which contain high levels of indigestible, water-soluble, non-starch polysaccharides, favour the proliferation of *C. perfringens* and predispose young chickens to NE, whereas diets poor in non-starch polysaccharides, such as corn-based diets, do not (Annett *et al*. 2002; Jia *et al*. 2009). Supporting this view, the current study showed that at 14 days post-hatch, chickens fed with wheat-based diet had more *C. perfringens* in the caeca than birds fed with corn-based diet. However, no difference in the abundance of *C. perfringens* was observed at 35 days of age.

In the current study, *Corynebacterium variabile* was detected in almost all birds but difference in its abundance between corn- and wheat-fed chickens was only evident at 35 days post-hatch. *Corynebacterium* spp. are commonly found in chicken litter (Schefferle 1966), therefore it is not surprising to have them detected in chicken GI tract. However, it remains to be determined why *C. variabile* was more abundant in birds fed with corn-based diet at 35 days of age but not at 14 days of age.

Two genus-level phylotypes, namely *Bacteroides* and *Escherichia*/*Shigella*/*Salmonella* cluster, were found to be associated with the diets. At both ages, *Bacteroides* were more abundant in chickens fed with wheat-based diet. With numerous genes encoding polysaccharide binding proteins and hydrolytic enzymes, *Bacteroides* spp. have a powerful carbohydrates utilisation system, which grants them the ability to utilise a wide range of carbohydrates available in the gut (Xu and Gordon 2003). Comparing to corn-based diet, the higher content of indigestible, water-soluble, non-starch polysaccharides present in wheat-based diet may serve as substrate for the growth of *Bacteroides* spp. allowing them to out-compete other bacteria that cannot utilise these non-starch polysaccharides.

Members of *Escherichia*/*Shigella*/*Salmonella* are common dwellers in chicken GI tract even though at low abundance. While the majorities are commensal bacteria, some of them are pathogenic and can cause diseases in chickens and human. We found that *Escherichia*/*Shigella*/*Salmonella* were more abundant in chickens fed with wheat-based diet at 14 days of age, whereas at 35 days of age, *Escherichia*/*Shigella*/*Salmonella* were found to be more abundant chickens fed with corn-

based diet. The underlying mechanism of such divergence at 14 and 35 days of age, however, remains to be elucidated.

Succession of caecal microbiome

Age is another factor that affects gut microbiome structure. Colonisation with microorganisms in the poultry gut occurs immediately after hatch and is followed by microbial succession from a transient community to a complex and climax microbiome (Lu *et al*. 2003; Brisbin *et al*. 2008). While the establishment of a typical microbiome in the small intestine takes approximately two weeks, a typical caecal microbiome takes up to 30 days to develop (Amit-Romach *et al*. 2004). In the current study, when caecal microbiome of the chickens fed the same diet were compared at the two ages, a clear separation was observed between chickens at 14 and 35 days of age, suggesting that birds at these two ages had distinct caecal microbiome structures (Figure 11.6).

