University of Reading

Exploring the effects of management strategies on the gut microbiome and metabolome of growing broiler chickens: an integrated metagenomic and metabolomic approach

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Abstract

Advances in genetic selection and intensification of management systems has led to the production of highly efficient broiler chickens. Attention is being turned to the role of gut microbes to further growth efficiency and improve animal health, particularly with the rise of antimicrobial resistance and the need to consider animal welfare and food safety. The microbiome is sensitive to management strategies, which may affect the growth and metabolism of the bird. Here, four studies are presented which explore the effects of antibiotic treatment with Linco-spectin and the subsequent use of Aviguard®, a mixed probiotic, diet in terms of protein source and the use of wheat or maize as a cereal base, and the supplementation of bedding with excreta from mature birds on the caecal microbiome and hepatic metabolome of broilers through population profiling metabolomic approaches. A final analysis of all datasets highlights consistent trends that appeared irrespective of treatment.

Alpha diversity increased between days 4 and 9 (P<0.001) and 15 and 22 (P=0.001) and was affected by all treatments but differences dissipated with age. Beta diversity also changed with age, but again differences were reduced by around two weeks of age, suggesting that diversity naturally reaches a plateau, irrespective of perturbations in early life. Age-related shifts in bacterial populations were observed in all studies with a reciprocal relationship between phyla *Firmicutes* and *Tenericutes* (P<0.001). The microbiome was consistently affected by management strategy, with evidence that different taxa are affected by diet, bedding and antibiotic/probiotic treatment. Energy-related metabolites were affected by treatment, but birds appeared to grow equally as well suggesting isofunctionality of the hepatic metabolome. Concentrations of short chain fatty acids (SCFA) increased between days 4 and 15 (P<0.001) and were affected by diet alone. Concentrations of all SCFA exhibited both positive and negative relationships with bacterial taxa.

Despite the plethora of treatment-associated differences in the microbiome, no single group of birds performed worse than another with a lack of a significant relationships

between differences in the caecal bacterial populations and differences in feed conversion ratio (r=0.386; *P*=0.270). It is hypothesised that the ability of a bird to thrive and grow is independent of the profile of its microbiome, so long as key metabolic activities are performed. This thesis is an overview that will provide a base to be utilised in exploring more specific relationships in depth in future work with methods and understanding of metagenomic-metabolomic relationships improved by the findings herein.

Declaration

I confirm that this is my own work and that the use of all material from other sources has been properly and fully acknowledged.

Alexandra A. Tonks

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List of abbreviations

AB	Antibiotic
AB+PRO	Antibiotic plus probiotic
ANOVA	Analysis of variance
APEC	Avian pathogenic *Escherichia coli*
B_0	Applied magnetic field
BCAA	Branched chain amino acid
BCVFA	Branched chain volatile fatty acid
CFU	Colony forming units
CON	Control
CP	Crude protein
D	Simpson's diversity index
DEFRA	Department for Environment, Food and Rural Affairs
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EMEA	European Agency for the Evaluation of Medicinal Products
EU	European Union
F:B	*Firmicutes*:*Bacteroidetes* ratio
FCR	Feed conversion ratio
FM	Fishmeal
GALT	Gut-associated lymphoid tissue
GHG	Greenhouse gas
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1
H'	Shannon's index
HMDB	Human Metabolic Database
HPLC	High performance liquid chromatography
Hz	Hertz
ICP-OES	Inductively coupled plasma optical emission spectroscopy
J	Coupling constant
LAB	Lactic acid bacteria
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis Effect Size
LS	Linco-spectin
LW	Liveweight
ME	Metabolisable energy
MHz	Megahertz

NAD^+/NADH	Nicotinamide adenine dinucleotide
NGS	Next generation sequencing
NIR	Near-infrared spectroscopy
NMR	Nuclear magnetic resonance
NSP	Non-starch polysaccharides
OECD-FAO	Organisation of Economic Cooperation and Development – Food and Agriculture Organisation of the United Nations
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
ppm	Parts per million
PYY	Peptide YY
QIIME	Quantitative Insights Into Microbial Ecology
RDP	Ribosomal Database Project
RF	Radiofrequency
rRNA	Ribosomal ribonucleic acid
SAA	Synthetic amino acid
SCFA	Short chain fatty acid
SD	Standard deviation
SEM	Standard error of the mean
TCA	Tricarboxylic acid
TMA	Trimethylamine
TMAO	Trimethylamine-*N*-oxide
TSP	Trimethylsilylpropanoic acid

1. Introduction

1.1. Broiler production

The global poultry industry is huge. Current figures from the OECD-FAO Agricultural Outlook (2018) report that, in 2017, the world produced over 118 million tonnes of poultry: for context, this mass is equal to roughly 16,239 Eiffel towers, or 134 Golden Gate bridges. These figures are predicted to rise to 131.5 million tonnes by 2026 in an attempt to feed the growing population of the planet (**Figure 1.1**).

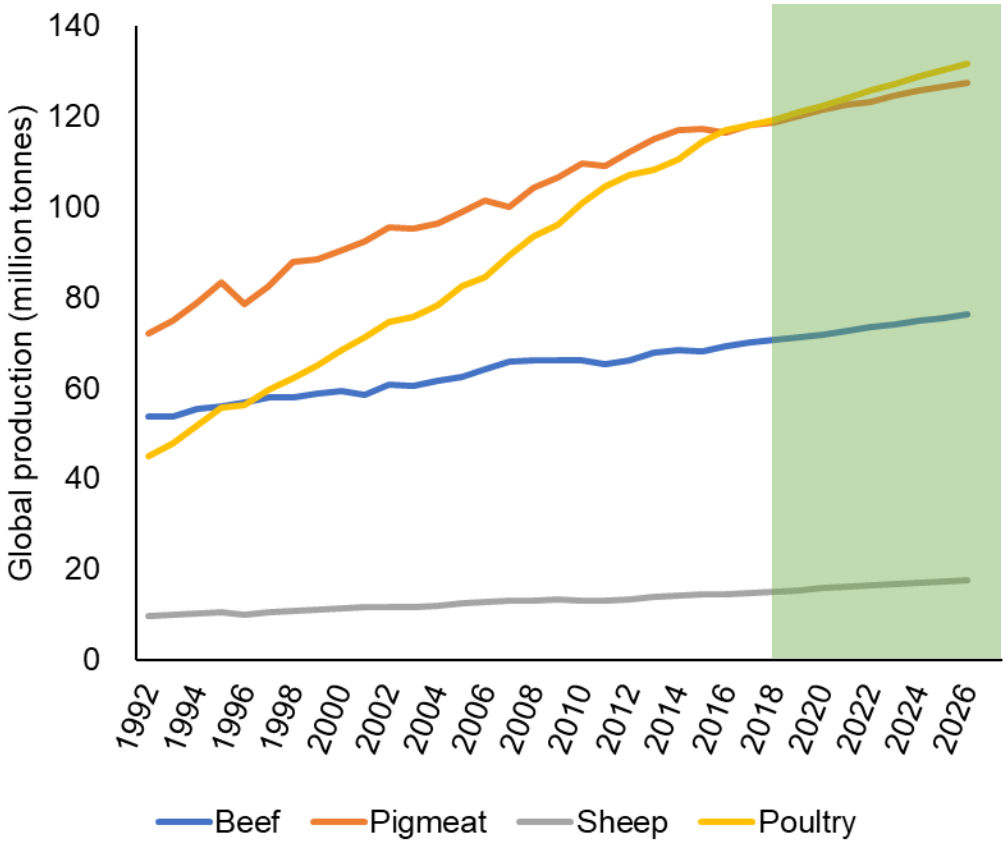


Figure 1.1 *Global meat production (million tonnes). Predicted figures for 2018-2026 are highlighted in green (OECD-FAO, 2018).*

For years pork was the most highly produced meat commodity in the world but was overtaken by poultry in 2016 when almost 117 million tonnes were produced globally. With the global population, and therefore demand for food, predicted to rise, the sustainability of livestock production is also of paramount importance: the water footprint and greenhouse gas (GHG) emissions of poultry production is much lower

than that of beef and pork (Mekonnen and Hoekstra, 2012; FAO, 2017), and is therefore considered to be a more sustainable industry (**Figure 1.2**).

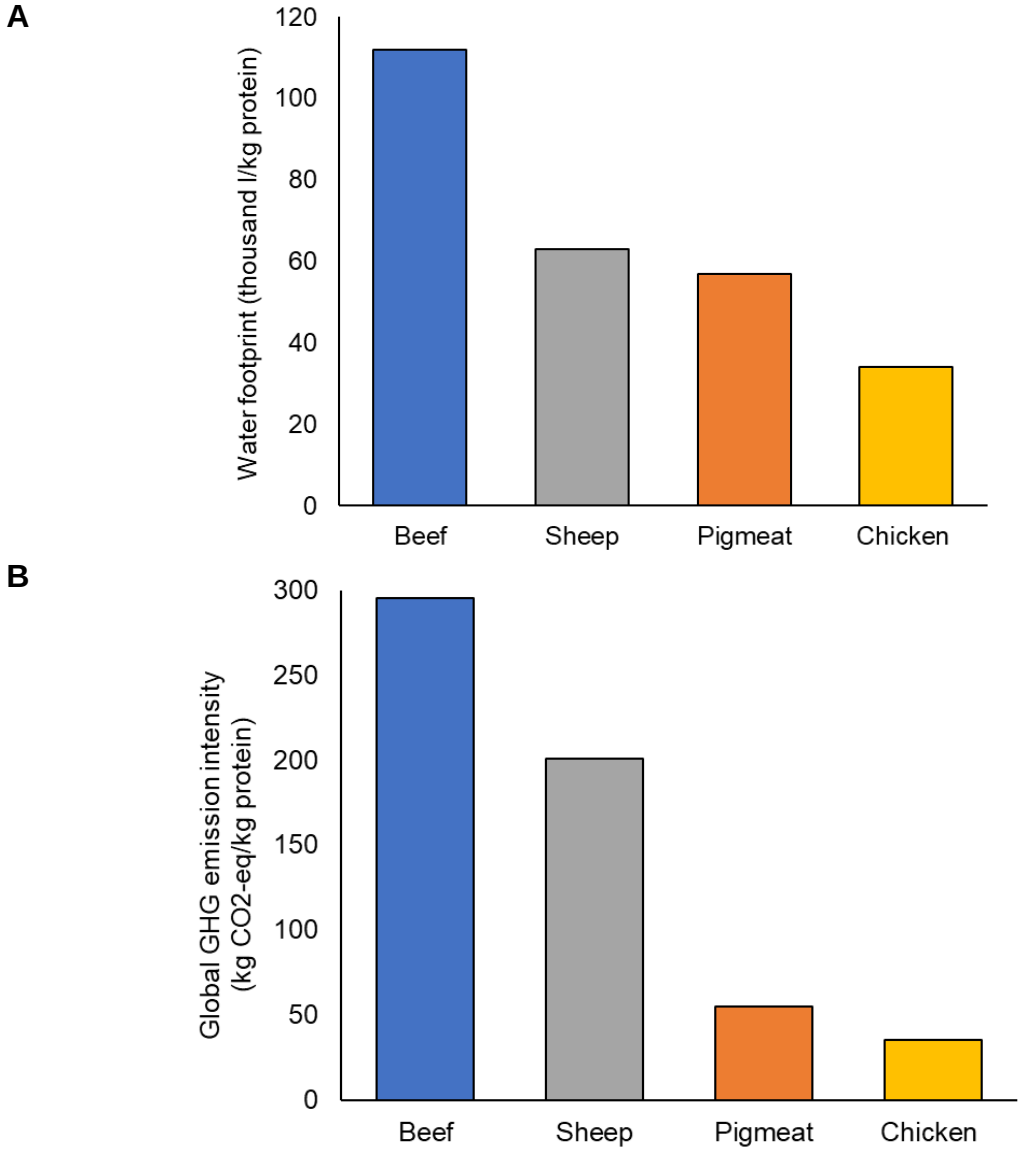


Figure 1.2 *Bar charts illustrating differences in the water footprints (**A**) and greenhouse gas emissions (**B**) per kg of protein from different meat sources (Mekonnen and Hoekstra, 2012; FAO, 2017).*

As well as being the most 'environmentally friendly' source of animal protein, production of poultry, in particular broiler chickens (*Gallus gallus domesticus*), is one of the most efficient industries in terms of feed conversion ratio (FCR). FCR is a measure of the mass of feed required to increase the body weight of an

animal by 1 kg and ranges from around 5.5 for beef animals (DEFRA, 2016) down to around 1.4 for broilers (Aviagen, 2017) in UK systems.

Production efficiency has significantly improved through selective breeding of highly productive birds and intensification of production systems, with evidence of a 500% increase in weight gain and 50% reduction in FCR over a period of around 50 years (Schmidt *et al.,* 2009; Zuidhof *et al.,* 2014). The photograph in **Figure 1.3** illustrates commercial broilers produced in the 1950s (left) and 2005 (right): each is the same age and were fed the same diet, but modern chicken (right) weighs over 4.5 times that of the traditional chicken (left).

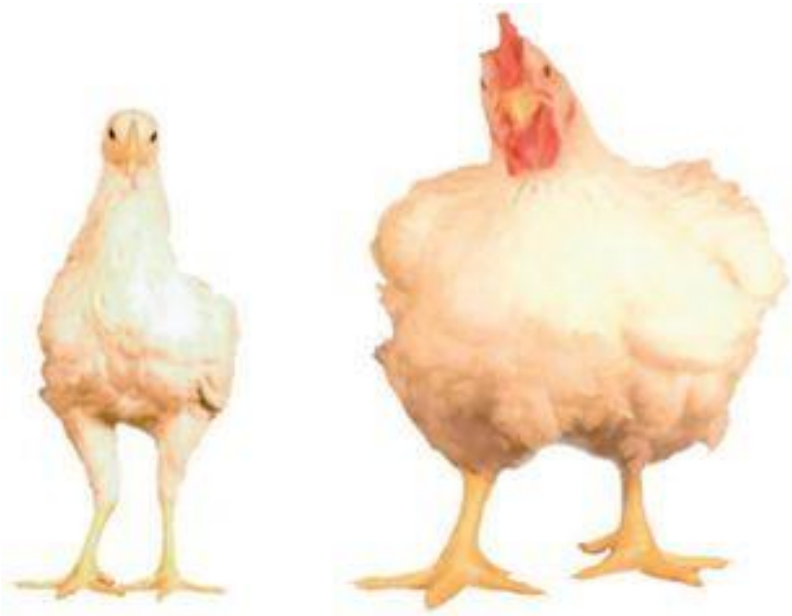


Figure 1.3 *Photograph showing traditional (circa 1950, left) and modern (circa 2005, right) broiler chickens: each is the same age and was fed the same diet (Zuidhof* et al., *2014).*

By day 28 of growth, the 1950s strain consistently weighed just 21% of that of the more modern strain. Given that it takes around 35 days to reach a target weight of s2.2 kg (Aviagen, 2017), it is predicted that it would have taken the 1950 strain over 100 days to reach a similar weight. In reality, the weight gain of a modern broiler begins

to plateau at around 70 days of age (Aviagen, 2017), so it unlikely that the 1950s strain would ever have reached the current target weight of around 2.2 kg.

An estimated 85 to 90% of the phenotypic improvement in production efficiency is accounted for by advances in genetic selection (Havenstein *et al.,* 2003), and the majority of intensively produced broilers are now of a very similar genetic makeup. Despite this, differences in the husbandry and management of birds have significant effects on bird performance (Mesa *et al.,* 2017). Of particular note is diet, since differences in the energy content of feed has been shown to significantly affect FCR (Abdollahi *et al.,* 2018). Birds are fed a cereal-based diet, such as wheat or maize, the nutritive values of which are a source of variation in efficient broiler production (Knudsen, 2014; Cardoso *et al.,* 2017).

It is hypothesised that the artificial selection of broilers for improved energy efficiency is reaching its limit (Tallentire *et al.,* 2018) and attention has recently turned to the role of gut bacteria in the maintenance of health and productivity. There is a plethora of evidence that the host forms symbiotic relationships with its gut microbiome, with the composition of said microbiome also affecting metabolic pathways through differential utilisation of feed components and alteration of gene expression (Palamidi and Mountzouris*,* 2018). It is anticipated that this will ultimately exert beneficial outcomes for both broilers and, indirectly, producers. Despite this evidence, the composition and dynamics of such a complex ecosystem, and how it responds to environmental stimuli are unclear. It is thought that, if said relationships could be characterised, that their impacts upon growth, metabolism and health could be explored and potentially exploited for the benefit of both the bird and the producer. To begin to explore the possibility of bacterial relationships within the gut, it is imperative to understand the physiology of the digestive system, how bacteria interact with the host, and what data concerning commonly identified bacterial species currently exist.

1.2. Digestive physiology of the chicken

A brief overview of the physiology of the broiler chicken is given here to provide context for later discussions. The anatomy of poultry provides an interesting environment for bacteria with several key differences to mammalian systems that are, perhaps, more widely understood. This section aims to highlight key points which need to be understood to explain the methodology and research aims. The labelled photograph in **Figure 1.4** illustrates the organs of the avian digestive tract – these will be now discussed in further detail.

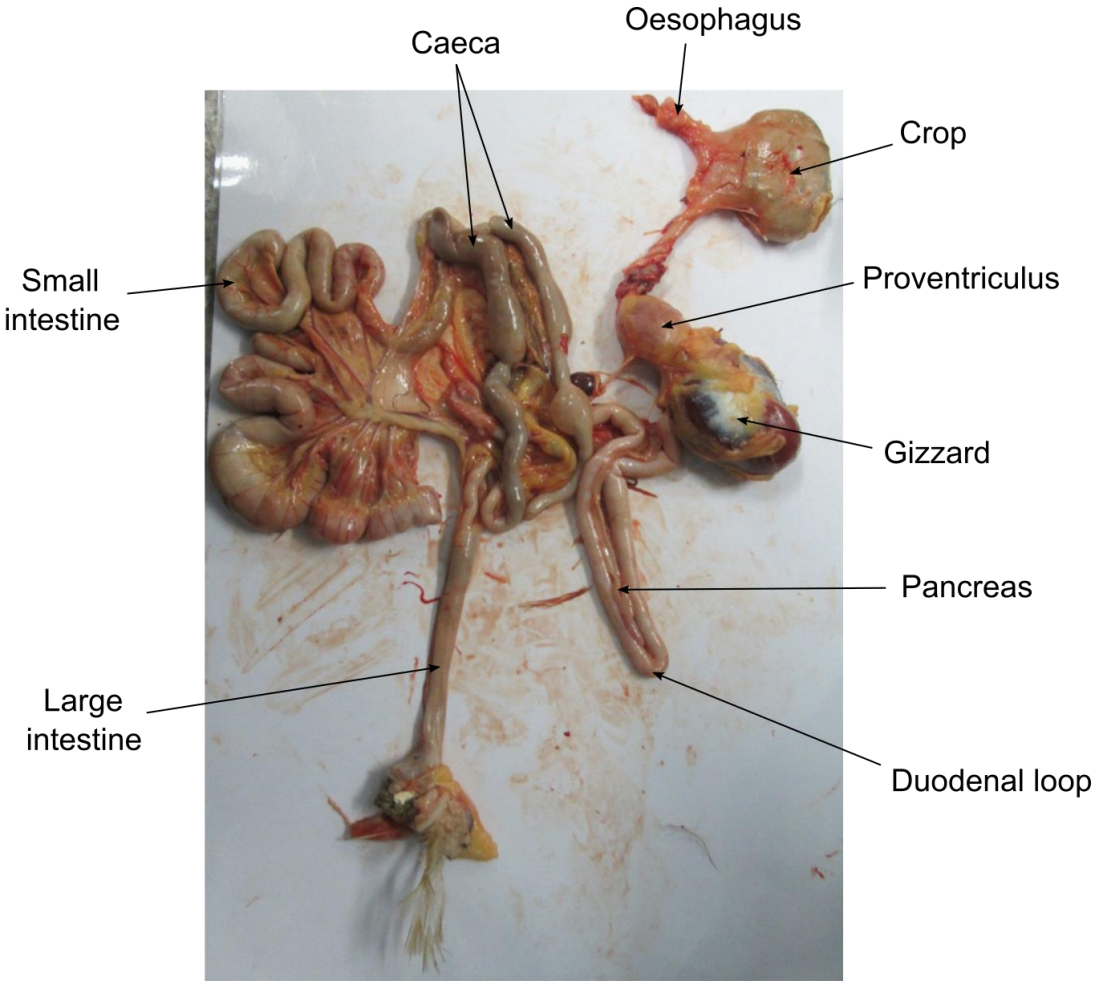


Figure 1.4 *Photograph illustrating the key digestive organs of an adult broiler chicken following dissection. Bacterial populations of the caeca, labelled at the top of the image, are the focus of this thesis. (Own image).*

1.2.1. Oesophagus and crop

The avian digestive system begins with the oesophagus, a long tube that connects the mouth to the remainder of the tract. The passage of feed down the oesophagus into the crop, a large pocket that primarily acts as a storage organ and allows feed to be held before the onset of digestion. It is thought that this has evolved from birds of prey with a requirement to move into open areas to hunt for their food but retreat to safety before digestion, acting as an evolutionary advantage.

1.2.2. Proventriculus

The crop is connected to the proventriculus, a penta-layered organ of highly vascularised tissues and glandular mucous membranes. Feed is moved from the crop to the proventriculus at intervals of 1 to 1.5 minutes with frequency decreasing after the entry of feed (Boorman, 1975). This controls the rate of passage of feed into the proximal digestive tract and acts as an appetite stimulant. Known as the 'true stomach' as a result of the secretion of hydrochloric acid from ducts at the surface of said glandular membranes, the proventriculus also produces digestive enzyme precursors such as pepsinogen (Boorman, 1975). Hydrochloric acid is required to dissolve minerals, including Ca^{2+} salts and phosphates, and to denature tertiary protein structures before proteolysis. Acidic conditions are also required for the conversion of pepsinogen to pepsin. The very low pH of digesta also acts as a physical barrier to pathogens, though it also creates the need for the thick, vascularised walls to prevent autodigestion.

1.2.3. Gizzard

Since chickens do not have teeth, the feed bolus requires mechanical digestion by another means. The gizzard is a muscular organ, around 2.5 cm thick, roughly biconvex, and contains insoluble particles such as small stones and grit against which the feed is ground (Cargill, 2013). The grinding power is supplied by two large muscles

that act as an analogous mechanism for the bird's teeth; the gizzard evolved this way as a way of reducing weight for flight. By breaking up the feed bolus, the surface area exposed to chemical digestion is increased, allowing digestive enzymes to act more efficiently. To prevent autolysis, membranous glands produce a keratinised liquid that hardens within the lining of the gizzard to replace tissue worn away by the grinding action of the organ (Boorman, 1975).

1.2.4. Small intestine

The small intestine is tripartite and comprises the duodenum (easily distinguished by a downwards loop), jejunum and ileum. As a result of the acidic pH of digesta arriving from the gizzard, the walls at the proximal end of the small intestine are thick with a protective barrier of mucous. The pancreas, which is located within the duodenal loop further protects the intestinal wall by secreting basic bicarbonate ions to neutralise digesta arriving from the proventriculus. The small intestine continues to the jejunum and ileum, though a distinctive division between these two compartments is somewhat lacking. It is generally accepted that the jejunum ends at Meckel's diverticulum, a small vestigial notch that originated from the yolk sac that fed the chick during incubation and early life.

A large proportion of digestion and absorption of nutrients takes place in the small intestine: proteins to amino acids; lipids to fatty acids and glycerol; complex carbohydrates from polysaccharides to smaller units and monosaccharides such as glucose. To aid digestion, the walls are highly muscular with a vascular system which maximises the efficiency of nutrient absorption into the blood and are covered in villi, small finger like protrusions, which increase diffusion surface area (Bell, 1971). Surrounding the folds of the small intestine is the mesentery – a thin double membrane that aids in the suspension of the jejunum and ileum from the abdominal wall. Being highly vascularised with extremely thin walls, the mesentery also brings a large volume

of blood into close proximity of the small intestine, further promoting nutrient absorption.

The digestion of some nutrients requires the input of additional substances. Lipids and fat-soluble vitamins (A, D, E and K) require bile released from the liver, *via* the gall bladder, for digestion. Bile acids are produced by hepatocytes and are required for the emulsification of hydrophobic lipids in order to dissolve them from the bolus into the digestive fluid which is rich in water-soluble lipase enzymes (Bowen, 2001).

The duodenum also contains a large amount of lymphoid tissue as an important part of the immune system: the lymph cells are used to store both T and B lymphocytes (Salmien *et al.*, 1998).

1.2.5. Large intestine

The large intestine is short in relation to other organs and its histology differs to that of the small intestine, due to a lack of villi. Since the majority of enzymatic digestion takes place in the small intestine, the production of enzymes is also far less pronounced in the large intestine, though water is reabsorbed to regulate the concentrations of ions in the blood.

1.2.6. Caeca

The caeca are two blind-ended sacs where nutrients that were not digested in the small intestine can be metabolised by microbes (Svihus, 2013). This is particularly important as the chicken requires more energy per unit of body mass than larger animals as a result of their high basal body temperature (Bell, 1971). Some animals are able to digest their feed more efficiently than others, whereas some rely more heavily upon gut bacteria for utilisation of feed components. The chicken’s diet is rich in carbohydrates and though many simple sugars are degraded enzymatically within

the gastrointestinal tract, some complex polysaccharides, such as cellulose, are indigestible without help from microbes. The importance of this process will be discussed in further detail in later sections.

1.3. Functions of gut bacteria

Bacteria are single-celled prokaryotic organisms and are so abundant that their global biomass is said to outweigh that of animals at a proportion of 35:1 at an estimation of 70 *vs* 2 gigatons of carbon respectively (Bar-On *et al.,* 2018). Chickens play host to a highly dense community of intestinal microbes that ranges from 10^7 to 10^{11} colony forming units (CFU) per gram of digesta (Apajalahti *et al.,* 2004). Since the gut transit time from ingestion to excreta is less than 3.5 hours (Hughes, 2008), the host generally selects bacteria that are able to rapidly colonise and/or adhere to the gut wall. The caeca harbour the most complex microbiome due to a slow rate of passage of digesta: unlike regular digesta, residency in the caecum can reach 24 hours with infrequent expulsions (Warriss *et al.,* 2004). As a research tool, they are easy to access within the abdominal cavity, are the location of the majority of bacterial fermentation and harbour the most complex bacterial populations in the avian gut: the caecal microbiome will therefore be the focus of this thesis.

The host forms a symbiotic relationship with its gut microbiome which contribute to beneficial interactions including nutrition and growth, intestinal physiology and immune function. These will now be discussed in more detail.

1.3.1. Performance, growth and nutrition

Since feed is one of the most expensive components of broiler production and productivity is driven by the ability of an animal to convert feed into body mass, recent attention has been turned to the role of the gut microbiome in production efficiency. As gut microbes enhance the ability of the bird to extract and utilise nutrients from its

feed, it is unsurprising that relationships between bacteria and performance have been identified (**Table 1.1**).

Table 1.1 *Summary of caecal bacterial taxa and observations associated with good performance (*e.g. *low FCR, high liveweight gain) in adult broiler chickens.*

Benefit	**Bacteria/observations**	**Reference**
Energy retention	*Ruminococcaceae, Lachnospiraceae, Clostridiaceae*	Mancabelli *et al.,* 2016
Low FCR	*C. lactatifermentans, R. torques*	Torok *et al.,* 2011
	Consistency between individuals	Rintiilä and Apajalahti, 2013
	Butyrate producers	Stanley *et al.,* 2013a
	High diversity	Stanley *et al.,* 2012
	Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae	Stanley *et al.,* 2016[1]
	Catabacteriaceae, unknown *Clostridiales, Faecalibacterium*	Stanley *et al.,* 2016[1]
	Lactobacillus, *Akkermansia*	Yan *et al.,* 2017
High body weight	*Lactococcus,* high bacterial diversity	Han *et al.*, 2016
Muscle deposition	*Bacteroidaceae, Ruminococcaceae, Prevotellaceae, Lachnospiraceae, Clostridiaceae*	Hou *et al.,* 2016

[1] Three trials were reported in the same article: different taxa were associated with low FCR in two of the three trials and are therefore reported separately.

These studies show that bacteria can be linked to a range of performance parameters though further work is warranted due to variations in results between experiments, perhaps as a result of diet, environment or genetics. It should also be noted that performance in terms of FCR is calculated on a flock-basis and though flocks may appear uniform, the abilities of individual birds to convert feed into body mass varies.

The chicken relies upon bacteria for the digestion of complex carbohydrates such as cellulose and arabinoxylans. Certain bacterial taxa are able to hydrolyse such complex carbohydrates to simple sugars which are then subsequently fermented to short chain fatty acids (SCFA) including acetate, propionate and butyrate (Hooper *et al.,* 2002; Eeckhaut *et al.,* 2011; Onrust *et al.,* 2015) (**Figure 1.5**).

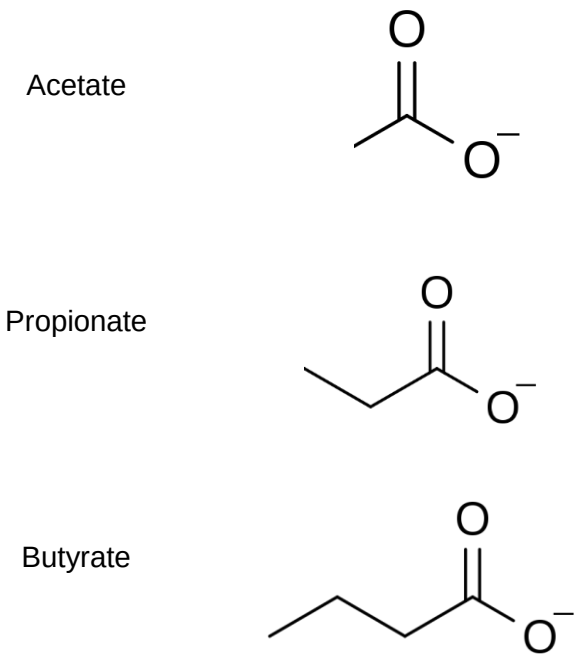


Figure 1.5 *Chemical structures of common SCFA produced from carbohydrate fermentation.*

It is suggested that around 20% of microbial genes are related to carbohydrate metabolism, including enzymes such as polysaccharide lyase which cannot be produced by the host (Qu *et al.,* 2008; Yeoman, 2012). It is important to maximise feed utilisation, and, through the metabolism of complex carbohydrates, nutrient wastage is reduced. SCFA are absorbed by the host and utilised as an energy source, amongst numerous other benefits (Van der Wielen *et al*., 2000; Hooper *et al.,* 2002; Tellez *et al.,* 2006).

1.3.2. Intestinal morphology and physiology

Following hatch, chicks must rapidly adapt to a carbohydrate-based diet, as opposed to the yolk sac that fed them during incubation (Gilbert *et al.,* 2010; Cheled-Shoval *et al.,* 2011). It is therefore highly important that the gut adapts to new nutrient sources to efficiently utilise feed. Microbes begin to colonise the gastrointestinal tract (GIT) immediately post-hatch: there is extensive evidence that this colonisation helps the gut to develop (**Table 1.2**).

Table 1.2 *Summary of bacterial attributes associated with gut physiology.*

Benefit	**Bacteria/observations**	**Reference**
Longer villi/deeper crypts	High bacterial load	Gabriel and Mallet, 2006 Forder *et al.,* 2007
	L. acidophilus, B. subtilis supplementation	Kim *et al.,* 2012
Enterocyte proliferation	SCFA – bacterial metabolites	Le Blay *et al.,* 2000 Blottiere *et al.,* 2003 Fukanuga *et al.,* 2003 Guilloteau *et al.,* 2010

Axenic birds generally exhibit smaller caeca with thinner walls, and shorter intestinal villi and shallower crypts than those in conventional birds: this reduces the absorptive surface area of the gut and the extent to which nutrients can be absorbed. As previously discussed, some gut bacteria in the small intestine and caecum can produce SCFA from the digestion of complex carbohydrates; evidence suggests that these SCFA, particularly butyrate, are the preferred energy source of colonocytes and they enhance the differentiation and proliferation of enterocytes (Le Blay *et al.,* 2000; Blottiere *et al*., 2003; Fukunaga *et al.,* 2003; Guilloteau *et al.,* 2010). Since the small intestine is the main site of nutrient absorption, an increase in surface area in response to gut microbes primes the host to maximise feed utilisation.

1.3.3. Immune function

The gut microbiome interacts with the host's immune system in terms of both physical and molecular barriers, with evidence that axenic birds are more susceptible to disease than conventional birds (O'Hara and Shanahan, 2006). Physical and molecular immune systems interact to provide protection from pathogens that would otherwise compromise the health, welfare and productivity of broilers whilst potentially raising issues concerning consumer food safety.

1.3.3.1. Competitive exclusion

Colonisation of the GIT by commensal bacteria has been shown to reduce the occurrence of disease (Seo *et al.,* 2000; Stern *et al.,* 2001; Nakamura *et al.,* 2002; Al-Zenki *et al.,* 2009). Probiotics (supplements of live bacteria) can prime the GIT to resist colonisation by pathogens including *Salmonella spp., Clostridium perfringens*, *Campylobacter jejuni* and avian pathogenic *Escherichia coli* (La Ragione and Woodward, 2003; Higgins *et al.,* 2007; Mountzouris *et al.,* 2009; Al-Zenki *et al.,* 2009; Saint-Cyr *et al.,* 2016b). This principle, known as competitive exclusion, is a physical process by which commensal microbes saturate ecological niches and the reduce the ability of pathogenic bacteria to colonise (Nurmi and Rantala, 1973).

Competitive exclusion relies upon the fact that two organisms that are in direct competition with each other for the same resource cannot sustainably coexist. Saturation of the gut by commensal microbes means that, if pathogens are ingested, the chances of them residing in the gut for long enough to establish stable colonies and induce a diseased state are highly compromised, since the luminal contents are continually moved through the GIT.

1.3.3.2. Mucous layer

The intestinal epithelium is coated by a mucous layer, which comprises mucin glycoproteins secreted by goblet cells (Forder *et al.,* 2012). This layer acts as a physical barrier between the lumen and the epithelium and reduces the extent to which potential pathogens can gain entry to the host (Brisbin *et al.,* 2008). An example of this is *Campylobacter,* a human pathogen whose virulence is attenuated by the intestinal mucous by preventing adherence and therefore invasion of the intestinal epithelium (Byrne *et al.,* 2007; Alemka *et al.,* 2010). Naughton *et al.* (2014), however, report that mucin glycans act as binding receptors for bacterial adhesion proteins, and may therefore alter gut microbial profiles by allowing certain species to adhere to, and colonise, the gut walls. This is particularly evident in *E. coli* (Bouckaert *et al.,* 2005).

The chemical composition of the mucous layer is also affected by bacteria. Mucins comprise a number of classes including sialylated and sulphated mucins: evidence indicates that sulphated mucins are more abundant in birds with low bacterial loads and are characteristic of an immature gut (Turck *et al.,* 1993). This suggests that gut microbes are important in the maturation of intestinal barrier function, particularly during early life when the acquired immune system is relatively immature and birds rely more heavily on physical barriers to prevent disease (Deplancke and Gaskins, 2001). Mechanisms behind the differing expression of sialylated or sulphated mucins are unclear, since differences in the expression of genes such as MUC2 have not been linked to differences in microbial status (Cheled-Shoval *et al.,* 2014).

1.3.3.3. pH reduction

The SCFA products of carbohydrate fermentation, particularly butyrate, can prime the host to resist a diseased state. Evidence suggests that the colonisation and shedding potential of *Salmonella* and *C. perfringens* are compromised by butyric acid (Van Immerseel *et al.,* 2004; Fernandez-Rubio *et al.,* 2009; Timbermont *et al.,* 2010;

Namkung *et al.,* 2011). Since these microbes are health, welfare and food safety concerns, their control is imperative and thus the roles of butyrate-producing bacteria in the promotion of broiler production are of importance.

1.3.3.4. Cellular immunity

Bacterial colonisation of the GIT commences at hatch, resulting in a low level of inflammation through cytokine expression and the rapid development of a mature immune system (Bar-Shira and Friedman, 2006; Crhanova *et al.,* 2011). The gut-associated lymphoid tissues (GALT), comprising the caecal tonsils and intestinal Peyer's patches, protect the chicken from enteric disease through both innate and adaptive responses. Furthermore, the development of the gut microbiome with age also interacts with the development of a mature immune system (Crhanova *et al.,* 2011), suggesting a relationship with the ability of the bird to resist infection.

Though few studies have characterised relationships with specific bacteria, the overriding evidence suggests that the microbiome plays a vital role in the immunological status of the bird. Such studies are summarised in **Table 1.3**.

Table 1.3 *Summary of bacterial attributes associated with modulation of the immune system.*

Immunological response	Relationship with microbiome	Reference
Heightened serum IgG/IgM	Probiotic containing *Lactobacilli*, *Bifidobacteria*, *Streptococcus*	Haghighi *et al.,* 2005
Altered T cell repertoire	Axenic *vs* conventional birds	Mwangi *et al.,* 2010 Oakley *et al.,* 2014b
Composition of proinflammatory cytokines	Phylum composition	Oakley and Kogut, 2016

1.4. Composition of the caecal microbiome

Over recent years, work has highlighted the complex nature of the caecal microbiome. Though variations between studies exist as a result of factors such as diet, bird age, feed additives and medication, a number of taxa are consistently found, suggesting a 'core' microbiome. **Figure 1.6** illustrates the relative abundance of caecal phyla of birds at around 36 days of age, from a range of published population profiling studies.

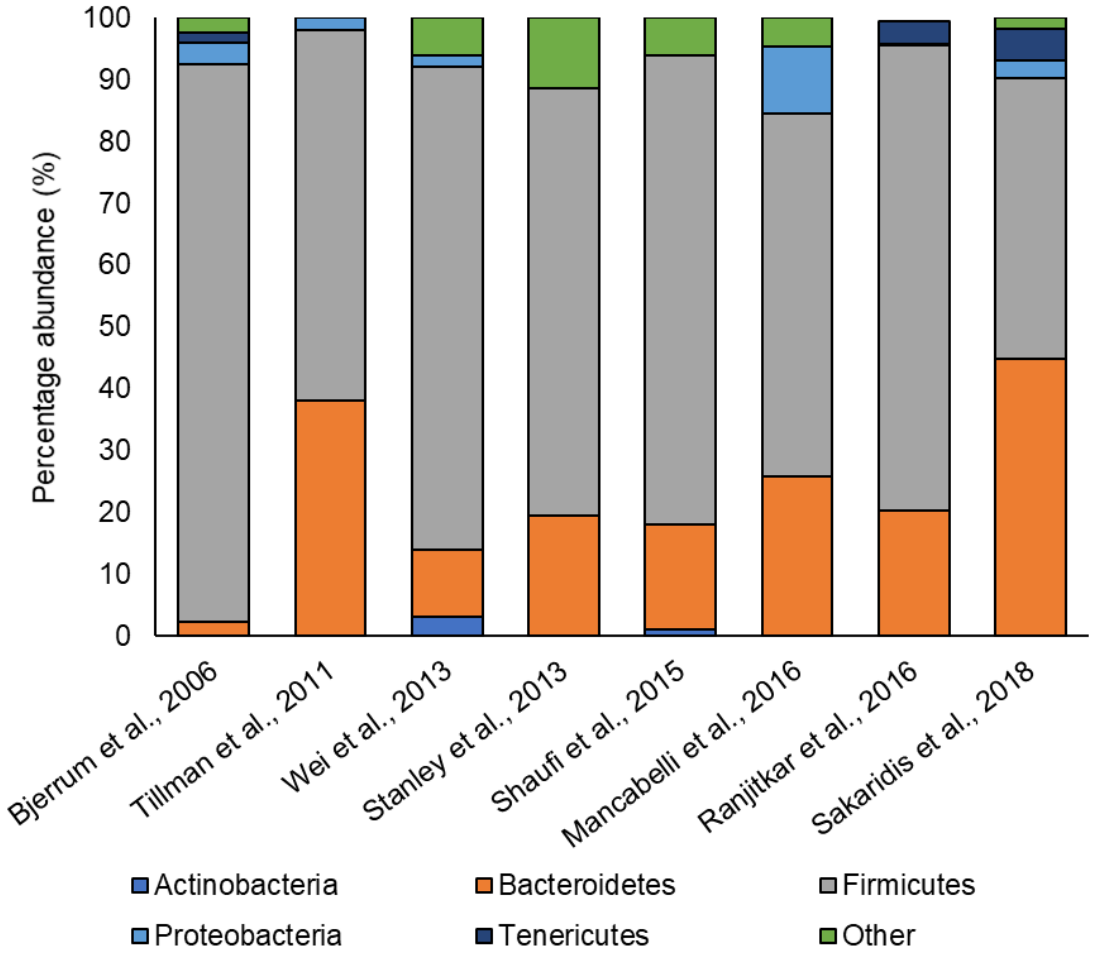


Figure 1.6 *Stacked bar chart summarising the relative abundances of phyla identified in the caecal digesta of adult (~35 days of age) broiler chickens. Birds from Bjerrum* et al. *(2006), Mancabelli* et al. *(2016) and Sakaridis* et al. *(2018) were raised on commercial broiler farms under conventional conditions. Birds from the remaining studies were housed in group pens at an experimental facility.*

With the exception of Bjerrum *et al.* (2006), *Firmicutes* and *Bacteroidetes* are consistently identified as the most abundant phyla. *Firmicutes* is a highly diverse phylum comprising generally Gram-positive taxa including *Lactobacillales*, *Clostridiales* and *Bacillales*. *Bacteroidetes* is less diverse than *Firmicutes*, and generally comprises Gram-negative taxa including *Bacteroidales*. The abundances of subordinate phyla, *Actinobacteria, Proteobacteria* and *Tenericutes*, appear to differ between studies.

Age plays an important role in the development of the gut microbiota, with distinctive differences between young and mature birds. An example of this is illustrated by Ranjitkar *et al.* (2016) where, although the hierarchal order of phyla remains relatively stable, the relative abundances of each differ (**Figure 1.7**).

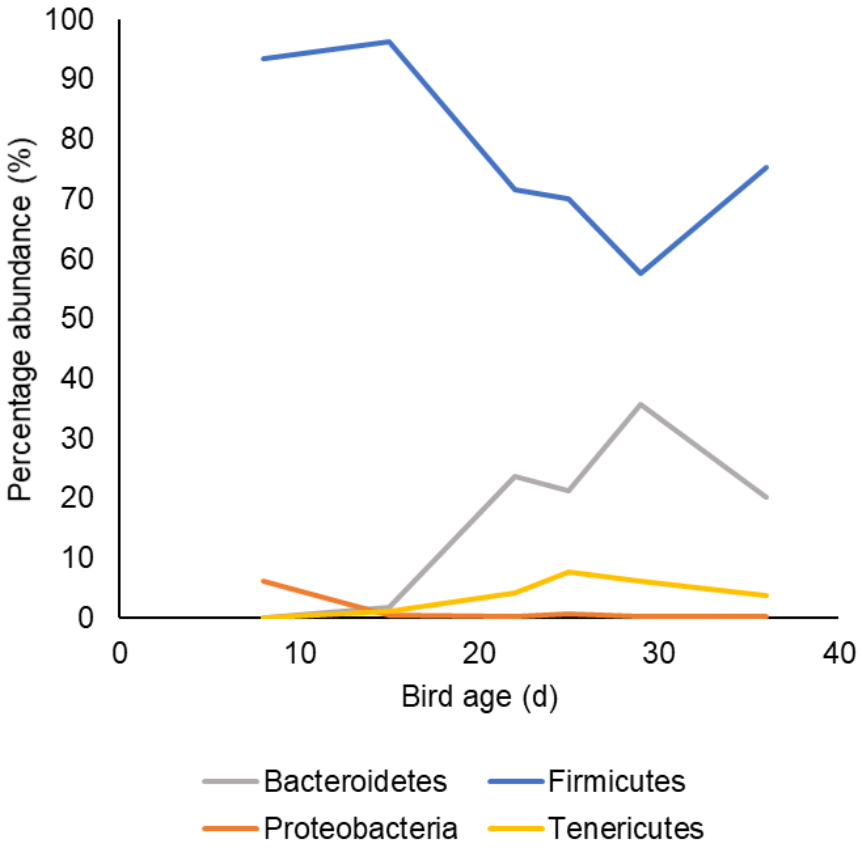


Figure 1.7 *Line plot illustrating the effects of age on levels of bacterial phyla in the caecal microbiome (Ranjitkar* et al., *2016).*

Firmicutes appear to exhibit a reciprocal relationship with *Bacteroidetes*, whilst *Proteobacteria* decrease to low levels by day 15 and *Tenericutes* peak at around day 25. The ratio of *Firmicutes* to *Bacteroidetes* is often explored, with suggestions of a relationship with bird weight (Salaheen *et al.,* 2017). Whilst these data by no means describe definitive levels of phyla, since there are large amounts of variation between studies, they effectively illustrate how the composition of the caecal microbiome develops with bird age.

There is also huge diversity in bacteria at lower levels of taxonomy. These taxa are influenced by a plethora of features including diet, bedding, the use of antibiotics, probiotics and prebiotics, feed additives and genotype. It would be impossible to compare each and every study, given how much variability exists between management strategies utilised in each one. The bar chart in **Figure 1.8** illustrates just one study (Ranjitkar *et al.,* 2016) to give an idea of some of the most common taxa. It should be noted that this is by no means a concrete representation of the caecal microbiome, and that other studies may report additional taxa to those highlighted by Ranjitkar *et al.* (2016).

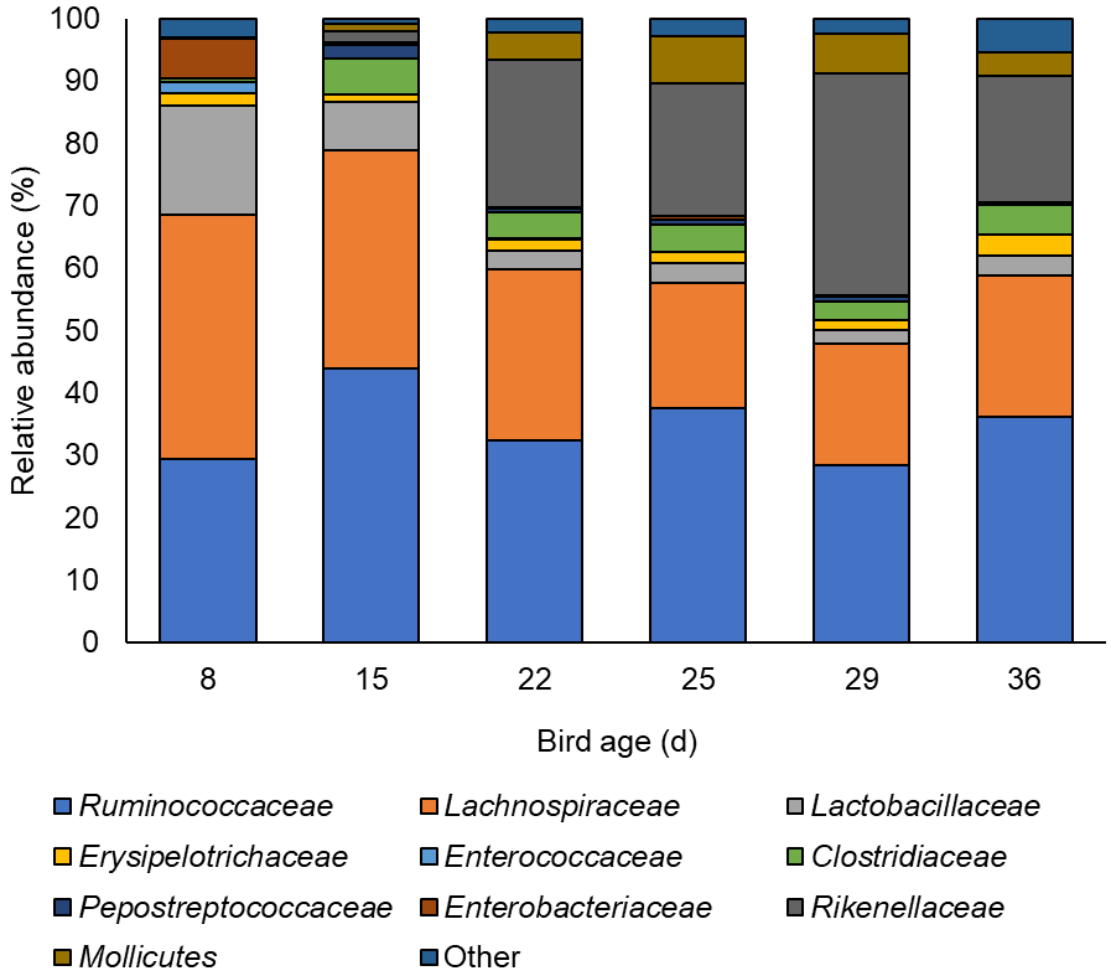


Figure 1.8 *Stacked bar chart illustrating an example of the composition of the caecal microbiome of growing broilers at family level (Ranjitkar* et al., *2016).*

The fact that there is so much variability in the caecal microbiome as a result of management strategy, coupled with the aforementioned evidence that the microbiome exerts beneficial effects on the host, poses the question as to what effect these differences may have on the host. It is currently unclear what relationships between taxa exist, and whether they are consistent when exposed to the effects of differing management strategies.