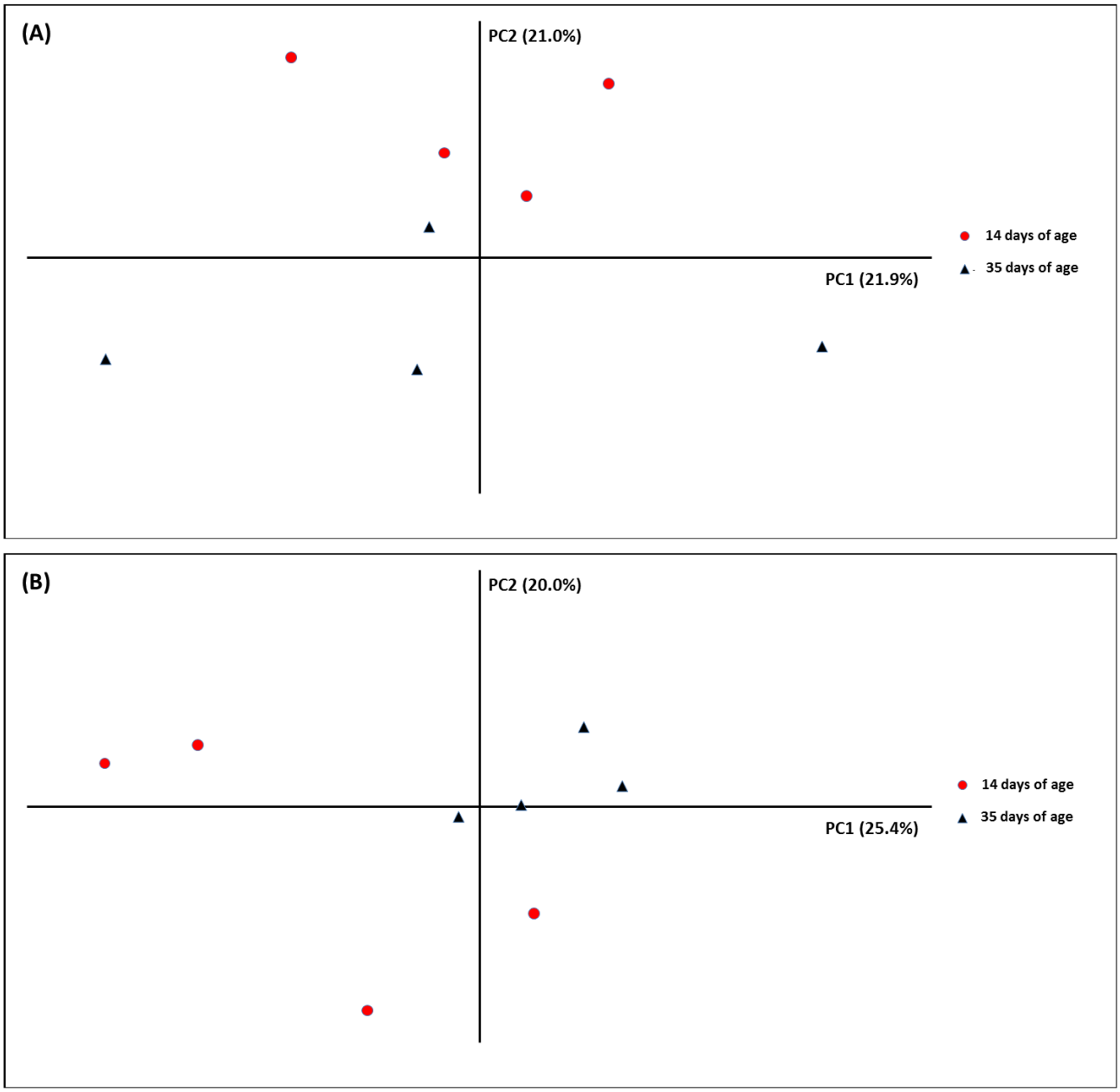

Figure 11.6. PCA plots showing the grouping of chickens fed with corn-based diet (A) and wheat-based diet (B) based on the structure of their gut microbiome.

PCA analysis showed clear separation between chickens fed with the two different diets and also between chickens at two different ages. Twenty phylotypes (either species-level or genus level) were further identified with different abundance in chickens fed different diets. *Akkermansia muciniphila* was found inversely associated with bodyweight, while *Bacteroides* was more abundant in chickens fed the wheat-based diet. *Escherichia, Shigella and Salmonella* were also more abundant in chickens fed the wheat-based diet at 14 days of age. Such a difference in gut microbiome revealed by PITChip3 may help to better understand the interactions between gut microbiome and diet and the risk associated with the two different cereal components, corn and wheat, used commonly in diets in the USA and Australia, respectively.

Conclusions

Based on the previous two versions of PITChip, here we developed the third version (PITChip3) of the poultry specific phylogenetic microarray. Compared to PITChip2, PITChip3 has several improvements/advantages: (1) instead of using only V3 region, probes on PITChip3 were designed from V1-V3 region, which can provide more accurate classification, (2) up to three unique probes were designed for a single target which may alleviate cross-hybridisation and false-positive or false negative issues, (3) no shared or multiple targets probes were used, therefore no ambiguous result will be generated. The utility of PITChip3 was tested using caecal samples from either corn-fed or wheat-fed birds, and it has shown to be a powerful yet easy to use high-throughput analytical tool for poultry gut microbiome study. A few groups (genera and species) of bacteria were found to be differentially associated with corn or wheat-based diets. These phylotypes may be partially responsible for the differences in growth performance and NE resistance seen in chickens fed with corn- and wheat-based diets.

Discussion of Results

Preamble

Improving growth performance in chickens has long been one of the most important goals in poultry research. Despite all the improvements in genetics, nutrition, disease control and husbandry over many decades, performance within and between flocks varies considerably despite intense genetic selection pressure for improved growth rate, feed efficiency and robustness, and this variation causes significant losses to the industry.