Some of the most commonly found taxa, along with factors that affect their abundance and suggestions of their roles in the gut will now be discussed in more detail.

1.4.1. Firmicutes

1.4.1.1. Ruminococcaceae

Ruminococcaceae is a family of bacteria from order *Clostridiales* and has been identified as one of the most abundant taxa in the chicken caecal microbiome (Ranjitkar *et al.,* 2016). It is a SCFA-producing family and comprises bacteria that can digest and ferment complex carbohydrates and are therefore important in the efficient use of feed and promotion of a healthy gut (Torok *et al.,* 2011; Stanley *et al.,* 2015, 2016). Relationships with performance are somewhat inconsistent between studies: Stanley *et al.* (2016) carried out a trial where the same experimental setup was repeated in triplicate and found elevated populations of *Ruminococcaceae* to be linked to good (low) FCR in just one replicate. On a similar thread, Ranjitkar *et al.* (2016) report numerous fluctuations with age, suggesting potential sensitivity to other taxa or environmental factors such as nutrient availability. It is hoped that some of these relationships may be elucidated in this thesis.

1.4.1.2. Lachnospiraceae

Lachnospiraceae is a family from order *Clostridiales* that has frequently been found in caecal digesta and has been linked to broilers with low FCR values (Stanley *et al.,* 2016). It is suggested that *Clostridium lactatifermentans*, from genus *Clostridium* within family *Lachnospiraceae*, exerts a range of beneficial effects on the host, including the fermentation of lactate to SCFA as an energy source and inhibition of the growth of *Salmonella* (van der Wielen *et al.,* 2002a,b,c; Hijova and Chmelarova, 2007). Ranjitkar *et al.* (2016) highlight a general decrease in relative abundance with bird age, with the largest difference observed in the first three weeks of life. As with *Ruminococcaceae*, populations have been linked to good performance, albeit inconsistently (Stanley *et al.,* 2016).

1.4.1.3. Bacillaceae

Bacillaceae is a family of Gram-positive rods that includes the genus *Bacillus.* They are spore-forming bacteria that can survive in harsh environments (Nicholson *et al.,* 2000) and therefore potentially persist between flocks. Species of *Bacilli,* in particular *Bacillus subtilis*, are often used as probiotics, though whether their beneficial effects are exerted as a result of colonisation by bacteria themselves or through the ingestion of spores is unclear (Barbosa *et al.,* 2005). Benefits appear to include an improvement in FCR (Harrington *et al.,* 2016), improved intestinal health and capacity for nutrient absorption (Aliakbarpour *et al.,* 2012), and a decrease in pathogen load (Jayraman *et al.,* 2013; Park *et al.,* 2018). It is hypothesised that these beneficial effects are exerted through competitive exclusion (Barbosa *et al.,* 2005).

1.4.1.4. Lactobacillaceae

Lactobacillaceae is a family of Gram-positive bacteria that is autochthonously found in the chicken gut. Their ability to ferment sugars to produce lactic acid results in their classification into the 'lactic acid bacteria' (LAB) group. These bacteria are often used as probiotics, with the potential to improve performance (De Cesare *et al.,* 2017), though effects very much depend upon strain and therefore results are fairly varied (Olnood *et al.,* 2015). Research also suggests that supplementation with *Lactobacillus plantarum* has the capacity to reduce levels of *Escherichia coli* in the caecum (Wang *et al.,* 2017), thereby improving bird health and food safety.

1.4.1.5. Enterococcaceae

Enterococaceae is a family of Gram-positive cocci and is also classed as LAB. Ranjitkar *et al.* (2016) found relative abundance to decrease with age, suggesting a temporal shift over time. Exploration of other gut compartments suggests that populations are higher in the ileum than the caecum, suggesting that these bacteria are sensitive to nutrient availability, since concentrations of dietary components are higher in the former (Lu *et al.,* 2003). Evidence suggests that bacteria from

Enterococcaceae have the potential to be used as a probiotic against the colonisation of *Clostridium perfringens* and occurrence of necrotic enteritis (Fasina *et al.,* 2016), and reduce levels of *E. coli* in the caecum (Cao *et al.,* 2013). The latter also found positive effects on intestinal morphology and performance, where dosing with *Enterococcus faecium* improved both villus height and bird weight.

1.4.1.6. Veillonellaceae

Veillonellaceae is a family from phylum *Firmicutes* that cannot utilise carbohydrates as an energy source and relies upon the fermentation of lactate to SCFA, particularly acetate, for energy production (Ng and Hamilton, 1971; Kwon *et al.,* 1997). This means that its survival may depend upon the presence of LAB to produce said lactic acid and is just one example of the complex relationships that exist within the caecal microbiome. Hinton *et al.* (1995) demonstrate that, when *Veillonella* are cultured with LAB such as *Lactobacillus acidophilus*, the growth of *Salmonella* spp. and *Escherichia coli* is inhibited, suggesting that such interactions are also important in host and human health.

1.4.1.7. Clostridiaceae

Faecalibacterium, a genus from family *Clostridiaceae*, is a gram-positive, non-sporing rod that has been found in the gut of numerous species including humans where their presence has been identified as an indicator of gut health (Benus *et al.,* 2010). The abundance of *Faecalibacterium* in broilers has been shown to increase with age (Donaldson *et al.,* 2017).

1.4.2. Bacteroidetes

1.4.2.1. Bacteroidaceae

Garcia *et al.* (2012) report that around 25% of bacteria from phylum *Bacteroidetes* belong to genus *Bacteroides*, a group of Gram-positive non-sporing bacilli. Their main nutrient source is host-derived and plant glycans and their abundance is sensitive to host diet, though there is evidence of resistance to antibiotics including ampicillin and tetracycline (Garcia *et al.,* 2012). Xiao *et al.* (2017) report that *Bacteroides* is the most abundant genus in the caecum, representing around 40% of the population.

1.4.2.2. Rikenellaceae

Ranjitkar *et al.* (2016) report that the entirety of phylum *Bacteroidetes* is attributed to *Rikenellaceae*, with levels increasing with age and peaking at day 29 before falling to day 36. This family is reported less frequently than others, perhaps as a result of reduced transfer between birds – Polansky *et al.* (2016) found evidence of *Rikenellaceae* in donor birds alone, with no colonisation in recipient birds. It is hypothesised that this is potentially a result of sensitivity to oxygen reducing environmental viability, and therefore the colonisation potential in a new host. Both Kim *et al.* (2012) and Daniel *et al.* (2014) found heightened levels in mice fed a high-fat diet, suggesting additional sensitivity to exogenous variables. It is therefore thought that these bacteria are highly variable between experiments and flocks, and that their presence or absence may reveal relatively little about the productive status of the bird.

1.4.3. Proteobacteria

Proteobacteria is often identified as one of the most abundant phyla in the caecal microbiome. Populations are generally high in young birds but decrease with age to be replaced by *Firmicutes* (Ranjitkar *et al.,* 2016; Awad *et al.,* 2016). The majority of this phylum is comprised of Enterobacteriaceae (class *Gammaproteobacteria*), such as *Escherichia coli* (Lu *et al.,* 2003). Though generally considered a commensal,

E. coli is an opportunistic pathogen meaning that it has the potential to induce a diseased state, however it has been suggested that high levels of *Escherichia* spp. in young birds may assist in the rapid development of the immune system (Awad *et al.,* 2016).

Also of note is *Salmonella*, a zoonotic food-borne pathogen from family Enterobacteriaceae that causes salmonellosis in humans and is a concern of food safety. The relatively recent development of control programmes has reduced the prevalence of poultry-derived salmonellosis, though rising antibiotic resistance may be a cause of future concern (Antunes *et al.,* 2016).

1.4.4. Tenericutes

Tenericutes is a phylum of bacteria containing the class *Mollicutes*, the name of which is derived from the Latin '*cutis*', meaning 'skin', and refers to the lack of cell wall. *Tenericutes* are frequently found in the caecal microbiome, though at lower abundances than *Firmicutes* and *Proteobacteria* (Witzig *et al.,* 2015; Awad *et al.,* 2016; Sakaridis *et al.,* 2018). Members of class *Mollicutes* are generally classified as unidentified order 'RF39'; their role in health and disease are unclear but have been found to be elevated in birds suffering from both necrotic enteritis (Stanley *et al.,* 2012) and high feed intake (Siegerstetter *et al.,* 2017).

1.4.5. Bacterial populations in alternative gut compartments

Though caecal bacterial populations are the focus of this thesis, it should be noted that said populations differ in alternative sites along the GIT. Han *et al.* (2016) report that six of 13 phyla are found at significantly different relative abundances between the caecum and ileum: *Bacteroidetes* are significantly more abundant in the in the caecum, whilst *Firmicutes* are significantly more abundant in the ileum. Furthermore, Xiao *et al.* (2017) report that *Lactobacillus,* whilst it was the most dominant genus in

all sections of the small intestine, was rarely found in the caecum, consolidating existing work (Bjerrum *et al.,* 2006; Dumonceaux *et al.,* 2006).

It is likely that different bacterial populations exert differential effects on the host, and may be affected differently by management strategies. It should therefore be remembered that, whilst the data presented in this thesis relate to caecal bacterial populations, this may not explain all effects of experimental treatments.

1.5. Interactions between management and the microbiome

Extensive evidence suggests that the microbiome is sensitive to a plethora of management strategies, including diet, bedding and the use of antibiotics and probiotics. Though other factors have been shown to alter the microbiome (Mesa *et al.,* 2017; Kers *et al.,* 2018; Borda-Molina *et al.,* 2018), these are the three strategies that will be explored in this thesis: existing literature will therefore be discussed in more detail.

1.5.1. Diet

The compositions and concentrations of nutrients that enter the gut act as substrates to fuel the growth of bacteria. By altering the composition of said nutrients, one may select for heightened growth of certain species whilst depressing the growth of others. Of particular note is the variation in diet composition as a result of cereal cultivars: evidence suggests differences in chemical composition of cereal grains from different genotypes, including barley, maize, oats and wheat (Rodehutscord *et al.,* 2015). These differences give rise to variations in metabolisable energy content, crude protein retention, and digesta viscosities (Pirgozliev *et al.,* 2003; del Alamo *et al.,* 2008), all of which are likely to affect bacterial populations. Evidence suggests that *Firmicutes*, in particular SCFA-producing families *Lachnospiraceae* and *Ruminococcaceae*, are affected by wheat and maize-based diets (Munyaka *et al.,*

2016), along with digesta pH and viscosity (Lunedo *et al.,* 2014). The composition of non-starch polysaccharides (NSP) such as arabinoxylans which are commonly found in cereal grains (Choct *et al.,* 1996) is also highly influential: the levels and compositions of said NSP alter digesta viscosity and nutrient digestibility and have been linked to heightened colonisation of *Clostridium perfringens*, the aetiological agent of necrotic enteritis (NE) (Pan and Yu, 2014).

Populations of *C. perfringens* have also been linked to the use of dietary animal proteins (Drew *et al.,* 2004). Apajalajti and Vienola (2016) highlight that lactic acid bacteria compete for dietary amino acids, but that excess protein entering the caeca can lead to putrefaction. The growth of certain species is highly reliant on the availability of amino acids (*e.g. Lactobacillus* spp. depend upon cysteine, leucine and methionine; Morishita *et al.,* 1981): differing protein sources offer differing compositions of amino acids, significantly altering the growth potential of certain taxa.

Studies exploring the influences of diet on the gut microbiome are summarised in **Table 1.4**.

Table 1.4 *Studies exploring the effects of diet on the gut microbiome of broilers.*

Dietary factor	**Reference**	**Breed/housing**
Cereal base	Munyaka *et al.,* 2016	Ross 308/group pens
*(*e.g. *wheat, maize, sorghum)*	Lunedo *et al.,* 2014	Cobb 500/individual cages
	Shakouri *et al.,* 2009	Cobb 500/group pens
	Bjerrum *et al.,* 2005	Ross 208/group pens
	Engberg *et al.,* 2004	Ross 208/group pens
	Hübener *et al.,* 2002	Unknown breed/housing
	Apajalahti *et al.,* 2001	Unknown/Commercial farms
Protein	Drew *et al.,* 2004	Hubbard × /group pens
*(*e.g. *animal* vs *plant)*	Knarreborg *et al.,* 2002	Ross 208/group pens

1.5.2. Bedding

In a natural system, a newly hatched chick would remain with its mother and ingest maternal bacteria to inoculate its gut. In industry, chicks are hatched into a relatively clean environment away from any adult birds and therefore do not have access to inoculum of an established microbiome from the mother. The environment into which the naïve chick is then placed therefore acts as a highly influential bacterial reservoir and alters the composition of the developing microbial populations in the gut. Management factors including bedding material (Torok *et al.,* 2009) and the reuse or supplementation of bedding material are highly influential. Birds frequently ingest bedding, which then acts as an inoculum for the gut: Cressman *et al.* (2010) highlight that the microbiome of broilers on reused litter comprises more bacteria of gut origin, whilst that from birds bedded on fresh litter comprises more bacteria of environmental origin. Similarly, Wang *et al.* (2016) highlight how the reuse of litter promotes a more rapid development of bacterial diversity with differences in the relative abundances of a range of taxa including *Lactobacillus*, *Ruminococcus* and *Faecalibacterium.* These studies show that exposure to bacteria from pre-existing flocks *via* bedding reuse or supplementation significantly alters the development of the microbiome in the recipient bird.

Studies exploring the effects of bedding on the gut microbiome are summarised in **Table 1.5**.

Table 1.5 *Studies exploring the effects of bedding on the gut microbiome of broilers.*

Management factor	**Reference**	**Breed/housing**
Bedding material	Torok *et al.,* 2009	Cobb 500/group pens
*(*e.g. *shavings, sawdust)*	Apajalahti *et al.,* 2001	Unknown/commercial farms
Bedding management	Wang *et al.,* 2016	Unknown/group pens
*(*e.g. *fresh bedding, reuse)*	Wei *et al.,* 2013	Unknown/group pens
	Cressman *et al.,* 2010	Unknown/pens and sheds

1.5.3. Antibiotics, probiotics and prebiotics

Until 2006, antibiotics were used as feed additives to promote animal growth. With rising antibiotic resistance (O'Neill, 2014), such use in the EU has now been banned, though antibiotics are still used therapeutically to treat bacterial diseases. Antibiotics are not species-specific, and evidence suggests that dosing alters both the rate of maturation and the composition of the commensal microbiome (Gao *et al.,* 2017). Xiong *et al.* (2018) report that the abundance of *Proteobacteria* is reduced by chlortetracycline, a tetracycline antibiotic, whilst *Lactobacillaceae* are reduced by amoxicillin, a β-lactam antibiotic (Schokker *et al.,* 2017). These studies suggest that different taxa may be affected by different classes of antibiotics, potentially as a result of differing mechanisms of action.

There is a pressing need to reduce the use of antibiotics to tackle a rise in antimicrobial resistance. Probiotics, supplementations of live bacteria designed to either colonise or alter the microbial composition of the gut, have also been highlighted as potentials to improve performance and health whilst avoiding the unnecessary use of antibiotics. Common probiotic species include *Lactobacillus* spp., *Bacillus subtilis* and *Streptococcus faecalis*: evidence suggests that supplementation with these strains accelerates the maturation of the microbiome (Gao *et al.,* 2017), compromises colonisation by pathogens (Pan and Yu, 2014), enhances the cellular architecture of the gut (Kim *et al.,* 2012), and improves the immune status of the bird (Haghighi *et al.,* 2005).

Also of note is use of prebiotics; these are dietary compounds including mannan-, xylo- and fructo-oligosaccharides and are defined as *"selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microflora that confer benefits upon host wellbeing and health"* (Gibson *et al.,* 2004). These effects also include improvement in performance and the immune responses of growing broilers (Ribeiro *et al.,* 2018; Biswas *et al.,* 2019). De Maesschalck *et al.*

(2015) report that supplementation with xylo-oligosaccharides increased the number of genes encoding butyrate production: since butyrate has been shown to improve the gut health of broilers (Onrust *et al.,* 2015) this is a suggested explanation for the mechanism of action of prebiotics.

Studies exploring the effects of antibiotics, probiotics, and prebiotics on the gut microbiome are summarised in **Table 1.6.**

Table 1.6 *Studies exploring the effects of antibiotics, probiotics, and prebiotics on the gut microbiome of broilers.*

Management factor	Reference	Breed/housing
Antibiotics	Xiong *et al.,* 2018	Unknown/group cages
	Schokker *et al.,* 2017	Cobb 500/group pens
	Salaheen *et al.,* 2017	Cobb 500/group pens
	Costa *et al.,* 2017	Cobb 500/group cages
	Mancabelli *et al.,* 2016	Various/commerical or free-range
	Wei *et al.,* 2013	Unknown/group pens
	Danzeisen *et al.,* 2011	Ross/group pens
Probiotics	Hayashi *et al.,* 2018	Cobb 500/group cages
	Gao *et al.,* 2016	Cobb 500/group pens
	Kim *et al.,* 2012	Ross/group pens
	Molnar *et al.,* 2011	Unknown/group pens
Prebiotics	Wang *et al.,* 2018	Arbor Ace/group cages
	Sarangi *et al.,* 2016	Venncobb/group pens
	De Maesschlack *et al.,* 2015	Ross 308/group pens
	Kim *et al.,* 2011	Ross/individual cages

1.6. Methods of profiling bacterial populations

Early attempts to characterise complex bacterial populations relied upon microbial culture, but many microbes were not identified due to difficulties growing them *in vitro*

(Salanitro *et al.,* 1974). Whilst many species were correctly identified prior to the advent of molecular tools, many were incorrectly categorised, since numerous, and often inappropriate, phenotypic tests had to be carried out for identification, increasing the probability of error. Hence a major issue in population analysis has been a lack of accurate tools that not only identify but also enumerate the diversity of species in a population. Next-generation sequencing (NGS) has taken precedence in speciation using complex algorithms to assess total genome and individual gene similarity. The same tools that have been used to define species are now commonly used methods of population characterisation, enabling researchers to characterise very complex communities with relative ease compared with early techniques.

There are two broad approaches. True metagenomics attempts to sequence DNA bearing organisms (bacterial, viral, fungal, protozoal) 'without' bias by a process of cloning and sequencing a complete cross section of all population members in a community sample. Population profiling on the other hand uses a surrogate or proxy method focussed on bacteria whereby a single gene common to all bacteria (and archaebacteria) is amplified by polymerase chain reaction (PCR). The millions of amplicons generated are individually sequenced, differentiated into similarity groups and counted. With an intestinal microbe density of up to 10^{11} CFU/g digesta (Barnes and Impey, 1972), NGS is now frequently used for microbiome analysis in the broiler chicken (Sergeant *et al.,* 2014; Fisinin *et al.,* 2016).

1.6.1. Theory of next-generation sequencing

NGS sequences nucleotide bases from the bacterial 16S ribosomal RNA (rRNA) gene. 16S is a highly conserved gene among bacterial species and can often taxonomically identify microbes to genus level (Chakravory *et al.,* 2007). The gene encodes an essential component of the 30S subunit of the bacterial ribosome and comprises 1500

base pairs grouped into alternating regions of highly conserved and hypervariable sequences, which are unique between taxa (**Figure 1.9**).

Figure 1.9 *Schematic structure of the 16S rRNA gene, illustrating alternating hypervariable (green) and conserved (grey) regions.*

By sequencing hypervariable regions of the 16S gene, reads can be compared against a genetic database such as the Ribosomal Database Project (RDP), allowing the taxonomic assignment of bacterial species. Since eukaryotes do not carry the 30S ribosomal subunit, only prokaryotic cells will be sequenced so bacterial nucleic acid does not need to be isolated from extracted DNA prior to sequencing. All nucleic acids are subjected to PCR with 16S-specific primers to amplify target sequences. NGS can be performed through a number of techniques, though Illumina® was used for bacterial profiling in the studies presented in this thesis.

Illumina® sequencing can be divided into three main stages (**Figure 1.10**). Firstly, a library of DNA is prepared: extracted nucleic acids are enzymatically fragmented and denatured to form single stranded DNA, motifs including adapter sequences and primer binding sites are added and DNA is then amplified through PCR. Secondly, amplified DNA fragments are loaded into a flow cell, which is coated with two oligomers, to which fragments bind *via* their adapter sequences. Bridge amplification produces distinct clonal clusters, or 'regions', of the same DNA fragment, which will increase signals given off during sequencing stages. Finally, fluorescently labelled deoxynucleotides (dNTPs) are washed over the flow cell. These bind to their complementary base and the characteristic wavelength and intensity of the resultant fluorescence is recorded. The process is repeated through a number of cycles and all reads are paired to reform double strands of DNA and aligned for taxonomic identification using a genomic database.

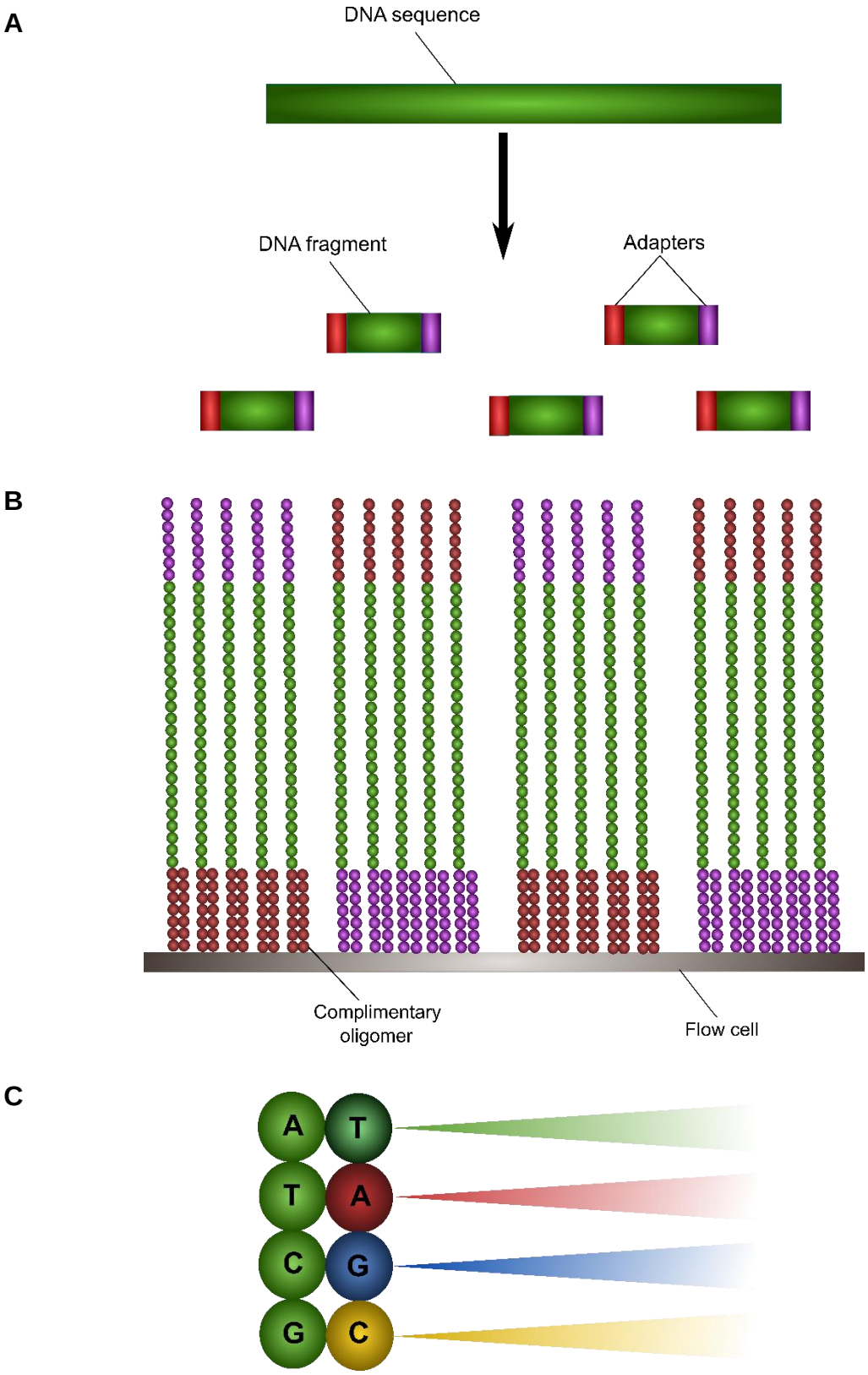


Figure 1.10 *Schematic illustration of the process of Illumina® sequencing. A) DNA is fragmented and bound to adapter sequences. B) Fragments bind to complimentary oligomers on a flow cell and are replicated through bridge amplification. C) Complimentary dNTPs bind to create a new DNA strand and give off fluorescence.*

1.6.2. Data analysis

Sequence reads are processed through software such as QIIME (Quantitative Insights Into Microbial Ecology), a bioinformatics pipeline which converts raw files into useable data including operational taxonomic units (OTUs). This process assigns taxonomic identities to sequences based upon pre-existing information. The number of DNA reads attributed to OTUs is quantified to give a qualitative assessment of taxa abundance, calculated as

$$Relative\ abundance\ (\%) = \left(\frac{Number\ of\ reads\ of\ target}{Total\ number\ of\ reads}\right) \times 100$$

This does not give an absolute value of the number of bacteria assigned to taxa but calculating percentage abundance allows the analysis of the relative composition and diversity of the bacterial community.

1.6.2.1. Alpha diversity

Alpha diversity, or species richness, quantifies the diversity of species within an ecosystem. QIIME outputs include measures of species diversity such as Chao1, though Chao (1984) noted that this measure is useful for datasets that are skewed towards low-abundance groups, as is often the case with complex bacterial communities.

Diversity can also be calculated from OTU data, through measures such as Shannon's index (*H'*) (Shannon, 1948). *H'* assumes that all species are present in a sample, causing issues if not all species within a community are represented, which is sometimes the case when analysing bacterial populations from different ecosystems. Alpha diversity will be explored through Simpson's Diversity Index (*D*) in this thesis. *D* is a dominance index which gives more weight to common taxa and indicates the

probability that individuals drawn at random will belong to different taxa (Simpson, 1949). *D* is calculated as

$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)}\right)$$

where *n* is the total number of organisms of a particular species, and *N* is the total number of organisms of all taxa.

1.6.2.2. Beta diversity

Beta diversity represents differences in the microbial profile between ecosystems. It is often explored through multivariate analyses such as principal component analysis (PCA). Since it is difficult to analyse data with numerous variables, data are orthogonally transformed into a new set of variables, known as principal components, through the rotation of the original data matrix to maximise the explained variance between communities. The first two to three principal components often explain the majority of variance within a dataset - by transforming data in this way the contribution of variables to overall variation can be investigated whilst removing background 'noise' that may not necessarily contribute greatly to differences between communities.

Principal components can be visualised through a score plot (**Figure 1.11**), whereby the distribution of datapoints across the matrix gives an indication of differences in microbial profiles. Datapoints can then be coded according to treatment group to visualise differences, where discrete clustering of datapoints suggests an effect of experimental treatment and points scattered across the matrix suggest no effect.

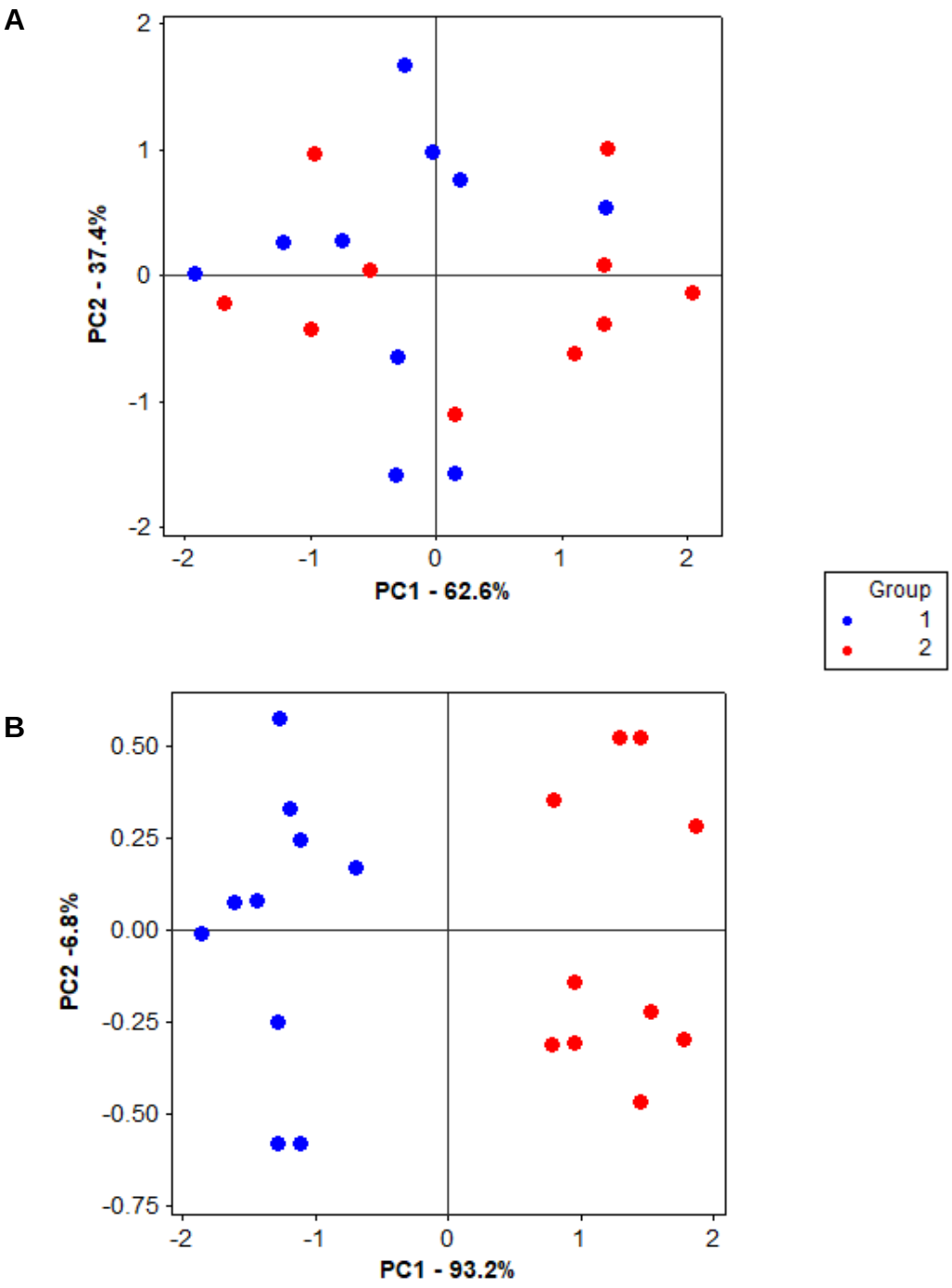


Figure 1.11 *Examples of PCA score plots for the first two principal components (PC) illustrating a lack of clustering (panel A) and discrete clustering (panel B) of data points. Percentages illustrate the proportion of total variation attributed to each PC within a model.*

1.6.2.3. Population analysis

There are no strict 'golden standards' for further analysis of data, though recent developments have seen the emergence of Linear discriminant analysis Effect Size (LEfSe), a web-based application that is designed for the analysis of complex microbial communities and will be utilised in this thesis (Segata *et al.,* 2010). This program is designed to highlight features, or biomarkers, that characterise differences between two groups of data both statistically and biologically. Since NGS data are complex and are often not normally distributed, a parametric test would not be appropriate for statistical analysis. Instead, a non-parametric Kruskal-Wallis sum-rank test is used to identify taxa that are differentially abundant between treatment groups. An unpaired Wilcoxon sum-rank text then investigates biological consistency by exploring distribution of data within treatment groups (Wilcoxon, 1945). Finally, linear discriminant analysis (LDA) gives an estimation of the effect size of differentially represented taxa and suggest the extent to which individual taxa explain differences in the microbiome between treatment groups. This analysis allows the investigator to identify which variables, in this case taxa, are most significantly affected by experimental treatment.

1.7. The microbiome and metabolism

Exploring the metabolic profiles of biological matrices is a highly useful method of investigating host responses to environmental changes. Polansky *et al.* (2016) characterised metabolic pathways and biological processes expressed by chicken caecal microbiota, summarised in **Table 1.7**. Though not an exhaustive list, this gives an idea of the complexity, scope and importance of the microbiome in metabolism.

Table 1.7 *Examples of metabolic characteristics and associated protein expression by caecal bacteria (Polansky* et al., *2016).*

Bacteria	Metabolic characteristic	Example protein expression
Firmicutes *(e.g. Anaerosties, Blautia, Dorea)*	SCFA production (mainly butyrate and propionate)	Acetyl-CoA acetyltransferase Acetyl-CoA carboxylase Butanol dehydrogenase Class IV alcohol dehydrogenase
Bacteroidetes *(e.g. Bacteroides, Alistipes, Prevotella)*	Lipid metabolism	Acyl carrier proteins Expression of pentose cycle Respiration of fumarate
	Polysaccharide degradation	α-amylase[1] α-1,2-mannosidase[1] Endo-1,4-β-mannosidase[1]
	Propionate production	Methylmalonyl-CoA epimerase Cobalamin-binding methylmalonyl-CoA mutase
	Butyrate production	Acetyl-CoA acetyltransferase Acetyl-CoA carboxylase Butanol dehydrogenase

[1] These enzymes were specifically expressed in *Bacteroidetes* and not *Firmicutes.*

Though metabolic profiles of an array of matrices in the broiler have been constructed (Le Roy *et al.,* 2016; Fathi *et al.,* 2017) the application of such knowledge is highly under-represented. Metabolic responses to heat stress in broilers have been explored by Shi *et al.* (2017), but comprehensive research into responses to other environmental stressors is lacking.

The caecal microbiome is highly complex, and evidence suggests a plethora of metabolic roles, but it is ultimately a physically separate system to the chicken itself; though the caecum is highly vascularised, metabolites must cross the epithelium and

enter into the bloodstream to have non-localised effects on the rest of the body. Conversely the liver is a highly metabolically active organ, is in direct contact with the physiology of the bird, and plays important roles including nutrient metabolism and transportation, detoxification and metabolism of drugs and processing of damaged blood cells. Exploring the metabolic profile of the liver may therefore enable researchers to explore the physiological effects of experimental treatments.

1.8. Methods of metabolomic profiling

The field of metabolomics is described by Lindon *et al.* (2000) as

"... the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification".

Since metabolites are the end products of cellular processes, researchers can explore the metabolic state of an organism and suggest effects of experimental variables. Nuclear magnetic resonance spectroscopy (NMR) has been utilised to explore the metabolic state of a number of species, including the chicken (Le Roy *et al.,* 2016).

1.8.1. Theory of ^{1}H nuclear magnetic resonance spectroscopy

Metabolomics frequently makes use of analytical techniques including NMR, but to understand how NMR allows such analyses, one must first understand the behaviour of atomic nuclei. Nuclei with an odd number of protons and/or an odd number of neutrons exhibit a property known as 'spin'. An example of this is ^{1}H, which has a single proton and no neutrons. 'Spin' has a value which can be either an integer (odd number of protons *and* neutrons) or a half-integer (odd number of protons *or* neutrons). With its single proton, ^{1}H is said to have a spin of ½.

Nuclei with spin generate magnetic fields which can be thought of in a similar way to a magnet with a north and south pole. The moment of said magnetic fields therefore depends upon the orientation of the nucleus: north to south or south to north. The number of orientations available to a nucleus, known as spin states, is defined as

$$2I + 1$$

where *I* is the spin value of the nucleus in question. In the case of ^{1}H, where *I* = ½, two spin states are possible (**Figure 1.12**).

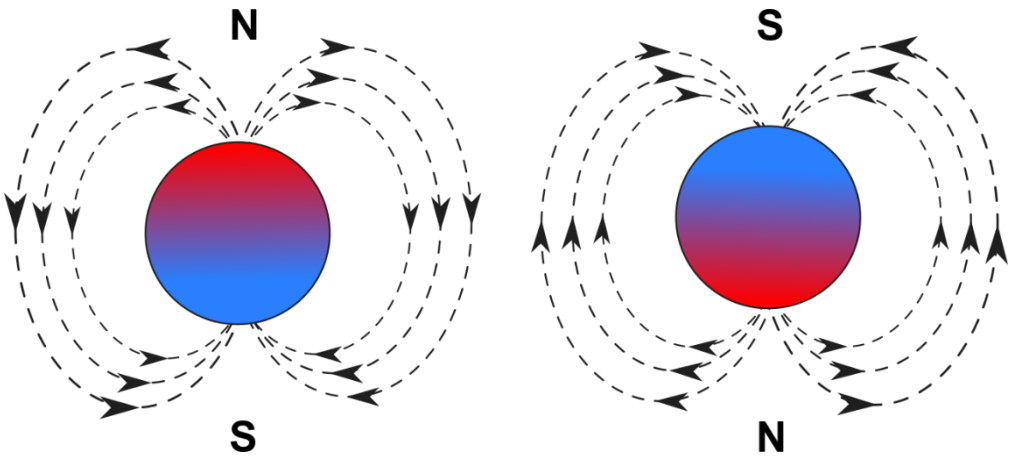


Figure 1.12 *Schematic illustration of magnetic fields surrounding a nucleus in two spin states.*

Under normal conditions nuclei freely orientate themselves, but the application of a magnetic field (B_0) splits nuclei between these two spin states: high energy (aligned against B_0) or low energy (aligned with B_0) (**Figure 1.13**). The difference between these two states, and therefore the energy required to promote nuclei from a low to high energy state, is known as ΔE.

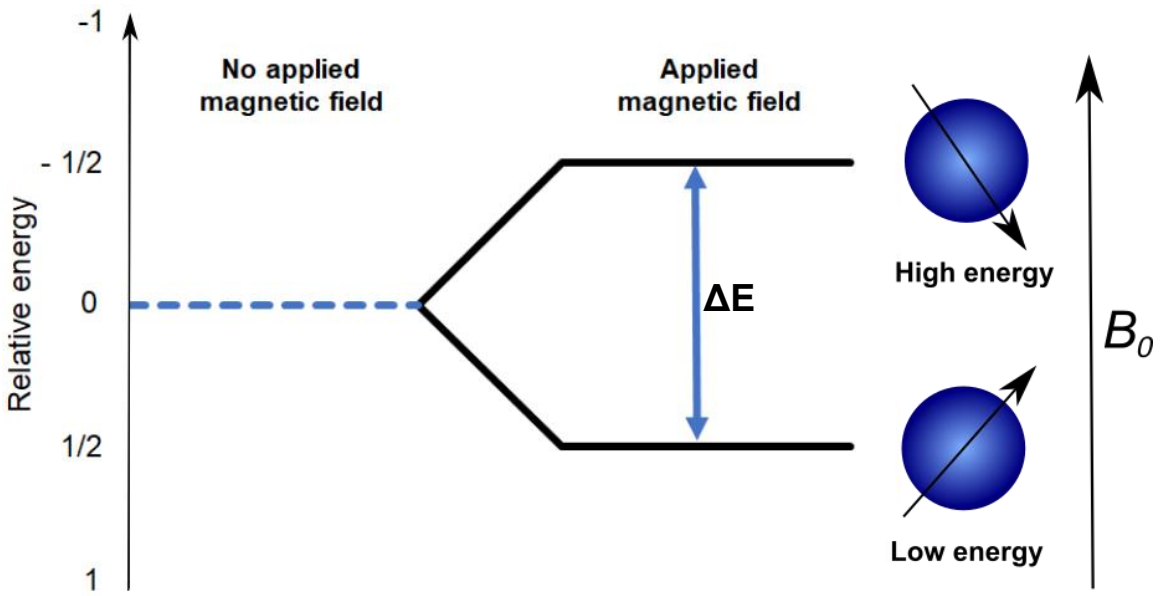


Figure 1.13 *Schematic illustration of the effects of an applied magnetic field* (B_0) *on spin states of nuclei where the difference in energy levels between spin states is denoted as ΔE.*

The energy required to promote a nucleus to its high energy spin state is provided by pulses of radiofrequency (RF) radiation. RF is absorbed at the frequency that matches ΔE and induces resonance which forms peaks in an NMR spectrum. Since different nuclei have different ΔE values, and therefore absorb differing amounts of radiation, numerous peaks appear in a spectrum. RF pulses are applied until there is an excess of nuclei in the high energy spin state, at which point the system is said to be saturated. The system is then allowed to relax and high energy nuclei return to their lower energy state; the system becomes unsaturated. The time for this relaxation is known as spin-lattice relaxation time, or T_1.

A second important property of a nucleus is its oscillating magnetic moment. A nucleus spins on its Z axis, the top of which might not totally align with B_0, and moves in a circle in the XY plane (**Figure 1.14**). This is known as precession.

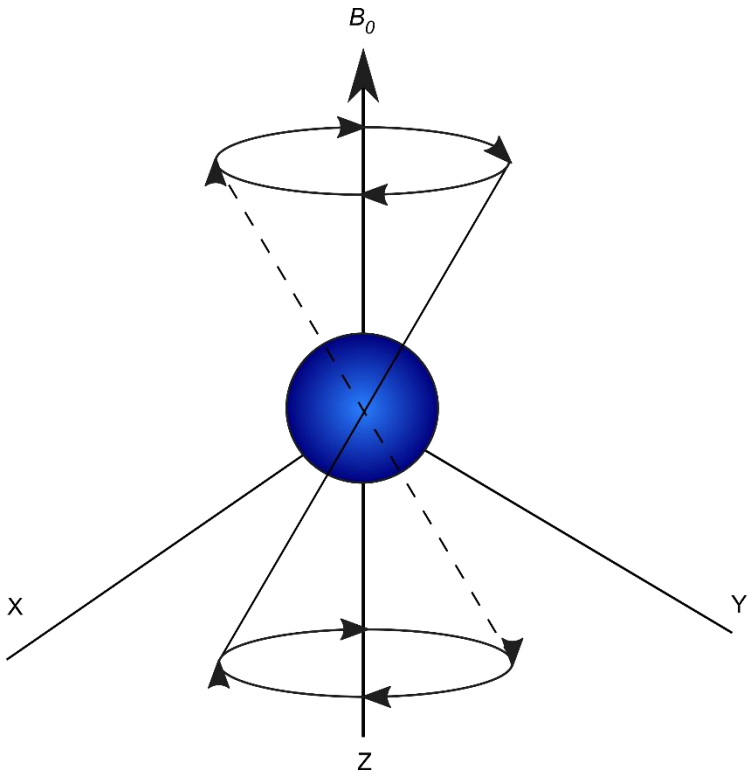


Figure 1.14 *Nuclei precess around the Z axis, to which B_0 is applied, creating a circular magnetic moment in the XY plane.*

Individual nuclei all precess at the same frequency but do so in a disordered manner, whereby each nucleus may be at a different point in its orbit around the Z axis. Taking an average of the positions of nuclei distributed throughout the XY plane, known as bulk magnetisation, gives the false impression that the magnetic moment is aligned with the Z axis. To avoid this, RF radiation 'knocks' precessions into alignment and forces all nuclei to precess coherently in the XY plane. RF radiation is withdrawn, and precession returns to a state of disorder over a period of time, known as spin-spin relaxation. The time taken for bulk magnetism to realign with the Z axis is defined as T_2 (**Figure 1.15**).

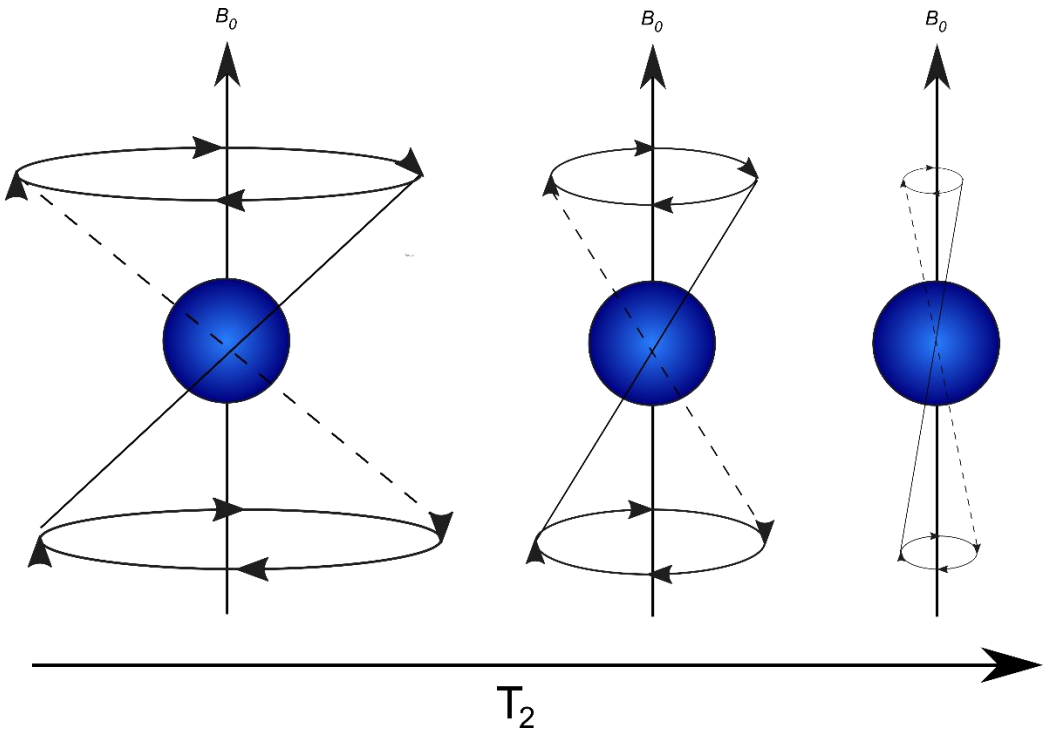


Figure 1.15 *Nuclei precess coherently in the XY plane following the application of RF radiation. T_2 is a measure of the time taken for nuclei to return to a state of disorder, or spin-spin relaxation, giving the impression that the bulk magnetism is aligned with the Z axis.*

The precession of bulk magnetism around the Z axis generates an oscillating electrical charge which is measured as an electrical current, or Free Induction Decay. Data are converted *via* Fourier Transformation to give peaks in a spectrum (**Figure 1.16**).