The main aims of this sub-projects were (1) customise an existing gut bacterial microarray chip for usage in Australia, (2) advance our understanding of gut microbes and host gene expression patterns underpinning bird performance, (3) identify feeding regimes (including probiotics) that can consistently facilitate the establishment of a healthy gut environment, (4) identify organisms with the potential to become new probiotics, and (5) advance our understanding of microbial changes during necrotic enteritis infection and identify dietary additives to reduce the severity of necrotic enteritis.

Development and validation of PITChip3, a gut bacterial microarray designed to detect microbes present in Australian conditions

The latest version PITChip3 was based on the previous two versions of PITChip, poultry specific phylogenetic microarrays, and included specific DNA probes derived from CRC-supported experiments conducted in Australia. Compared to PITChip2, PITChip3 has several improvements/advantages: (1) instead of using only V3 region, probes on PITChip3 were designed from V1-V3 region, which can provide more accurate classification, (2) up to three unique probes were designed for a single target which may alleviate cross-hybridisation and false-positive or false negative issues, (3) no shared or multiple targets probes were used, therefore no ambiguous result will be generated. The utility of PITChip3 was tested using a small number of samples from the study in Chapter 11, and it has shown to be a powerful yet easy to use high-throughput analytical tool for poultry gut microbiome study. A few groups (genera and species) of bacteria were found to be associated with corn or wheat-based diets. These phylotypes may be partially responsible for the differences in growth performance and necrotic enteritis resistance seen in chickens fed with corn- and wheat-based diets.

PITChip3 can have some advantages over other high-throughput techniques. Firstly, the microarray can provide a uniformed, robust assessment of individual populations of complex microbiomes. Secondly, nucleic acid samples can be directly hybridised with the microarray chip, avoiding inherent PCR bias, which has been shown to compromise the assessment of

microbiomes. Finally, PITChip3 can be custom fabricated or printed in house at relatively low cost (less than AUD$350 per microarray chip), updated quickly when new probes need to be added, and analysis of microarray data is much less resource demanding.

However, during the life of this sub-project, DNA sequencing technologies have advanced at such a rapid rate that it now possible to analyse hundreds of 16S mRNA sequences from gut bacteria for less than AUD$5 per sample in less than one day. Furthermore, the current technologies identify what is actually present in the samples, which make these technologies the preferred choice of most if not all researchers conducting the latest studies on gut microbial ecology.

Host genes and gut microbes that are consistently linked to high and low-performing birds

Gene expression

We concluded that the statistically significant gene expression differences that are observed in the duodenum and other tissues are unlikely to be directly linked to differences in performance and hence will not provide useful tools to monitor the effects of dietary manipulation for productivity gain or to provide potential gene markers for marker assisted breeding for improved performance. We speculate that the differences in gene expression seen in each trial may be correlated to factors other than FCR performance. For example, the expression differences might be established by slight variations in environmental conditions, differences in gut microflora, or other minor perturbations in the experimental setup or source flock of chickens. After review of the project in March 2012, it was agreed that there would be no further work on gene expression, and that all future efforts would focus on gut microbial profiles.

Gut microbes

In all three trials described in Chapter 1, growth rate and feed conversion of the chickens were comparable to the breeder's expectations (Cobb-Vantress 2013), however, within each trial there were differences in the performance parameters of individual birds and so relatively high and low performance birds could be identified. The current analysis performed here was aimed at determining whether in the face of this sort of microbiota variation there were common changes in the phylotype profiles that characterised the enhanced performance birds in the different trials. In each of the three trials, in which birds were fed the same commercial broiler diet, there where bacterial phylotypes that were differentially abundant between the high and low performance birds. However, it was found that some phylotypes associated with high performance in one trial were completely absent in another trial. With the variation in background microbiota between trials there were no OTUs that were consistently associated with good or bad performance across all three trials. We concluded that there are numerous consortia of gut microbes that can support high performance in meat chickens.

Reliability and reproducibility of feed additives and specific feeding regimes that influence the gut microbiota

A series of three similar experiments were performed (Chapter 2). In each experiment two diets were used. These were for Trial 1 (1) Control and (2) Control with zinc bacitracin (50 ppm active ingredient, for Trial 2 (1) Control and (2) Control with avilamycin (15 ppm active ingredient), and for Trial 3 were (1) wheat-based diet and (2) sorghum-based diet with the same nutrient specifications as the wheat-based diet.