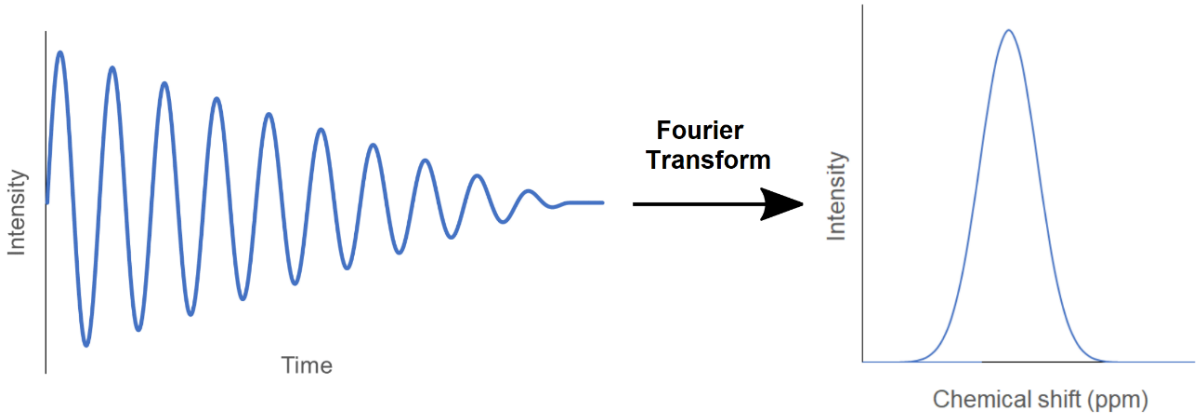
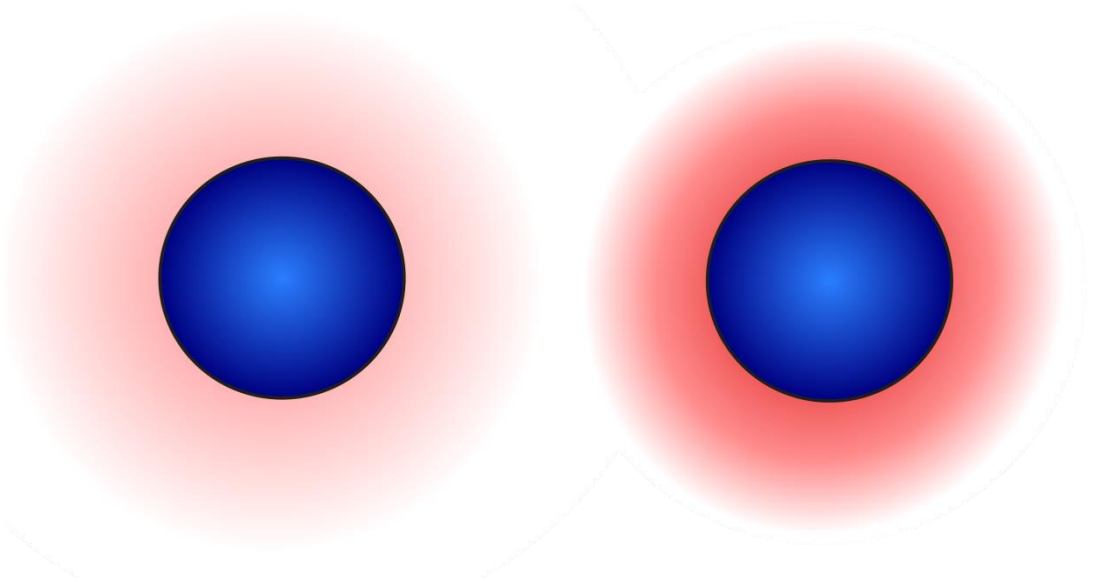


Figure 1.16 *Precession of bulk magnetism is measured as an electrical current (FID) and is converted via Fourier Transformation to give peaks in an NMR spectrum.*

1.8.1.1. Chemical shift

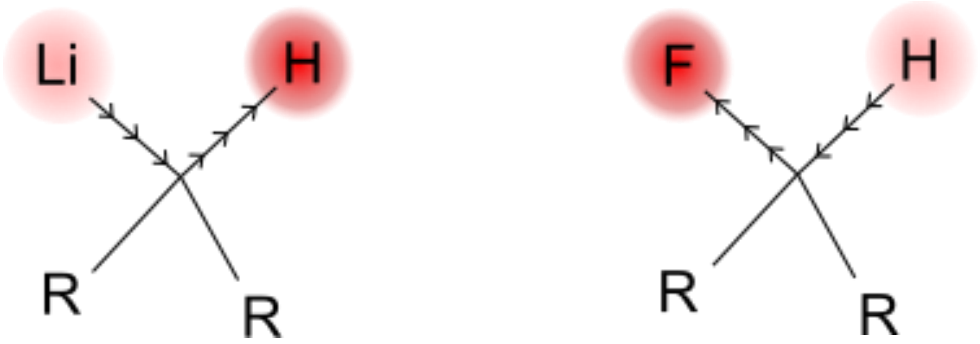
Nuclei of the same isotope experiencing the same B_0 resonate at an identical frequency. A nucleus is surrounded by electrons, each of which have their own magnetic field that opposes B_0, exerting a shielding effect. The perceived magnetic field is therefore lower than B_0, and the extent to which it is perceived varies depending upon the environment (**Figure 1.17**). In areas of low electron density, the nucleus is relatively unshielded and exposed to more of B_0 resulting in a high resonance frequency and a peak to the left of the spectrum. Conversely in areas of high density, the nucleus is relatively shielded resulting in reduced perceived B_0, a low resonance frequency and peaks to the right of the spectrum.



Low electron density = **low** shielding	**High** electron density = **high** shielding
High exposure to B_0	**Low** exposure to B_0
Left of spectrum (**high** ppm)	**Right** of spectrum (**low** ppm)

Figure 1.17 *Illustration of how electron clouds surrounding nuclei can alter the extent to which B_0 is perceived, affecting peak position on spectra.*

Inductive effects from nearby electronegative and electropositive atoms can also alter electron density (**Figure 1.18**). Highly electronegative atoms (such as fluorine) withdraw electron density, reducing shielding and increasing resonance frequency. Conversely, highly electropositive atoms (such as lithium) donate electron density, increasing shielding and lowering resonance frequency.



Li is highly **electropositive**	F is highly **electronegative**
H **shielded** by induction	H **deshielded** by induction
Low exposure to B_0	**High** exposure to B_0
Right of spectrum (**low** ppm)	**Left** of spectrum (**high** ppm)

Figure 1.18 *Electron density, and therefore perceived B_0, is affected by the electronegativity of nearby atoms.*

Magnetic anisotropy explains why aromatic compounds tend to be found at higher frequencies. Such molecular systems are surrounded by electron clouds that create currents above and below rings. B_0 is aligned with these currents on either side of the ring, resulting in a localised deshielding effect and an increased perception of B_0 known as paramagnetic deshielding (**Figure 1.19**).

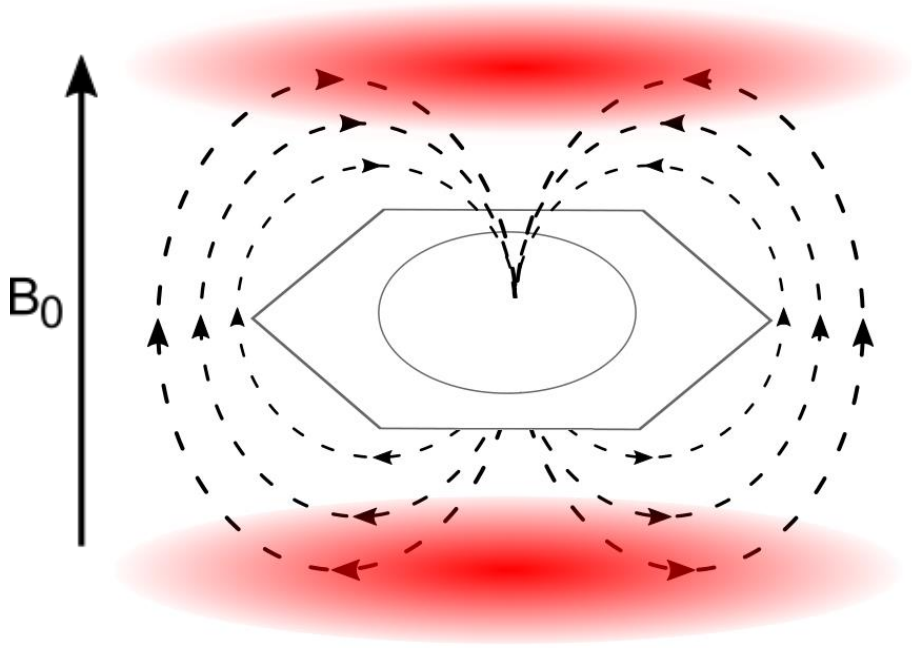


Figure 1.19 *Electron clouds surround aromatic rings and generate magnetic fields which have a localised deshielding effect since they are aligned with B_0.*

The combination of these effects means that not all nuclei within a molecule perceive B_0 to the same extent meaning that a spectrum will often contain multiple peaks originating from the same molecule.

1.8.1.2. Peak splitting

Spectra also give information about neighbouring nuclei through the shape, or splitting pattern, of a peak. The number of nuclei, or protons in the case of this thesis, on neighbouring atoms splits peaks based upon the equation n+1, illustrated in **Figure 1.20**.

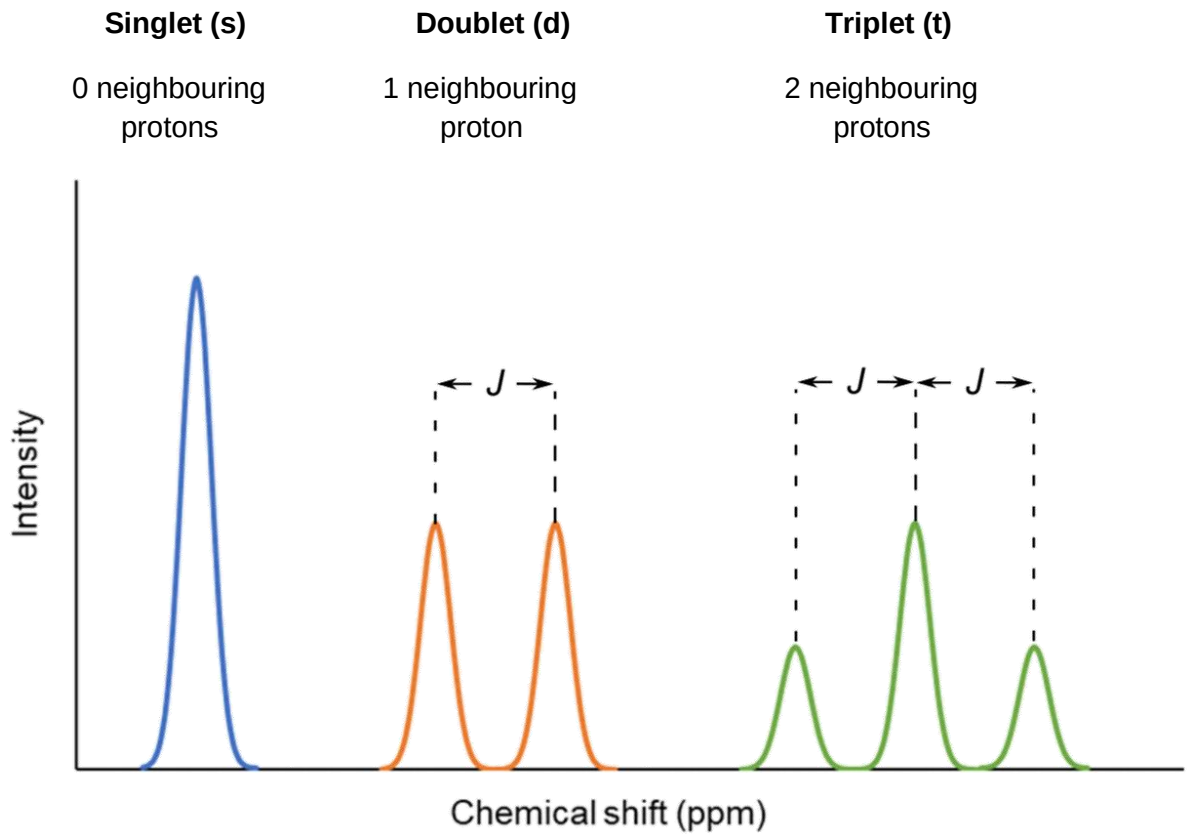


Figure 1.20 *Schematic illustration of a singlet, doublet and triplet peak and their associated J values.*

In addition to splitting patterns, a value known as the coupling constant (*J*), also illustrated in **Figure 1.20**, gives information leading to the assignment of peaks. *J* constants differ depending upon the dihedral angles between protons and are calculated by multiplying the difference between maximum peak height positions (ppm) by the frequency of the spectrometer in MHz. The Karplus equation states that maximum *J* constants are attributed to bond angles of 180°, whilst minimum *J*

constants are attributed to bond angles of 90°. Application of this knowledge supports increased accuracy when assigning molecular identities to peaks in a complex spectrum and can also be used to distinguish between isomers of a molecule (*e.g.* anomers of a sugar ring).

1.8.2. Data analysis

Statistical analysis of NMR data is notoriously complex, though recent developments in metabolomics have seen a rise in analytical techniques including orthogonal projections to latent structure discriminant analysis (OPLS-DA). This type of discriminant analysis constructs a model to explain variation between spectra, and gives rise to three important variables: R^2Y, Q^2 and *P* (**Table 1.8**).

Table 1.8 *OPLS-DA variables used to explore the robustness of models used to classify spectra into experimental treatment groups and explain differences in metabolic profiles.*

Variable	Explanation
R^2Y	A measure of 'goodness of fit'. The model is assessed for its ability to distinguish between experimental groupings between spectra with respect to the Y axis (peak intensity). Values range from 0 to 1, where 1 indicates a perfectly fitting model and 0 indicates no fit at all.
Q^2	A measure of 'goodness of prediction'. The model is assessed for its ability to classify random data into predefined experimental treatment groups. This shows the true power of the model to classify treatments based upon spectral data. Values should ideally be >0 and similar to R^2 to suggest classification potential.
P	A *P*-value is obtained through permutations whereby treatment groups are randomly assigned to spectra in the model to explore whether separations forced by R^2Y are true. A high *P*-value suggests that separation between treatment groups is no better when treatments are randomly assigned to spectra, than when the 'correct' treatments are assigned to their according spectra.

To visualise the statistical analysis of the NMR spectra, a coefficient correlation plot can be constructed. This plot resembles an NMR spectrum, but colours peaks dependent on differences in intensities between treatment groups and therefore predicts which peaks may account for differences in metabolic profiles predicted by OPLS-DA models. 'Warm' colours such as reds and oranges suggest that peaks are biased towards one treatment group over another, whereas 'cool' colours such as blues and greens suggest no difference. These peaks can then be characterised to suggest molecular identities based upon existing literature and metabolomic databases. An example of this is given in **Figure 1.21**.

The dark red colour in panel A suggests that the intensity of a singlet peak at δ = 2.93 ppm differed between two experimental treatment groups. Panel B shows the raw spectra from which this difference was predicted – there appears to be two distinct clusters of peaks suggesting differences in peak intensities and therefore metabolite concentrations.

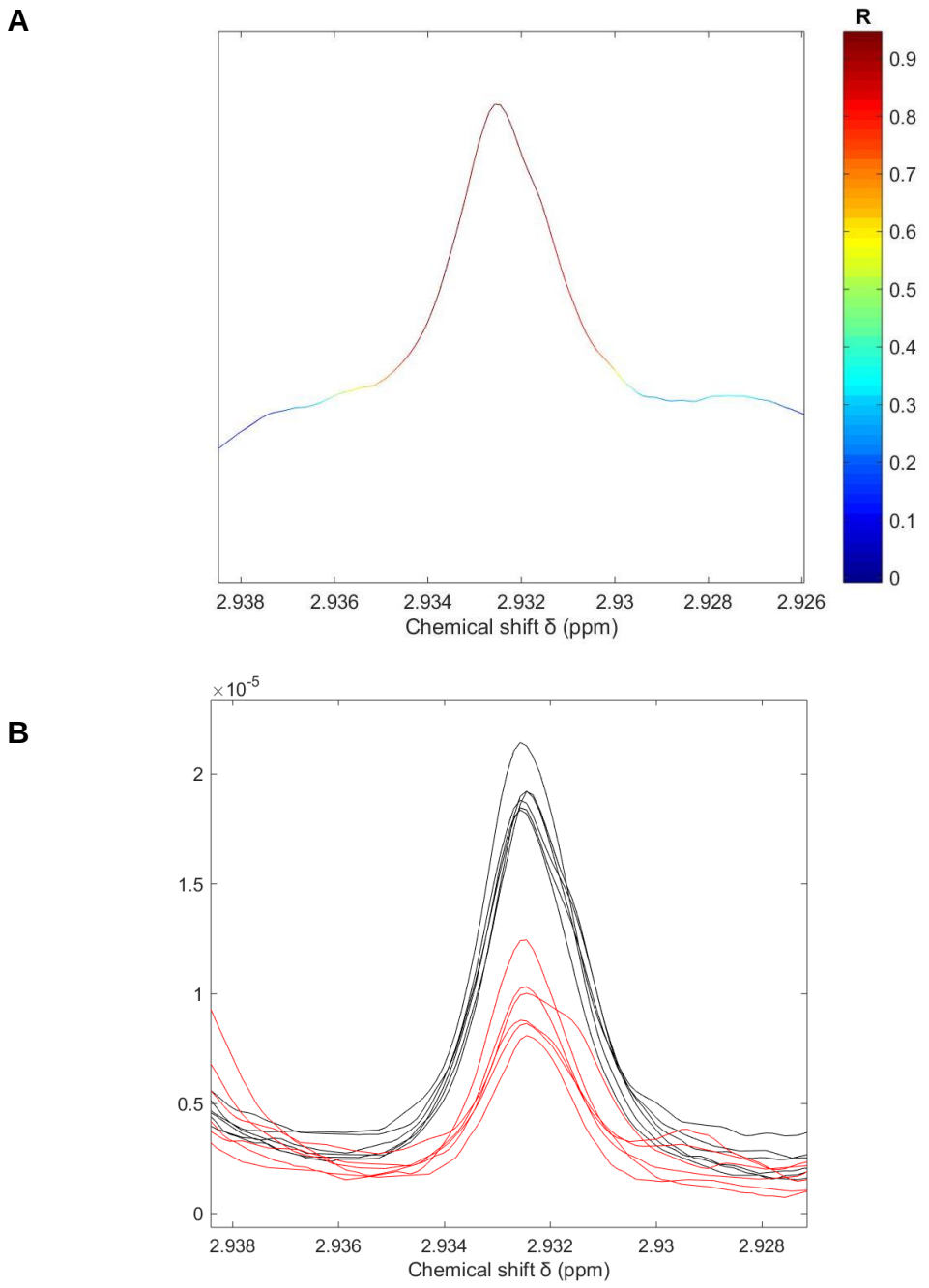


Figure 1.21 *Example of a differentially represented peak in a correlation plot (A) and the correlating peaks from raw spectra where red and black peaks illustrate differing treatment groups (B).*

An NMR sample typically contains an internal standard of a known concentration that gives rise to a singlet peak at low ppm, such as trimethylsilylpropanoic acid (TSP), which is set to δ = 0.00 ppm. Concentrations of target molecules can therefore be estimated through the mathematical integration of peaks in raw spectra and incorporation into the following equation

$$\left(\frac{\boldsymbol{C_{Target}}}{\boldsymbol{C_{Standard}}}\right) = \left(\frac{\boldsymbol{I_{Target}}}{\boldsymbol{I_{Standard}}}\right) \times \left(\frac{\boldsymbol{N_{Target}}}{\boldsymbol{N_{Standard}}}\right)$$

where C is concentration, I is integration value, and N is the number of protons represented by the integrated region. Concentrations per gram of starting material can then be calculated using the equation

$$Concentration\ per\ gram = \left(\frac{C_{Target}}{Mass\ of\ starting\ material\ (mg)}\right) \times 1000$$

1.9. Aims and objectives

The literature presented here gives an overview of the roles of the gut microbiome, why they might be important in health and productivity, and how the caecal microbiome of broiler chickens is comprised. It is evident that said microbiome is of paramount importance in ensuring that broilers develop healthily and efficiently, and that the composition of their caecal microbiome interacts with a plethora of pathways. With a rise in antimicrobial resistance, it is becoming ever more important to find ways to either avoid or reduce their use and furthering our understanding of the gut microbiome may help to formulate solutions to reduce the impact of agriculture on the development of antimicrobial resistance whilst ensuring that pathogen loads are controlled to ensure food safety.

It is unclear how the microbiome responds to environmental stimuli such as management strategies, how bacteria interact with one another, and what effect this has on host metabolism. Variations in the microbiome exist as a result of a plethora of environmental factors (Pedroso *et al.,* 2006; Torok *et al.,* 2009). These differences can exert either positive or detrimental effects upon bird health and growth, though clear interactions between microbes, host and metabolism are still to be elucidated. If bacterial dynamics can be fully understood, it is anticipated that physiological responses to changes in the gut microbiome can be explored and potentially manipulated through management strategies to positively influence the status of the bird.

A comprehensive overview of the microbiome and relationships between bacterial taxa irrespective of how birds are raised have not yet been elucidated: this thesis therefore aims to explore the influence of a range of management strategies on the performance, caecal microbiome and hepatic metabolome of growing broiler chickens. A final analysis of all data gathered throughout this thesis will explore persistent trends across the presented studies, with an aim to highlight which bacterial taxa are most commonly affected by management, whether there are consistent relationships between taxa, and whether these differences translate into alterations in performance and the metabolic profile of the liver. It is anticipated that these trends can be investigated in future work to elucidate any physiological effects that the most commonly affected bacteria may exert on the host, and to utilise findings to enhance bird performance, health and welfare.

The following management strategies will be explored:

1. **Medication:** unmedicated birds compared with antibiotic treatment and subsequent administration of a mixed probiotic.
2. **Protein source:** fishmeal compared with soybean meal supplemented with synthetic lysine and methionine.
3. **Diet cereal base:** wheat compared with maize.
4. **Bedding:** fresh shavings compared with shavings supplemented with excreta from mature adult birds.

Aim 1: Explore the effects of management strategies on the caecal microbiome

- Determine the effects of management strategies on measures of alpha and beta diversities of the caecal bacterial populations.
- Determine the effects of medication, protein source, dietary cereal base and bedding supplementation on the composition of caecal bacterial populations.

Aim 2: Explore the effects of management strategies on the hepatic metabolome

- Determine the effects of medication, protein source, dietary cereal base and bedding supplementation on the profile of the hepatic metabolome.
- Determine the effects of on the concentrations of SCFA and lactate in the liver.

Aim 3: Explore the relationships between bacterial taxa and hepatic metabolites

- Suggest relationships between caecal bacterial populations and concentrations of hepatic metabolites for application in future work.

It is hypothesised that:

1. Bacterial populations will be sensitive to the effects of management strategy, particularly in young birds.
2. There will be differences in hepatic metabolic profiles as a result of differences in bacterial populations.
3. Relationships between bacterial abundances and concentrations of hepatic metabolites exist and will provide information regarding microbiomic-metabolic interactions.

2. Materials and methods

2.1. Animal studies

Four animal studies were conducted to explore the effects of management strategy on their caecal microbiome and hepatic metabolome. Since physiological responses to said management strategies were required, it was necessary to undertake work *in vivo*. Birds were housed and raised under the recommendations of welfare and husbandry from the Ross 308 broiler management handbook (Aviagen, 2014). When birds were slaughtered for sampling, they were treated humanely and appropriate methods for their body weight were carefully selected to minimise any pain and suffering. Birds were not dissected until all movement had fully ceased. If it was noticed that, at any point, a bird was unwell or suffering for a prolonged period of time (*e.g.* not eating, notably smaller than all other birds), it was humanely euthanised to prevent unnecessary suffering. Slaughter of birds was only carried out by licenced personnel.

All birds were sourced as day old Ross 308 chicks from PD Hook Hatcheries (Bampton, Oxfordshire, UK) and housed in an environmentally controlled experimental room at the Centre for Dairy Research at the University of Reading (Reading, UK). All birds, except those used in study 1, were sexed males, and were not tested for pathogens before the onset of the trials. Specific housing conditions and experimental treatments for each study are discussed below.

2.1.1. Study 1: Effect of medication - antibiotic and probiotic treatment

2.1.1.1. Birds and housing

216 chicks were obtained, as hatched, and allocated to one of 12 1 × 1.5 m pens (n=18 birds/pen) within a single controlled room and bedded on EnviroBed^{TM} (EnviroSystems, Preston, UK) bedding. A solid 0.6 m high cardboard barrier was set up around the perimeter of each pen to prevent cross-contamination.

Feed was offered from shallow trays on the floor until day 10, when it was transferred to suspended feed hoppers level with the birds' backs. A commercial wheat-based starter diet (Chick Crumb, Countrywide Farmers, Twyford, UK) was offered from days 1-9 which contained 6-phytase (500 FTU/kg), endo-1,3(4)-beta-glucanase (1500 VU/kg) and endo-1,4-beta-xylanase (1100 VU/kg) as digestibility enhancers, and lasalocid sodium (90 mg/kg) as a coccidiostat. Grower and finisher diets were formulated and manufactured as a meal (Target Feeds Ltd, Whitchurch, UK) and offered from days 10-21 and 22-29 respectively. The ingredient and nutrient compositions of diets, analysed by Sciantec Analytical, are summarised in **Table 2.1**.