The microbial profiles told a very similar story to that described in Chapter 1. That is, the birds in each trial had distinctly different microbiota and there was a considerable variation from bird to bird within each trial. There were operational taxonomic units (OTUs) that were in differential abundance in the high and low performance birds fed each diet but because of the between trial variation in overall microbiota composition none of these differential OTUs were seen in all diet groups across three experiments conducted in a similar manner. The results from three similar experiments further demonstrated that there is extensive inter- and intra-trial variation in the microbiota composition of birds, as was the case described in Chapter 1. This required a re-evaluation of the best way to proceed with future experiments in this project.

PITChip3 analysis of caecal contents showed clear separation between chickens fed either wheat-based or corn-based diets and also between chickens at two different ages (Chapter 11). Twenty phylotypes (either species-level or genus level) were further identified with different abundance in chickens fed different diets. *Akkermansia muciniphila* was found inversely associated with bodyweight, while *Bacteroides* was more abundant in chickens fed the wheat-based diet. *Escherichia, Shigella and Salmonella* were also more abundant in chickens fed the wheat-based diet at 14 days of age. Such a difference in gut microbiome revealed by PITChip3 may help to better understand the interactions between gut microbiome and diet and the risk associated with the two different cereal components, corn and wheat, used commonly in diets in the USA and Australia, respectively

Identification of culturable microbes with potential to be developed and commercialised as new probiotics, identified under Australian conditions, targeted at improving performance

Inoculation of newly hatched chickens by gross microbial transfer from healthy chickens

Our experiments described in Chapters 1 and 2 demonstrated that there is extensive inter- and intra-flock variation in the gut microbiota composition of birds. In other words, the supposedly sterile guts of the hatchlings initially have been seeded by different bacteria from the environment.
In these two experiments (Chapter 3), we took a more direct approach by coating egg shells during incubation with caecal contents from healthy chickens in experiments described in Chapters 1

and 2. We attempted to simulate how the hen inoculates her eggs during incubation and her chicks post-hatch in an attempt to achieve consistent gut microbiota within and between batches of chickens.

In general, attempts to influence colonisation of the gut microbiota by presentation of caecal organisms from healthy chickens to hatching chickens were unsuccessful. A possible explanation for failure is that bacteria in frozen and thawed caecal contents were not viable. We concluded that more direct methods of inoculation of newly hatched chickens required further study, and that inoculation should utilise highly characterised species of bacteria known to colonise and proliferate in newly hatched chickens.

Isolation, culture and characterisation of potential probiotic organisms

We isolated and identified many hundreds of different gut bacterial species from healthy chickens (from experiments described in Chapters 1 and 2), and have shown that these can colonise and proliferate in the gut of naive chickens vaccinated orally on day of hatch (Chapter 5).

Influence of potential probiotic organisms on growth rate and feed efficiency of meat chickens

Essential characteristics of a probiotic product are, firstly, that it can colonise and proliferate in the gut of the chicken under a wide set of conditions encountered on commercial chicken farms, then have beneficial influence on productivity, health and welfare of chickens, and, lastly achieve consistent results from one flock to the next.

Published research in this area features similar combinations of bacterial species rather than pure cultures of a single species, possibly because combinations are better able to colonise and proliferate whereas monocultures tend to become competitively excluded by mixed populations of native species derived randomly from the chickens' environment. For this experiment we examined two live bacterial cultures each comprised of five different *Lactobacillus* species and one *Enterococcus* species, but different strains of each species in each culture. These organisms were originally isolated from previous experiments described in Chapters 1 and 2, then cultured and identified by sequencing (Chapter 5).

The selected combinations of these organisms did not influence productivity, health or welfare of chickens during a 41-day experimental period as indicated by live weight gain, feed intake, feed conversion and livability. The two consortia of bacterial cultures given as a single dose to newly hatched chickens colonised and proliferated in the caeca over the first two weeks, but then markedly reduced in abundance, despite these introduced bacterial species successfully colonising in previous chicken experiments (Chapter 5). Possible reasons for failure to thrive include mutual competitive

exclusion amongst introduced bacterial strains, or by competition from naturally-occurring organisms also able to support high productivity, as was subsequently observed in the experimental flock. The potential of these probiotic organisms to enhance growth rate and feed efficiency of meat chickens under commercial conditions remains to be demonstrated.

Key predisposing factors and microbial changes that occur during necrotic enteritis (NE) infection

The present study (Chapter 7) elucidated the role of predisposing factors, i.e., *Eimeria* infection and fishmeal, together with *C. perfringens* in the introduction of NE disease in an experimental challenge model. The results indicated that fishmeal was not a prerequisite for the successful induction of NE into meat chickens fed an industry standard diet. However, it appears that *Eimeria* infection is important for this challenge model to produce clinical NE with associated gut damage and morbidity in meat chickens.

The factors predisposing birds to NE were studied further (Chapter 8) to determine the effects of fishmeal and *Eimeria*, and the causative agent *C. perfringens* on the dynamics of volatile fatty acids (VFA) and lactic acid, and intestinal histomorphometry. These changes may be important for the onset of NE but may be only the synergetic responses to the micro environmental stress. Based on the intestinal VFA responses to the treatments, it was confirmed that *Eimeria* is more important than fishmeal in predisposing birds to NE, thus the application of *Eimeria* in NE challenge model provides more consistency in inducing the disease under experimental conditions.

A further experiment (Chapter 9) examined the effects of various feed additives to reduce severity of lesion scores when birds were challenged in the NE model described in Chapters 7 and 8. Birds fed products A (mixture of xylanase 40,000 U/g and amylase at 350 g/t), C (mixture of different organic acids at 1500, 1000 and 500 g/t in starter, grower and finisher respectively) and D (protease (75,000 U/g, 200 g/t) demonstrated lower mean lesion scores in the jejunum and overall small intestine compared to that of the group with NE challenge and without zinc bacitracin treatment. Product B (subtilisin 12,000 U/g supplemented with xylanase and amylase 300 g/t) was ineffective in reducing lesion score. Although the feed additives did not improve performance overall, some of them reduced the impact of necrotic enteritis when birds were challenged.

Implications

- Efficient and cost effective methods of analysing the phylogenetic composition of gut microbiota have been established and widely used on experimental broiler flocks. During the course of the project the technology used for such analysis has rapidly advanced and the latest, most advanced methods have been applied, allowing the simultaneous analysis of hundreds of samples. This has facilitated the rapid advance in our understanding of the structure, complexity and variability in gut microbiota; providing a technological and knowledge base that can now be deployed to address gut health and productivity issues within the industry.
- The poultry specific phylogenetic microarray, PITChip3, includes specific DNA probes derived from CRC-supported experiments conducted in Australia. Probes on PITChip3 can provide more accurate classification of the gut microbiota than earlier versions of PITChip, which may alleviate cross-hybridisation and false-positive or false negative issues.
- PITChip3 can be custom fabricated or printed in house at relatively low cost (less than AUD$350 per microarray chip), updated quickly when new probes need to be added, and analysis of microarray data is much less resource demanding than 16S rRNA gene-based next generation sequence (NGS) analysis.
- However, during the life of this sub-project, NGS technologies have advanced at such a rapid rate that it now possible to analyse hundreds of gut samples, using 16S mRNA sequences, for approximately AUD$8 per sample. Furthermore, the current technologies identify all the significant bacterial populations present in the samples, rather than relying on an array with probes only from previously identified bacteria, which make these technologies the preferred choice of most, if not all, researchers conducting the latest studies on gut microbial ecology.
- Gene expression differences observed in the duodenum and other tissues are unlikely to be directly linked to differences in performance and hence will not provide useful tools to monitor the effects of dietary manipulation for productivity gain or to provide potential genes for marker assisted breeding for improved performance.
- Numerous different consortia of gut microbes, arising naturally from the local environment, can support high performance in meat chickens.
- However, extensive inter- and intra-flock variation in the gut microbiota composition of birds indicate haphazard colonisation. It is hypothesised that current hygiene practices on breeder farms and hatcheries, result in the lack of uniformity or consistency in establishment of a healthy gut microbiota.
- Conversely, the health and hygiene conditions that can lead to dysbiosis remain undetermined.