Table 2.1 *Ingredient and nutrient composition of diets fed in study 1. Since the starter diet was a commercial product, a detailed ingredient composition is not available. The methods by which nutrients were analysed are stated in parentheses (NIR, near-infrared spectroscopy; ICP-OES, inductively coupled plasma optical emission spectroscopy; HPLC, high performance liquid chromatography).*

	Starter Days 1-9	**Grower Days 10-21**	**Finisher Days 22-29**
Ingredient composition (g/kg)			
Maize	-	624	643
Soya Hi Pro	-	310	280
L-lysine HCl	-	2.3	1.7
DL-methionine	-	2.9	2.6
L-threonine	-	1.3	1.1
Soya oil	-	10.0	24.0
Limestone	-	12.5	12.0
Monocalcium phosphate	-	13.5	12.5
Salt	-	3.0	3.0
Sodium bicarbonate	-	1.5	1.5
Vitamin and mineral premix	-	4.0	4.0
Titanium dioxide	-	5.0	5.0
Celite	-	10.0	10.0
Nutrient composition (g/kg unless specified)			
Oil (NIR)	35.0	37.1	51.3
Crude protein (NIR)	180.0	208	194.2
Fibre (NIR)	45.0	26.5	25.9
Ash (NIR)	55.0	66.3	63.3
ME (MJ/kg)	-	122.9	127.1
Total phosphorus (ICP-OES)	5.2	6.5	6.1
Available phosphorus (ICP-OES)	-	4.5	4.2
Total lysine (HPLC)	8.9	13.1	11.7
Available lysine (HPLC)	-	12.0	10.8
Methionine (HPLC)	3.5	6.1	5.6
Threonine (HPLC)	-	9.3	6.7
Tryptophan (HPLC)	-	2.2	20.6
Calcium (ICP-OES)	-	9.1	8.6
Sodium (ICP-OES)	1.5	1.8	1.8
Valine (HPLC)	-	9.3	8.7
Vitamin A (IU) (HPLC)	8000	135	135
Vitamin D3 (IU) (HPLC)	3000	5	50
Vitamin E (IU) (HPLC)	-	100	100

2.1.1.2. Experimental treatments

The 12 pens were assigned to three treatments, summarised in **Table 2.2**: control, antibiotic (AB) and antibiotic followed by probiotic (AB+PRO). Control pens (n=6) were offered unmedicated water on an *ad libitum* basis for the entirety of the study. Linco-spectin (LS; Zoetis, NJ, USA) was administered to the remaining six pens in drinking water offered from bottles from days 1-3 with increasing concentrations appropriate for bird age as advised by a veterinary professional. Each pen received 270 mL medicated water split between two drinkers, based upon the estimation that each bird (n=18 per pen) would drink around 15 mL in a 24-hour period. Any remaining stock solution was discarded at the end of each day. Of the antibiotic-treated pens, half remained without further intervention (AB, n=3) whilst the other half were dosed on day 5 with Aviguard® (MERCK, NJ, USA) at a concentration of 1.67 mg/mL as per the manufacturer's recommendations (AB+PRO, n=3). Unless medicated, all water was offered from a suspended drinker connected to the mains water supply.

Table 2.2 *Treatments and medication given to birds throughout the study. (LS, Lincospectin; AB, Linco-spectin days 1-3; AB+PRO, Linco-spectin days 1-3 plus Aviguard® day 5).*

Day	Control	AB	AB+PRO
1	-	LS 2.8 mg/mL	LS 2.8 mg/mL
2	-	LS 3.7 mg/mL	LS 3.7 mg/mL
3	-	LS 4.6 mg/mL	LS 4.6 mg/mL
4	-	-	-
5	-	-	Aviguard® 1.6 mg/mL
6-29	-	-	-

2.1.1.3 Data and sample collection

Birds were weighed upon arrival (day 1), following the cessation of LS treatment (day 4) and on days 10, 15, 22 and 29 thereafter. The mass of feed allocated to each pen was recorded at days 10, 15 and 29. Feed intake was subsequently calculated by

subtracting the mass of feed refused at days 15, 22 and 29 for the calculation of feed conversion ratio (FCR) on an as-fed basis.

Birds were randomly selected for biological sampling at days 4, 9, 15 and 29, where selected birds were weighed and killed. The gastrointestinal tract was exposed and contents from both caeca were emptied and then pooled in cryotubes:

- **Day 4:** one pool of caecal content from each control and AB pen (n=6 per treatment). Three birds were sampled per pool, since little caecal content was obtained from birds at this age.
- **Days 9, 15 and 29:** one pool from each control pen and two pools from each AB and AB+PRO pen (n=6 per treatment). Two birds contributed to each pool.

Since the caeca harbour the most complex bacterial populations they were chosen as the organ of focus for bacterial population analysis. Other biological sites (*e.g.* the small intestine) and the environment (*e.g.* diet and bedding), were not analysed to allow more focussed analysis of the caeca and to reduce the complexity of the study design.

A section of tissue from the end of the right lobe of the liver, approximately 3-4 cm in length, was also taken from one bird per caecal pool at all ages for metabolomic analysis. It was ensured that the sample was sufficiently large enough to sample 100 mg of 'deep' tissue (*i.e.* avoiding externally exposed tissue). All samples were snap frozen in liquid nitrogen, temporarily stored on dry ice for transportation and were then stored at -80 °C pending analysis. All sampled birds were sexed internally from day 9 onwards for use as a covariate when analysing performance data; this was not determined at day 4 since birds were too small to accurately assign sex.

2.1.2. Study 2: Effect of diet - protein source

2.1.2.1. Birds and housing

32 male Ross 308 broiler chicks were obtained from PD Hook Hatcheries Ltd (Bampton, Oxfordshire, UK). Upon arrival (day 1) birds were weighed, blocked and allocated to one of two brooding pens to acclimatise. On day three, birds were transferred, in pairs, to one of 16 small animal cages where they were housed for the remainder of the trial. All birds were bedded on wood shavings where feed and water were provided on an *ad libitum* basis from suspended hoppers.

2.1.2.2. Experimental treatments

One of two isoenergetic starter diets offering identical levels of lysine and methionine, the first two growth limiting amino acids, was offered, with differing protein sources (n=8 cages per diet): fishmeal (FM) or soybean meal supplemented with synthetic lysine/methionine (SAA). The ingredient and nutrient compositions of diets, analysed by Sciantec Analytical, are presented in **Table 2.3**.

Table 2.3 *Ingredient and nutrient composition of diets (FM, fishmeal; SAA, soya plus synthetic lysine/methionine) fed in study 2. The methods by which nutrients were analysed are stated in parentheses (NIR, near-infrared spectroscopy; ICP-OES, inductively coupled plasma optical emission spectroscopy; HPLC, high performance liquid chromatography).*

	FM	**SAA**
Ingredient composition (g/kg)		
Ground maize	300	300
Vegetable oil	48	56
Ground wheat	208	208
White fishmeal	100	0
Synthetic L-lysine	0	3.68
Synthetic DL-methionine	0	1.92
Soya (48% CP)	276	340
Praire meal	50	50
Trace element/vitamin supplement	5	5
Dicalcium phosphate	5.92	15
Limestone	8.64	13.56
Salt	1.36	3.12
Nutrient composition (g/kg unless specified)		
Crude protein (NIR)	272	246
Apparent metabolizable energy (MJ/kg)	12.6	12.6
Total lysine (HPLC)	14.4	14.4
Available lysine (HPLC)	12.9	12.9
Total methionine (HPLC)	5.0	5.4
Available methionine (HPLC)	4.6	5.1
Threonine (HPLC)	10.3	8.8
Calcium (ICP-OES)	9.6	9.6
Total phosphorus (ICP-OES)	7.0	6.9
Available phosphorus (ICP-OES)	4.8	4.8
Sodium (ICP-OES)	1.6	1.4
Magnesium (ICP-OES)	1.5	1.5

2.1.2.3. Data and sample collection

Birds were weighed upon arrival (day 1), at the point at which they were transferred from brooding pens to experimental cages (day 3), and subsequently at days 7, 14 and 17. Allocated feed was weighed at days 3 and 17 for the calculation of FCR.

One bird per pen (n=8 per treatment) was randomly selected and killed at day 17: contents from both caeca were pooled whilst a section of tissue from the end of the right lobe of the liver, approximately 3-4 cm in length, was taken. All samples were snap frozen in liquid nitrogen, placed on dry ice for transportation and stored at −80 °C pending analysis.

2.1.3. Study 3: Effect of diet - cereal base

2.1.3.1. Birds and housing

84 male Ross 308 broiler chicks were obtained from PD Hook Hatchery (Bampton, Oxfordshire, UK). Birds were wing-tagged, weighed, randomly allocated to one of two 1 × 1.5 m brooding pens and bedded on clean wood shavings. At 15 days of age, birds were weighed and randomly allocated to one of a dozen 1 × 1.5 m trial pens according to their diet type (six pens per diet with seven birds per pen). Lighting was provided *via* incandescent lights with 23 hours continuous light per 24-hour period for the first seven days, followed by 18 hours continuous light in each 24-hour period for the remainder of the study.

2.1.3.2. Experimental treatments

Birds were offered either a wheat or maize-based diet and fresh water from suspended feed hoppers on an *ad libitum* basis. Diets were specially formulated by Target Feeds (Whitchurch, Shropshire, UK) as follows: starter days 0-14, grower/finisher days 15-35. The ingredient and nutrient compositions of diets, analysed by Sciantec Analytical, are presented in **Table 2.4**.

Table 2.4 *Ingredient and nutrient composition of diets used in studies 3 and 4. The methods by which nutrients were analysed are stated in parentheses (NIR, near-infrared spectroscopy; HPLC, high performance liquid chromatography).*

	Starter (days 1-14)		Grower/finisher (days 15-35)	
	Wheat	**Maize**	**Wheat**	**Maize**
Ingredient composition (g/kg)				
Barley	40	40	40	40
Maize	-	515	-	560
Wheat	500	-	550	-
Soyabean meal	320	320	265	270
Rapeseed meal	42	42	42	42
Soyabean oil	50	50	65	65
L-lysine HCl	1	1	1	1
DL-methionine	3.45	2	2.42	2.42
L-threonine	2.05	2	2.02	2.02
Sodium bicarbonate	2.5	2.5	2.5	2.5
Salt	2	2.5	2.5	2.5
Limestone	12	8	8.56	8.56
Poultry vitamins/minerals	2	2	2	2
Dicalcium phosphate	20	18	17	17
Nutrient composition (g/kg unless stated otherwise)				
ME (MJ/kg)	12.8	12.7	13.4	13.4
Crude protein (NIR)	244	230	216	194
Oil (NIR)	64.5	62.3	92.8	84.5
Sugar (as sucrose) (NIR)	46.6	55.6	46.2	34.8
Starch (NIR)	369	377	378	422
Alanine (HPLC)	8.9	8.6	8.6	8.7
Arginine (HPLC)	14.2	13.9	13.7	11.7
Aspartic acid (HPLC)	21.5	20.6	19.9	17.9
Cystine (HPLC)	3.5	3.6	3.7	2.8
Glutamic acid (HPLC)	47.7	47.1	45.8	32.0
Glycine (HPLC)	9.2	8.8	9.0	7.8
Histidine (HPLC)	5.5	5.4	5.3	4.6
Isoleucine (HPLC)	9.6	9.3	9.0	7.8
Leucine (HPLC)	16.3	15.9	15.6	15.0
Lysine (HPLC)	15.5	13.8	11.4	11.0
Methionine (HPLC)	6.4	7.0	5.6	4.7
Phenylalanine (HPLC)	11.1	10.7	10.6	8.9
Proline (HPLC)	14.1	13.7	12.7	10.1
Serine (HPLC)	10.6	10.4	10.3	8.5
Threonine (HPLC)	9.6	9.2	10.1	8.9
Tyrosine (HPLC)	5.5	4.6	5.0	4.2
Valine (HPLC)	10.1	9.9	9.6	8.4
Tryptophan (HPLC)	1.9	1.9	2.1	1.7

2.1.3.3. Data and sample collection

At days 1, 15 and 22, the mass of feed offered to each pen was recorded. At days 15, 22 and 35, the mass of allocated feed remaining in each pen was weighed for the estimation of feed consumption. Birds were weighed at all time points and feed conversion ratio (FCR) was calculated. Daily feed intake and daily liveweight gain per bird were also estimated by dividing the total feed intake and weight gain per pen by the number of bird days, *i.e.* the number of birds per pen multiplied by the number of calendar days.

At days 15, 22 and 35, six birds per treatment (n=1 bird per pen) were randomly selected and killed by cervical dislocation. From each bird both caeca were removed, emptied, and pooled to give a total of six samples per treatment group and a section of tissue from the end of the left lobe of the liver, approximately 3-4 cm in length, was taken. All samples were immediately snap frozen in liquid nitrogen, transported on dry ice and stored at −80 °C pending analysis.

2.1.4. Study 4: bedding supplementation

2.1.4.1. Bird husbandry and treatments

84 male Ross 308 broiler chicks were obtained from PD Hook Hatcheries (Bampton, Oxfordshire, UK). Birds were wing-tagged, weighed and randomly allocated to one of two 1 × 1.5 m brooding pens. At 15 days of age, all birds were weighed and randomly allocated to one of 12 1 × 1.5 m experimental pens according to their treatment group (six pens/treatment with seven birds/pen). Clean, fresh water was supplied by nipple drinkers and the same wheat-based diets that were used in study 3 (**Table 2.4**) were fed on an *ad libitum* basis; hoppers were set such that the feed was level with the birds' back.

Lighting was provided *via* incandescent lights with 23 hours continuous light per 24 hours for the first seven days, followed by 18 hours continuous light per 24 hours for the remainder of the study. The room was heated by blown air from the ceiling, with a temperature for the room of 30 ˚C for the first three days, reducing to 28 ˚C after three days, and then by 1 ˚C every three days until a temperature of 20 ˚C was attained.

2.1.4.2. Experimental treatments

Birds were assigned to one of two bedding types: clean wood shavings (control), or clean shavings supplemented with 50 g litter from healthy adult layers (supplemented). These excreta were collected from a previous experiment and stored in sealed buckets until the onset of the present study.

2.1.4.3. Data and sample collection

At days 1, 15, 22 and 35, the weights of all birds, feed offered, and feed refused were recorded for the assessment of bird performance in terms of liveweight and feed conversion ratio (FCR). At days 15, 22 and 35, six birds per treatment (n=1 bird/pen) were randomly selected and killed for sampling: the content of both caeca was collected, and a section of tissue from the end of the left lobe of the liver, approximately 3-4 cm in length, was taken. All samples were snap frozen in liquid nitrogen, placed on dry ice for transport and stored at −80 °C pending analysis.

2.2. Assessment of bird performance

Bird liveweight and pen feed intake were recorded at various points within each study for the assessment of bird performance. Pen liveweight gain was calculated through the addition of individual liveweight gain for each bird in the pen and was used to calculate pen feed conversion ratio (FCR) as follows

$$FCR = \frac{Pen\ feed\ intake\ (g)}{Pen\ weight\ gain\ (g)}$$

Daily liveweight gain and daily feed intake were also calculated by dividing pen feed intake or pen liveweight gain by the number of 'bird' days (*i.e.* the number of birds in the pen multiplied by the number of days in the period of interest). Calculating these parameters on a bird day basis allows the comparison of feed intake and weight gain between pens with differing numbers of birds if, for example, mortality was higher in one pen than another.

The effects of experimental treatment on all performance data were analysed through ANOVA in Minitab 17, where means were separated by Tukey's test and statistical significance was denoted as $P<0.05$.

2.3. Bacterial profiling

2.3.1. Next-generation sequencing

DNA was isolated from caecal digesta using either a PowerSoil® DNA Isolation kit (Chapters 3 and 4; Thermo Fisher Scientific, MA, USA) or a GeneAll Exgene™ Stool SV kit (Chapters 5 and 6; Cambio Ltd, Cambridge, UK), following the manufacturer's instructions[1]. All DNA samples were tested for concentration and quality on a NanoDrop® nd-1000 spectrophotometer (NanoDrop Technologies Inc., DE, USA): a 260:280 ratio of 1.80 ± 0.15 with a minimum concentration of 10 ng/µL was deemed acceptable for downstream application. Any samples at a concentration below this threshold were subsequently concentrated through solvent evaporation to reduce volume. All DNA samples were normalised to a standard concentration of 10 ng/µL and loaded into a 96 well plate.

[1] The PowerSoil® DNA isolation kit was discontinued part way through sample analysis. The GeneAll Exgene SV kit was recommended by the manufacturer as an alternative – DNA obtained *via* this new kit was tested and was of comparable quality to that obtained from the PowerSoil® kit.

Sequencing was carried out at the Animal and Plant Health Agency (APHA, Surrey, UK). Aliquots of extracted DNA were amplified with universal primers for the V4 and V5 regions of the 16S rRNA gene using primers U515F (5'-GTGYCAGCMGCCGCGGTA) and U927R (5'-CCCGYCAATTCMTTTRAGT), designed to amplify both bacterial and archaeal ribosomal RNA gene regions (Ellis *et al.,* 2013). Forward and reverse fusion primers consisted of the Illumina overhang forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and reverse adapter (5'-GTCTCGTGGGCTCGGAGATGTGTAATAAGAGACAG) respectively.

Amplification was performed with FastStart™ HiFi Polymerase (Roche Diagnostics Ltd, West Sussex, UK) using the following cycling conditions: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 35 s, 72 °C for 1 min, followed by 72 °C for 8 min. Amplicons were purified using 0.8 volumes of AMPure® XP magnetic beads (Beckman Coulter, CA, USA). Each sample was then tagged with a unique pair of indices and the sequencing primer, using Nextera® XT v2 Index kits (Illumina, CA, USA), and KAPA HiFi HotStart ReadyMix (Kapa Biosystems, MA, USA) using the following cycling conditions: 95 °C for 3 min, 12 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 5 min. Index-tagged amplicons were purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter, CA, USA) and the concentration of each sample was measured using the fluorescence-based PicoGreen® assay (ThermoFisher Scientific, MA, USA). Concentrations were normalised before pooling all samples, each of which would be subsequently identified by its unique multiplex identifier. Sequencing was performed on an Illumina® MiSeq with 2 x 300 base reads according to the manufacturer's instructions (Illumina®, Cambridge, UK).

2.3.2. Data processing and statistics

Sequence reads were processed according to the microbiome-helper pipeline (https://github.com/mlangill/microbiome_helper/wiki/16S-standard-operating-procedure). Briefly, paired end reads were merged based on overlapping ends using

PEAR (http://sco.h-its.org/exelixis/web/software/pear/), before filtering the data for base-calling quality with a cut-off of 95%. The processed sequences were then classified using the pick open reference OTUs process implemented in QIIME v1.9.0 against the Greengenes 16S rRNA gene database.

Any samples containing <10% of the average number of reads for the dataset were excluded from analysis. The relative abundances of bacterial families were calculated as a percentage of total reads and were and analysed through LEfSe where $\alpha<0.05$ and an LDA threshold of 2.0 were applied (Segata *et al.,* 2011). Simpson's Diversity Index (*D*) was calculated for each sample to explore alpha diversity - differences between treatment groups were analysed through ANOVA in Minitab 17, where Tukey's test for pairwise comparisons highlighted differences between means where statistical significance was denoted as $P < 0.05$. Beta diversity was explored through weighted PCA, where score plots for the first two principal components were constructed to visualise differences in the profiles of the caecal microbiomes of birds from different treatments.

2.4. Metabolomics

2.4.1. ^{1}H NMR

100 ± 5 mg of each liver sample was weighed and added to 1000 µL 2:1 (v/v) MeOH:H_2O. Tungsten carbide beads (3 × 3 mm, Qiagen, Hilden, Germany) were added to each tube and samples were lysed at 50 oscillations/s for 10 min in a TissueLyser (Qiagen, Hilden, Germany). Samples were centrifuged at 13,000 rpm for 10 min at 5 °C in a Heraeus Megafuge 16R (ThermoScientific, USA). The supernatant was transferred to a clean tube and concentrated in a Concentrator Plus speed vacuum (Eppendorf, Hamburg, Germany) at 45 °C until dry. The pellet was then reconstituted in 600 µL 9:1 (v/v) D_2O:H_2O containing 0.05% (w/v) trimethylsilylpropanoic acid (TSP) as a reference, and 0.2 M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4). Samples were centrifuged at 8000 rpm for 10 min to remove any debris.

600 µL of the supernatant was transferred into an NMR tube with a diameter of 5 mm and stored at 5 °C pending analysis.

Metabolic profiling was performed using ^{1}H NMR spectra measured by an Avance III HD 700 MHz spectrometer (Bruker). A standard one-dimensional NOESYPR1D pulse sequence (pulse length of 16.96 µs and total acquisition time of 2.17 s) with water presaturation applied during relaxation delay (3 s) and a mixing time of 0.01 s was used. For each sample, 128 scans were recorded, each with 65 k data points and a spectral width of 16 ppm.

2.4.2. Data processing and statistics

NMR spectra were Fourier Transformed and manually phased, calibrated to TSP (δ = 0.00 ppm) and stacked using MestReNova (2013 Mestrelab Research). Stacked spectra were imported into MATLAB v.9.1 (R2013b, The MathsWorks Inc.) where data outside of 0.5 < δ < 10 ppm and the signals for D_2O (δ = 4.80s) and any residual methanol (δ = 3.34s) from tissue extractions were removed. Data were normalised using median-based probalistic quotient methods (Korrigan Toolbox) and OPLS-DA models were constructed. $R^2Y > 0.8$ was indicative of a good model, with $Q^2 > 0.5$ indicating good predictive ability. PCA score plots and OPLS correlation plots were also produced to visualise differences in the metabolic profile between treatment groups.

Differentially represented peaks (‘warm’ colours) and peaks assigned to SCFA (acetate, propionate and butyrate) and lactate (**Table 2.5**) were integrated in MATLAB. The concentrations of metabolites per gram of starting material were calculated by comparing against TSP of a known concentration. Concentrations were analysed through ANOVA in Minitab 17, where Tukey’s test for pairwise comparisons highlighted differences between means where $P < 0.05$.

Table 2.5 *^{1}H NMR peaks assigned to short chain fatty acids and lactate, based upon the Human Metabolome Database. (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet).*

	Peak position (ppm)	Splitting pattern	Coupling constant, *J* (MHz)
Acetate	1.91	s	-
Propionate	1.04	t	7.68
	2.17	q	7.66
Butyrate	0.88	t	7.39
	1.55	m	-
	2.14	t	7.33
Lactate	1.40	d	7.00

2.5. Multi '-omics' correlations

Correlations between microbial taxa and metabolite concentrations, as identified through OPLS-DA and peak integrations were investigated in R-studio. The minimum threshold for significant relationships was obtained from a table of critical values for Pearson's *R*, where $\alpha < 0.05$. Should an *R* value meet this value, the null hypothesis was rejected since a correlation would occur by chance just 5% of the time. The resulting *R* values were plotted as a heat map in MATLAB to illustrate relationships between the caecal microbiome and hepatic metabolome.

3. Study 1 - Effect of medication: antibiotic and probiotic treatment

3.1. Introduction

Until 2006, antibiotics were routinely used as growth promoters in poultry production systems (Broom, 2017). Due to rising levels of antimicrobial resistance, such usage has now been banned in the EU, but antibiotics are still utilised to prevent and cure bacterial infections. Though it depends upon the class and dose of antibiotic and the age of the bird, numerous authors have found an alteration in bacterial load and diversity following antibiotic treatment (Zhou *et al.,* 2007; Danziesen *et al.,* 2011; Lozupone *et al.,* 2012; Lin *et al.,* 2013) with some species struggling to recover following cessation of dosing (Stanley *et al.,* 2014).

Producers are turning to alternative products to enhance production efficiency and reduce the incidence of disease, including probiotics which are defined as *"viable microorganisms used as feed additives, which could lead to beneficial effects in broilers by improving microbial balance or properties of the indigenous microflora"* (Fuller, 1989). An array of bacterial species including *Lactobacillus* and *Streptococcus* have been found to improve performance (Nusairat *et al.,* 2018; Tayeri *et al.,* 2018), reduce pathogen load (Saint-Cyr *et al.,* 2017; Nusairat *et al.,* 2018), induce beneficial histological changes (Khatun *et al.,* 2017; Manafi *et al.,* 2018) and enhance immunological efficacy (Wang *et al.,* 2018). Evidence suggests that antibiotic prophylaxis can also alter the composition of the gut microbiome, and therefore have downstream effects on factors such as physiology, and immunity (Belote *et al.,* 2018).

3.2. Aims

The present study aimed to:

- Investigate the influence of dosing growing Ross 308 broiler chicks with a mixed probiotic, Linco-spectin (LS), either alone or followed by a mixed probiotic, Aviguard®, upon the gut microbiome.

- Explore the effects of treating birds either with LS alone, or LS followed by Aviguard® on the profile of the hepatic metabolome.
- Explore relationships between hepatic metabolites and caecal bacteria altered by antibiotic/probiotic treatment.

LS was selected as the antibiotic of choice for this study since it is a mixture of lincomycin, a lincosamide, and spectinomycin, an aminoglycoside. These antibiotics are given as a mixture since each targets Gram-positive and Gram-negative bacteria respectively, and therefore affect a wide range of potential pathogens. Since the study aimed to explore the effects of post-antibiotic administration of Aviguard®, a separate probiotic control group was not included. Physical experimental constraints also limited study space at the time of the experiment, meaning that it was impossible to house additional pens to explore a fourth treatment group.

3.3. Results and discussion

3.3.1. Performance

Effects of treatment on performance parameters are summarised in **Table 3.1**. FCR, daily feed intake and daily liveweight gain were calculated on a pen basis (n=6/treatment). Liveweight was calculated on an individual bird basis, though the number of replicate units varied with the progression of the study, since birds were sacrificed for biological sampling at days 4 (four birds/pen), 9, 15 and 29 (two birds/pen). The number of individual birds per pen therefore declined over time. Daily feed intake and FCR were not calculated between days 1 to 10, since feed was offered from shallow trays on the floor and it was therefore difficult to accurately measure feed consumption.

AB birds weighed less at days 10 and 15 than controls and AB+PRO birds, and gained significantly less weight during this period. AB birds also consumed less feed, resulting in a similar FCR value to controls. Although AB+PRO birds gained the same amount

of weight as controls during this time period, they consumed slightly less feed and therefore their FCR tented to be lower (*P*=0.079). AB birds also tended to gain less weight than controls and AB+PRO birds between days 15-22, although this was not associated with a difference in either feed intake or FCR. There was no effect of treatment on bird performance between days 22-29.

Table 3.1 *ANOVA exploring the effects of treatment (AB, Linco-spectin days 1-3; AB+PRO, Linco-spectin days 1-3 + Aviguard® day 5) on bird liveweight, where individual birds were replicate units, and daily liveweight gain, daily feed intake and FCR, where pens were replicate units (n=6/treatment). Statistical significance was denoted as P<0.05 and means were separated by Tukey's test.*

	Treatment group				
	Control	**AB**	**AB+PRO**	**SEM**	***P*-value**
Liveweight (g)					
Day 1	42.9	42.2	-	0.29	0.252
Day 4	72.3	68.2	-	1.16	0.090
Day 10	158	155	159	2.2	0.757
Day 15	351	328	350	4.3	0.080
Day 22	771	711	787	12.0	0.072
Day 29	1305	1280	1350	20.4	0.423
Daily feed intake (g)					
Days 10-15	50	46	48	0.7	0.108
Days 15-22	89	83	88	1.9	0.459
Days 22-29	122	118	122	2.4	0.766
Daily liveweight gain (g)					
Days 10-15	38^{a}	34^{b}	38^{a}	0.4	0.030
Days 15-22	59	52	59	1.1	0.078
Days 22-29	77	76	78	1.8	0.929
FCR (g/g)					
Days 10-15	1.328	1.338	1.243	0.0142	0.079
Days 15-22	1.522	1.602	1.535	0.0227	0.408
Days 22-29	1.582	1.553	1.575	0.0101	0.552

Gao *et al.* (2017) found an improvement in FCR in birds dosed with chlortetracycline and salinomycin, caused by an increase in liveweight gain. The opposite effect on liveweight gain was observed in the present study, suggesting that the antibiotic mixture or factors such as diet or stress may influence the effects of antibiotics. That said, the conditions were well controlled but FCR was a little lower than anticipated for industry standards suggesting diet and/or stress may have been influential. The effects of probiotics on performance reported in the literature are varied, with inconsistent evidence of improvements but also a lack of effect being reported in different studies (Gao *et al.,* 2017; Wang *et al.,* 2017). The data in the present study suggested that any marginal reduction in performance associated with the use of LS was ameliorated by Aviguard®. However, none of the differences observed were statistically significant and any trends observed would need considerably larger studies to generate the necessary statistical power to test this possibility.

It should be noted that a mixture of both male and female birds were used in this study: Aviguard® appeared to have more of an effect on the liveweights of males, with trends noted during the grower phase at days 15 (P=0.054) and 22 (P=0.066), suggesting an interaction between bird sex and treatment. It is generally accepted that male birds grow faster than females (Aviagen, 2017) - since birds were randomly placed at day 1, there may have been differences in the proportion of males and females within each treatment group, subsequently affecting average liveweight. The use of single sexed birds will be considered for future work.

This trial was primarily designed to investigate the effects of treatment on gut health, rather than performance, thus the growth data as discussed above were a little lower than industrial expectations. Numerous factors may have affected growth efficiency, including an abrupt change in feed base from wheat to maize at the end of the starter phase. The trial was also conducted alongside another study to reduce costs, introducing a number of variables despite best efforts to ensure barriers between study

groups. Birds were also housed differently to those in a commercial environment, with a reduced stocking density and increased interaction with investigators during weighing and feed changes. Despite this, all birds were effectively treated identically in this study and so the results between treatment groups can still be compared justifiably. What cannot be truly compared, however, is the performance data recorded in this study with industrial standards, where expected FCR stands slightly lower than was observed in the present study, at roughly 1.4 (Aviagen, 2017). The effects of LS and Aviguard® should also be investigated in a commercial environment to confirm these data.

3.3.2. NGS: alpha diversity

Unfortunately, a number of samples failed the sequencing process: at day 9, only three antibiotic samples were successfully analysed, despite a total of 6 samples per treatment being sequenced. This reduces the reliability of results since sequencing outputs may not be truly representative of the treatment group, and variation between samples may have more of an effect on the statistical significance of results compared with a larger dataset. This should be noted when interpreting the presented data.

Alpha diversity, measured by Simpson's Diversity Index, is illustrated in **Figure 3.1**.

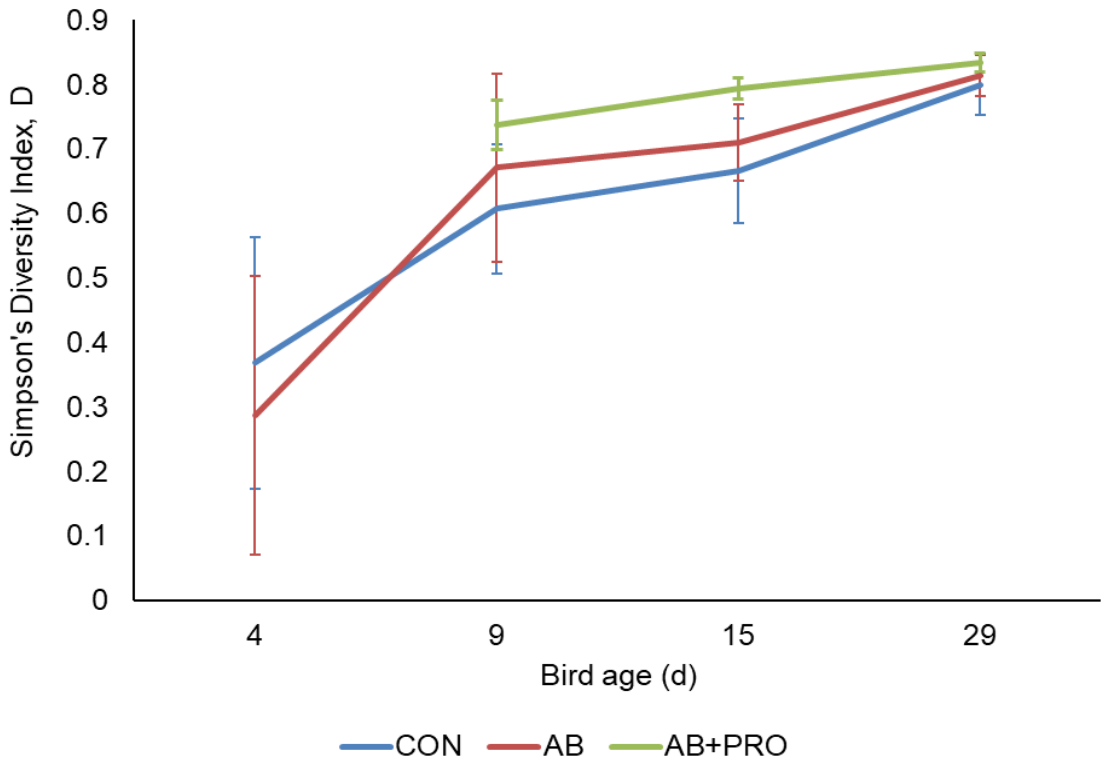


Figure 3.1 *Line plot illustrating the effects of age and treatment on alpha diversity, reported as Simpson's diversity index. Error bars represent standard deviation with respect to treatment. (CON, control; AB, Linco-spectin days 1-3; AB+PRO, Linco-spectin days 1-3 + Aviguard® day 5).*

Alpha diversity significantly increased with age in all treatment groups (P<0.001), a widely-reported, and therefore expected, effect (Ranjitkar *et al.,* 2016). Diversity increased between days 4 and 9 in all birds (controls, P=0.027; AB, P=0.063). Though diversity increased by similar amounts between days 9 and 15 in all treatment groups, significance was only observed in AB+PRO birds (P=0.027). Significance was not observed in controls or AB birds (P=0.319 and 0.571, respectively) because of wide variation between individual birds, suggesting a stabilising effect of Aviguard® on measures of alpha diversity.

LS treatment numerically reduced average diversity by 0.081 in comparison with controls at day 4, though between-bird variation reduced statistical significance (*P*=0.599). Langdon *et al.* (2016) reported that a plethora of antimicrobials reduce bacterial diversity, making this numerical finding unsurprising. A lack of significant differences between AB and controls from day 9 onwards (*P*>0.05) suggests a 'replacement' effect following the cessation of antibiotic treatment, an observation concurrent with findings by Grazul *et al.* (2016). The subsequent administration of Aviguard® following LS treatment significantly increased diversity in comparison with controls at days 9 and 15 (*P*=0.014 and 0.019, respectively), again concurrent with Grazul *et al.* (2016) and suggesting that a mixed probiotic allowed diversity to recover at a slightly accelerated rate than if left to recover naturally through the provision of replacement microbes. Diversity was equivalent in all birds by day 29 (*P*=0.390), suggesting that the effects of LS and Aviguard® on comparative diversity were not long-lasting. Given these birds would achieve table weight by 35-42 days, it would seem probable that the diversity observed at day 29 would likely be maintained until slaughter.

3.3.3. NGS: beta diversity

Beta diversity was explored through unsupervised principal component analysis. Discrete clustering of data points in the first two principal components is illustrated in **Figure 3.2** and suggests that the general profile of the caecal microbiome was altered by age, irrespective of treatment. Overlapping of data points from days 9 and 15 in controls and AB birds suggests less of an effect of age than in AB+PRO birds, where very discrete clustering was observed. It is hypothesised that Aviguard® had something of an acceleratory effect on the rate of development of bacterial diversity. Despite these differences between treatment groups, these plots clearly illustrate the effects of bird age on bacterial diversity and successional nature of the gut microbiome.

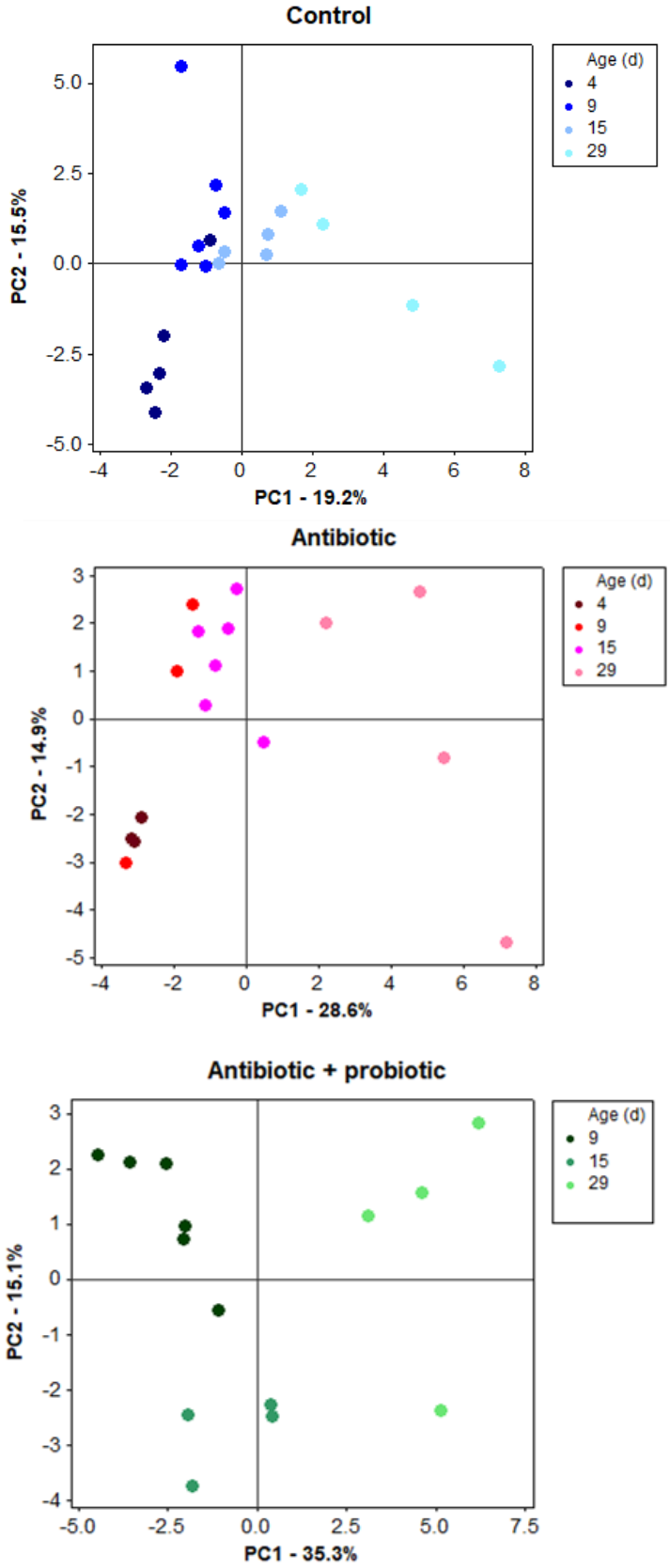


Figure 3.2 *PCA score plots illustrating the effects of bird on beta diversity of caecal bacterial populations of growing broilers, profiled by Illumina® sequencing. Differential clustering of datapoints suggests differences in the profiles of caecal bacterial populations. (Antibiotic, Linco-spectin days 1-3; Antibiotic + probiotic, Linco-spectin days 1-3 + Aviguard® day 5).*

The effects of treatment on beta diversity are illustrated in **Figure 3.3.**

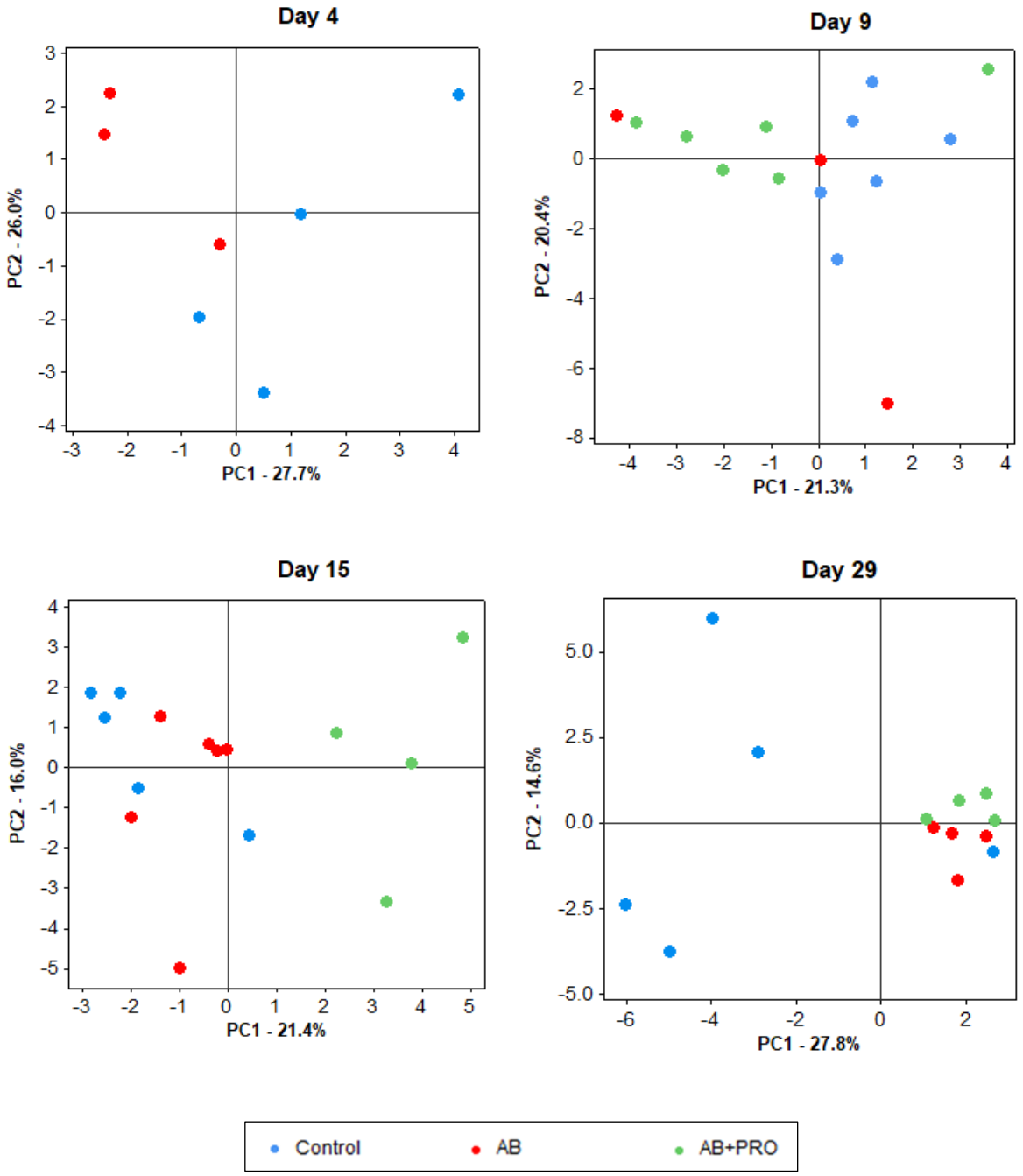


Figure 3.3 *PCA score plots illustrating the effects of treatment on beta diversity of caecal bacterial populations of growing broilers, profiled by Illumina® sequencing. Differential clustering of datapoints suggests differences in the profiles of caecal bacterial populations. (AB, Linco-spectin days 1-3; AB+PRO, Linco-spectin days 1-3 + Aviguard® day 5).*

LS appeared to alter the profile of the caecal microbiome at day 4 compared with controls, a difference that continued to day 9 and was exacerbated by the subsequent administration of Aviguard®. By day 15, controls and AB birds appeared to be similar with both groups clustering and exhibiting differences to AB+PRO. By day 29, however, AB and AB+PRO clustered differently from controls. Though work specifically investigating the effects of LS and Aviguard® in the broiler chicken has not been carried out, Gao *et al.* (2017) report that dosing with *Lactobacillus plantarum* as a probiotic increases, but antibiotics decrease, the rate of maturation of the intestinal microbiome of broilers. Though the effects of sequentially dosing with an antibiotic and a probiotic were not assessed, their results generally support the observations made in this study.

The data presented here suggest that administration of LS initially altered the profile of the microbiome, though it appears that AB birds were able to recover to match that of controls by day 15. Despite this, differences reappeared by day 29, suggesting that antibiotic prophylaxis in young birds altered the capacity for development of the microbiome in later life. Aviguard® did not restore the profile of the microbiome to match that of controls at any point, though matched that of AB birds by day 29. LS provided an opportunity for the microbiome to develop beyond that of controls, an effect that may have been accelerated by Aviguard®. It appears that, if given the opportunity to develop naturally, a bird dosed solely with LS will eventually reach a similar microbial profile of a bird subsequently dosed with Aviguard®, beyond that of a bird withheld from any treatment.

Despite these general observations, the scattering of data points within a treatment group represented variation between birds, perhaps caused by differences in bacterial intake. It was assumed that all birds would consume the same volume of medicated water, though it is likely that variation between individuals existed and therefore that birds were dosed inconsistently. In an ideal system, birds would be dosed individually,

though this would involve individual housing which was not possible in the current system due to experimental constraints and the necessity for young birds to huddle in a flock.

Previous investigations into the relationships between diversity and performance have been inconsistent, with suggestions that the microbiome of highly performing birds may be varied or consistent between individuals (Geier *et al.,* 2009; Torok *et al.,* 2011; Stanley *et al.,* 2012). Given the diversity of organisms, there is likely to be much metabolic redundancy shared across the microbiome such that loss of one organism with its metabolic contribution could be readily substituted by another unrelated organism that possessed similar metabolic functionality. Liveweight gain was significantly reduced in AB birds between days 15-22, though inconsistent clustering of datapoints in **Figure 3.3** suggests a lack of relationship with beta diversity. These findings suggest that variation in the caecal microbiome may not be linked to growth efficiency. Indeed, this concept could be inverted to suggest that many different caecal microbiota can achieve the same overall growth outcome.

3.3.4. NGS: population analysis

NGS data were analysed through LEfSe to highlight taxa that were differentially abundant between treatment groups. These taxa are highlighted in **Appendix 1**.

3.3.4.1. Day 4

LEfSe analysis indicated that there were few differences between the caecal microbiomes of controls and AB birds at day 4 (**Figure 3.4**). LS significantly reduced the relative abundance of *Lachnospiraceae* (0.3% *vs* 12.1%; *P*=0.025) where all genera were equally affected with *Ruminococcus* largely contributing to the difference observed in family abundance. The effects of LS on *Lachnospiraceae* are undocumented, but these results suggest sensitivity to antibiotic treatment.

LS also appeared to reduce levels of *Ruminococcaceae*, *Erysipelotrichaceae* and *Clostridiaceae* and increase levels of unknown *Clostridiales*, although these differences were not statistically significant, due in large part to the high levels of variation between birds. This may be a result of differences in LS dosing, since medicated water was administered on a pen, rather than individual, basis, and it was therefore merely assumed that all birds consumed an equal amount of antibiotic.