- The faecal microbiota were qualitatively similar to caecal microbiota but quantitatively different. Non-invasive collection of faecal samples can used to detect some shifts and responses of caecal microbiota. However, choice of sampling site remains critical in experimental design as faecal microbiota do not provide a complete indication of caecal community structure.
- The use of *Lactobacillus* isolates as probiotics must be approached with some caution. Comparison of the gut microbiota composition of high and low performance birds indicates that although some *Lactobacillus* species are associated with superior performance, other Lactobacilli, even of the same predicted species, are associated with poor performance.
- Other probiotic candidates, for example, some *Clostridia* members involved in degradation of plant material, cellulose utilisation and short chain fatty acid production, are associated with good growth performance.
- On the other hand, there is mounting evidence from PITChip and NGS analyses that certain specific organisms are associated with poor feed conversion but without causing overt disease or health issues. It will be useful for industry to know what the identity of these undesirable organisms are, and to be provided with practical advice and ways to exclude these organisms from the developing gut microbiota in newly-hatched chickens.
- Many hundreds of different gut bacterial species isolated from healthy chickens were cultured successfully. These organisms were identified by DNA sequencing then shown to colonise and proliferate in the gut of naive chickens vaccinated orally on day of hatch. An initial small scale trial indicated productivity improvements associated with some bacterial consortia.
- However, our first attempt to demonstrate benefits in terms of improved performance and flock homogeneity by oral inoculation of potential probiotics failed. The two consortia of bacterial cultures given as a single dose to newly hatched chickens colonised and proliferated in the caeca over the first two weeks, but then markedly reduced in abundance, possibly because of mutual competitive exclusion, or by competition from naturally-occurring organisms also able to support high productivity. The potential of these probiotic organisms to enhance growth rate and feed efficiency of meat chickens under commercial conditions remains to be demonstrated.
- Because of the differences between the initial small scale trials (RMIT and CQU) and the larger trial at SARDI it is hypothesised that potentially probiotic bacteria cannot improve the productivity of birds that are already operating at peak performance. It is probable that probiotics will only be effective in birds that are growing sub-optimally – they may alleviate gut dysbioisis, immune dysfunction, or improve digestion and SCFA production but cannot advance the gut health of birds that are already very healthy.
- The NE challenge model to test alternatives to in-feed antibiotics was simplified to usage of *Eimeria* as a predisposing factor without need to feed a high level of fishmeal prior to infection with *C. perfringens* in chickens fed an industry standard diet.

- Some feed additives based on commercial enzyme products and organic acids had ameliorating effects on gut damage in birds challenged with *Clostridium perfringens*.

Recommendations

- The analysis of the gut microbiota of carefully controlled and monitored experimental broiler flocks has clearly shown that there are correlations between the abundance of certain bacteria and growth performance. This work has demonstrated that microbiota with different compositions can equally support high productivity birds. The analysis of what constitutes a “good” microbiota should now be extended to a large scale survey of commercial growers throughout the industry. Such analysis will build a more complete picture of the underlying trends in microbiota composition and its relationship to performance, allowing the targeting of methods for growers to achieve optimal gut health through microbiota manipulation via nutritional management or direct feed microbial additions.
- Breeder flocks are regularly tested for presence of *Salmonella* species. Development and availability of cost-effective methods based on DNA sequencing allows for regular surveillance of much more detailed gut microbial ecology of individual breeder flocks, and related down-stream effects on progeny health and performance.
- This approach could be extended to surveillance of gut microbial ecology on meat chickens farms achieving either superior or inferior performance on a regular basis with a view to establishing bench marks in gut microbial health. Then informed decisions could be made on which under-performing farms required upgrading of health and hygiene practices.
- Comparisons are needed on gut microbial composition of birds in conventional and free range farming systems. The comparisons should be made between farms sourcing chickens from the same hatchery and fed the same diets provided from the same feed mill in order assess the relative degrees of uniformity on a field scale.
- The microbiota analysis methods can be deployed to determine the effectiveness of many of the phytogenic, prebiotics and probiotic products that are offered to the industry. It would be possible to establish whether the products change the gut microbiota in a favourable manner.
- The pure cultures of gut bacterial species isolated from healthy chickens have potential for development into commercial probiotic products, not only for application in Australia, but also as exportable commodities. We suggest that the Australian Poultry CRC and its successors holding the intellectual property seek expressions of interests from commercial partners to plan and fund the required research to identify viable combinations of the many bacterial species able to consistently colonise the chicken gut soon after hatch, and have beneficial effects on subsequent flock performance and uniformity.
- *In ovo* application of these pure cultures should be explored, along with administration of probiotics via other routes and at other times during the life of the flock. For example, encapsulated probiotics could be provided via drinking water to offset gut dysbiosis and performance losses arising from clinical and subclinical disease challenges, and other stresses

incurred on flocks such as through mechanical breakdowns, and by temporary feed withdrawal during partial harvesting of the flock.

- The beneficial effects of some feed additives based on commercial feed enzyme products and organic acids to ameliorate the detrimental effects of NE warrants further investigation under different growth conditions. Understanding of the mechanisms underlying the improvement will be beneficial to the application of these additives as alternatives to in-feed antibiotics in the poultry industry.

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