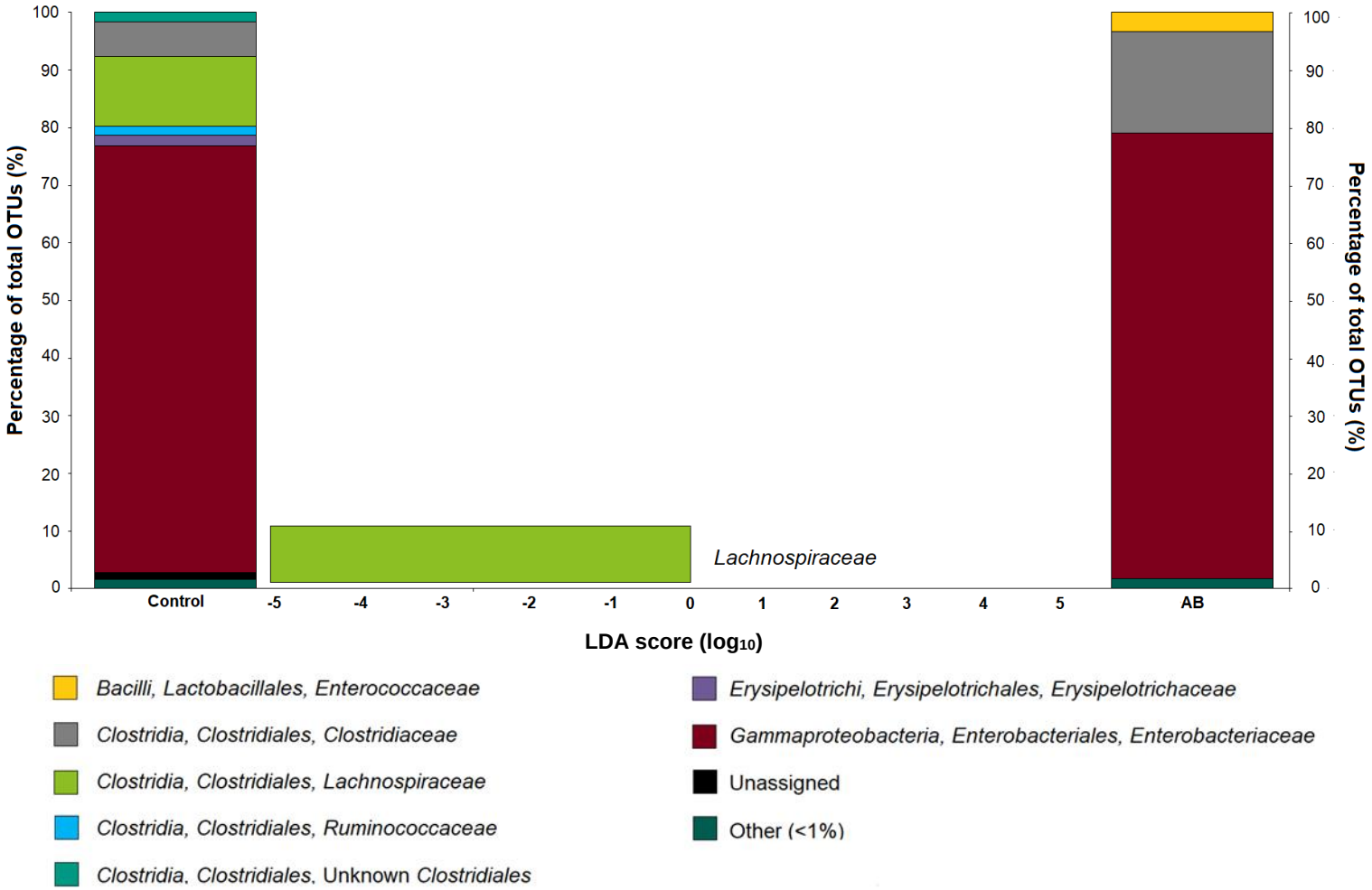


Figure 3.4 *NGS data comparing controls and AB at day 4.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

3.3.4.2. Day 9

Significant differences in taxa abundance were observed between controls and treated birds (**Figures 3.5 and 3.7**), but not between AB and AB+PRO (**Figure 3.6**), meaning that Aviguard® did not immediately alter the composition of the caecal microbiome following LS treatment. The stacked bar chart comparing AB and AB+PRO in **Figure 3.6** suggests an effect of treatment, but high levels of variation between samples reduced statistical significance. This may be a result of differences in dosing, since it was impossible to guarantee that all birds were dosed equally in the experimental setup.

The relative abundance of *Ruminococcaceae* was significantly depleted in AB birds compared with controls (13.0% compared with 26.8%; P=0.046). Aviguard® increased levels to 22.2%, though this was not significantly different from either controls or AB due to variation between birds. This suggests that bacteria were unable to fully and consistently recover following antibiotic treatment.

Alcaligenaceae, a family from class *Betaproteobacteria*, were found in LS-treated birds alone and were found at significantly higher levels in both AB and AB+PRO than controls (P=0.010 and P=0.011, respectively). It is thought that LS may have depleted other species, providing a niche in which *Alcaligenaceae* could reside. These bacteria also accounted for 3.5% of OTUs in Aviguard® itself, suggesting the probiotic as the source of these bacteria. Levels tended to be slightly, but not significantly, higher in AB+PRO than Aviguard®, suggesting that bacteria were able to colonise and begin to proliferate in the caeca.

Enterobacteriaceae*,* from class *Gammaproteobacteria*, appeared to be elevated in AB birds compared with controls, but inconsistencies between biological repeats (*i.e.* individual birds) resulted in a lack of statistical significance. Enterobacteriaceae were attributed to just 0.03% of OTUs in Aviguard®, but an average 19.2% in AB+PRO

birds. This suggests that these bacteria are likely to be of environmental origin, particularly since there was a lack of difference between AB and AB+PRO. It is likely that LS treatment reduced the abundance of other taxa, allowing Enterobacteriaceae to colonise in their place. This is perhaps of concern as many members of this family are opportunistically pathogenic. Is it possible that early life exposure to LS, or perhaps any antibiotic, may permit an unintended increase in pathogen content in the gut with avian pathogenic *Escherichia coli* (APEC) just one example of a natural resident of the chick gut that is an opportunistic pathogen.

Families from the order *Lactobacillales* were affected by treatment. *Enterococcaceae* were reduced, albeit insignificantly, from 1.1% to 0.3% by LS treatment but were not affected further by administration of Aviguard®. Relative abundances of *Lactobacillaceae* were not significantly different between controls and AB birds (1.6% and 2.4% respectively). These bacteria were absent at day 4 so it is thought that their ability to colonise was not affected by antibiotic prophylaxis. *Lactobacillaceae* were found at almost identical relative abundances in AB+PRO birds (3.1%) and Aviguard® itself (3.2%), suggesting that the bacterial population of *Lactobacillaceae* in the probiotic efficiently colonised in the caecum.

Though found at low levels, *Bifidobacteriaceae* were found at significantly higher proportions in AB+PRO than controls (0.07% compared with 0.002%; P=0.029). Since these bacteria were found at intermediate levels of 0.04% in AB samples, it is hypothesised that LS treatment created an opportunity for colonisation which was exacerbated by the ingestion of *Bifidobacteriaceae* from Aviguard®. Similarly, *Veillonellaceae*, identified at an average of 12.1% in Aviguard®, were only found in AB+PRO birds, although at much lower proportions at just 0.7% (P=0.041). This suggests that some bacteria were unable to fully colonise the caeca, perhaps due to restrictions on nutrient availability or colonisation sites.

The stacked bar chart in **Figure 3.5** shows a numerical increase in *Clostridiaceae* in birds treated with LS compared with controls (10.8% compared with 0.3%), although this observation was not statistically significant because of a single AB sample where reads accounted for 28.7% of total OTUs. Despite *Clostridiaceae* being the most abundant family sequenced from Aviguard® at an average of 20.9%, the proportions observed in AB+PRO birds were unaffected by probiotic treatment.

'Unassigned' bacteria that could not be taxonomically identified by the Greengenes database were only found in treated birds. Though accounting of 0.2% of OTUs identified in Aviguard® , these bacteria were found in both AB and AB+PRO birds and may therefore have been of environmental origin. Since the identities of these bacteria could not be assigned, their biological effects could not be hypothesised.

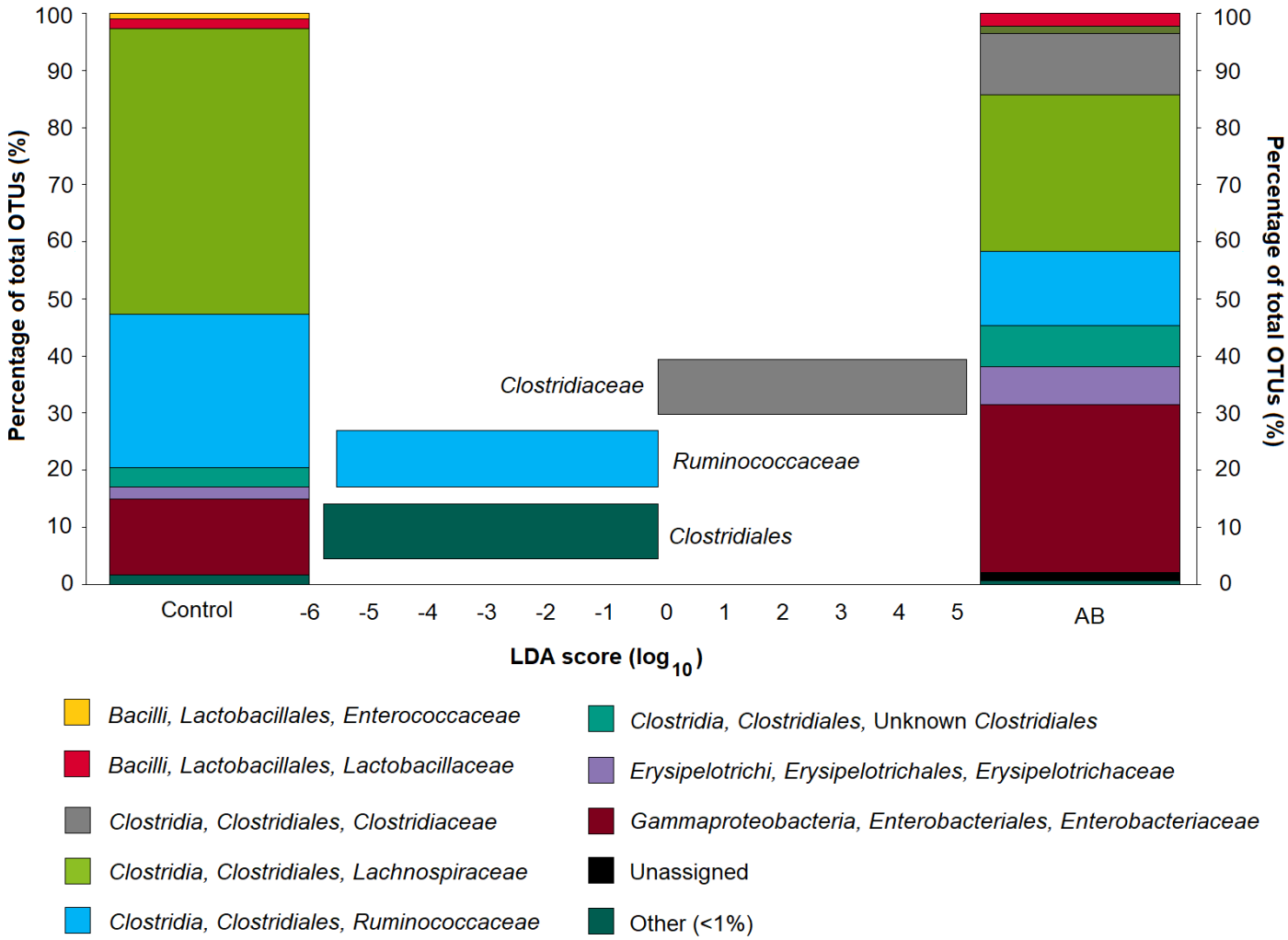


Figure 3.5 *NGS data comparing controls and AB at day 9.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

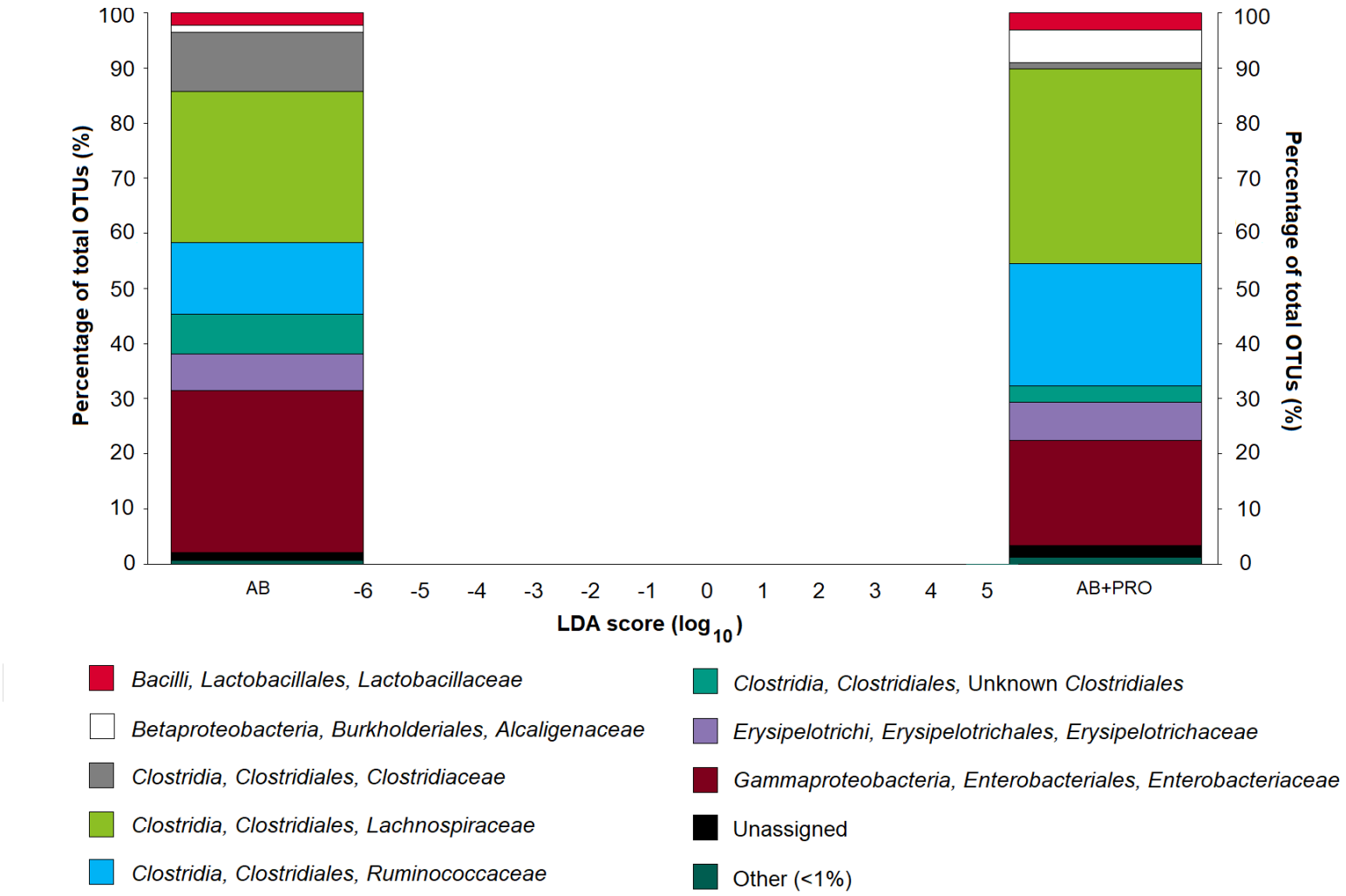


Figure 3.6 *NGS data comparing AB and AB+PRO at day 9.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

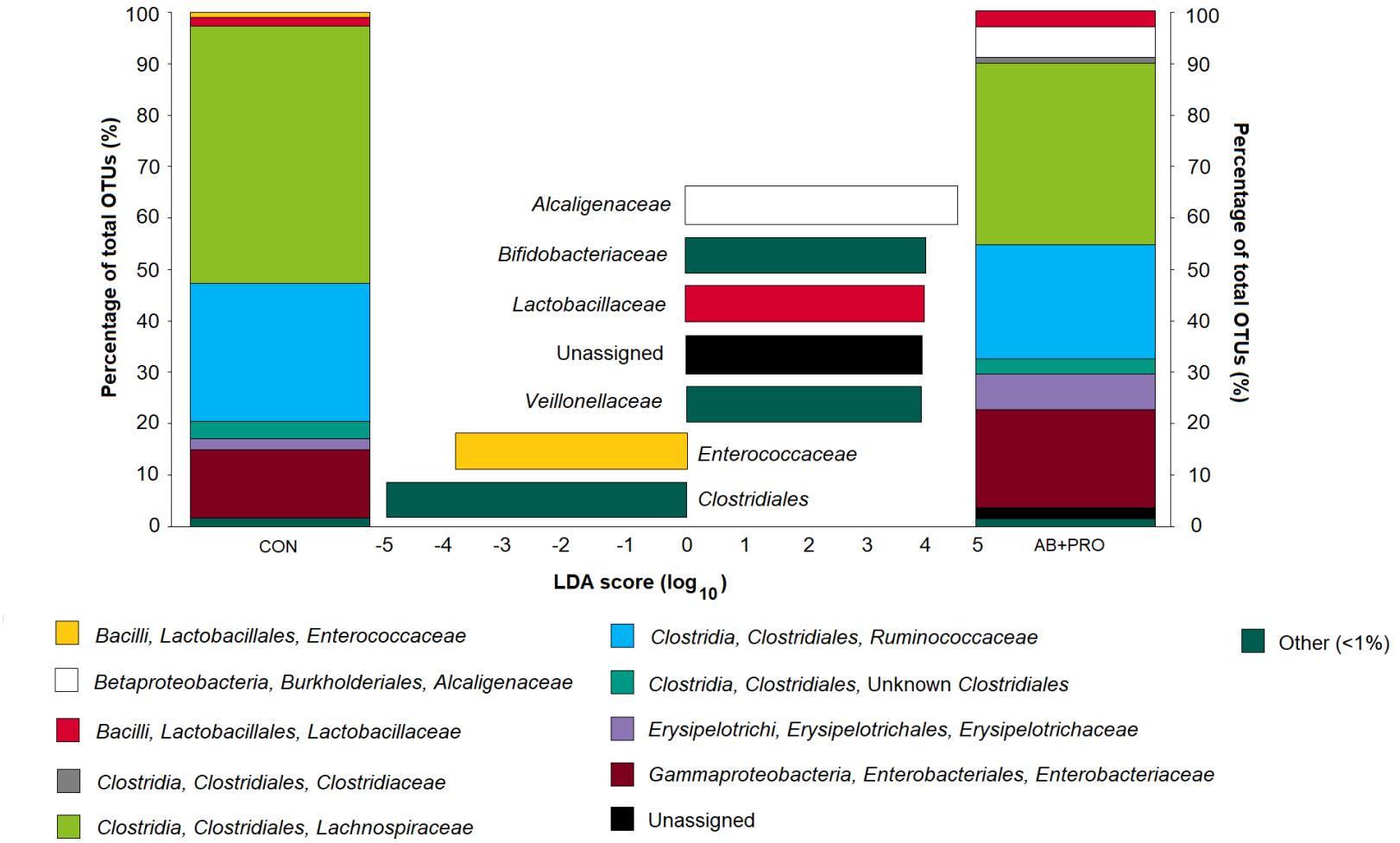


Figure 3.7 *NGS data comparing controls and AB+PRO at day 9.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

3.3.4.3. Day 15

As at day 9, *Ruminococcaceae* were significantly depleted in AB birds at an average of 36.8% compared with 50.0% in controls (*P*=0.019; **Figure 3.8**). This suggests that the effects of LS were not reversed following the withdrawal of LS. Despite accounting for 37.9% of OTUs in Aviguard®, its subsequent administration furthered this depletion to an average of 24.5% (*P*=0.014; **Figure 3.9**), suggesting that probiotic treatment exacerbated the effects of LS. This is a somewhat counterintuitive finding for which explanations are difficult to suggest. *Ruminococcaceae* are a highly diverse group – it is possible that different, non-isofunctional, taxa were selected in those supplemented by Aviguard® and thereby failed to colonise. This is worthy of further analysis.

Levels of *Alcaligenaceae* were biased towards AB+PRO birds compared with both controls (*P*=0.010) and AB (*P*=0.014) and were similar to those found in Aviguard®. Unlike day 9, however, these bacteria were found in AB as well as AB+PRO, suggesting that Aviguard® may have merely advanced bacterial colonisation. Their absence in controls suggests that colonisation depends upon antibiotic treatment.

Enterobacteriaceae were found at similar levels in controls and AB birds but were depleted by Aviguard® (*P*=0.019). Since Enterobacteriaceae accounted for just 0.03% of OTUs in Aviguard® itself, it is hypothesised that caecal levels are highly sensitive to change and fluctuate depending on exposure and competition from other taxa. Given the discussion above on LS enhancing Enterobacteriaceae in early life, the impact of Aviguard® may be highly protective against pathogenic types of that family.

A number of differentially abundant taxa were found at particularly low levels, including *Bacteroidaceae* (0.25% in AB compared with 0.02% in controls; *P*=0.011) and *Rikenellaceae* (0.2% in AB compared with 0.05% in controls; *P*=0.028). Some taxa including *Eubacteriaceae* and *Veillonellaceae* were found in AB+PRO alone, suggesting that bacteria were of probiotic origin.

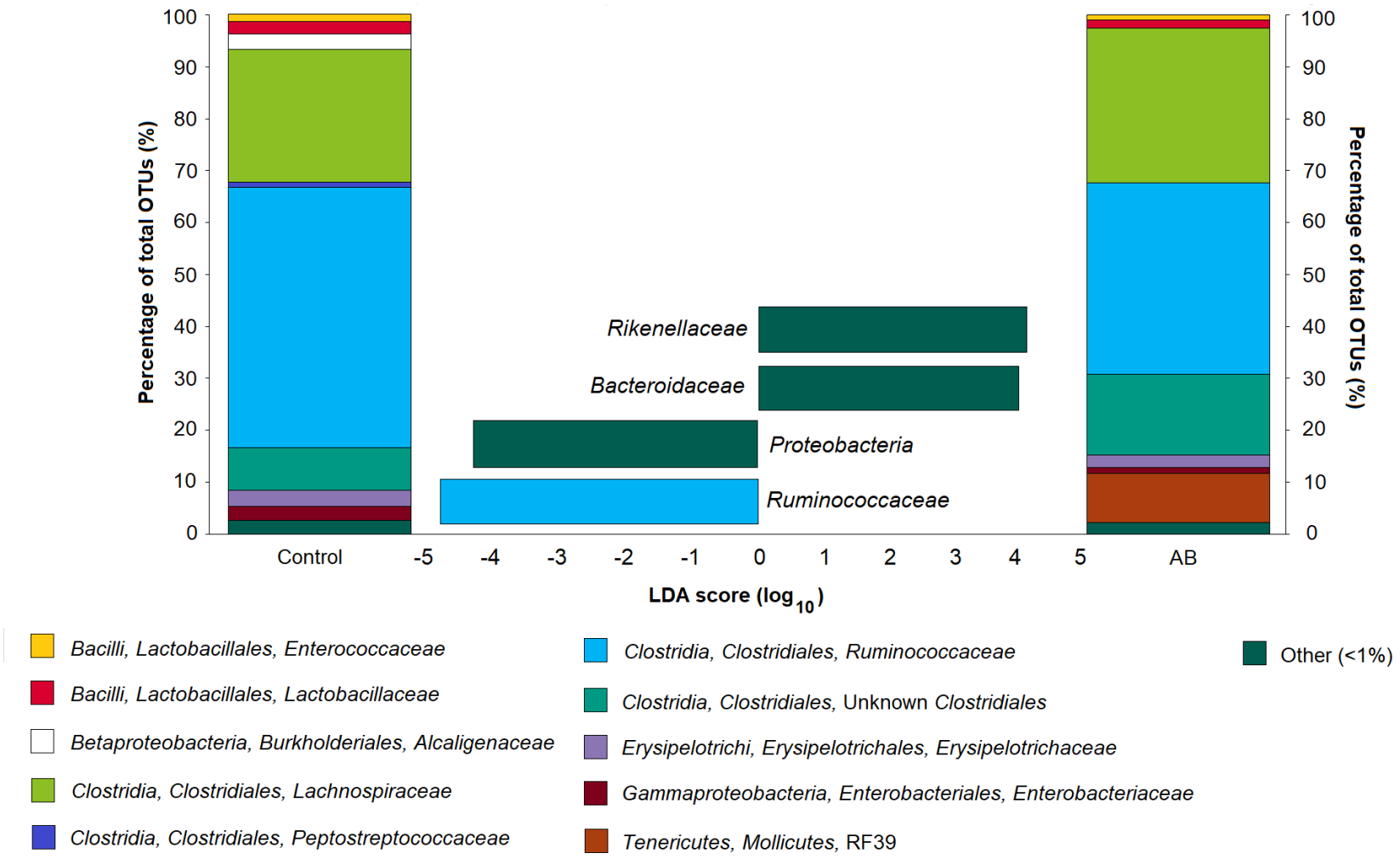


Figure 3.8 *NGS data comparing controls and AB at day 15.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

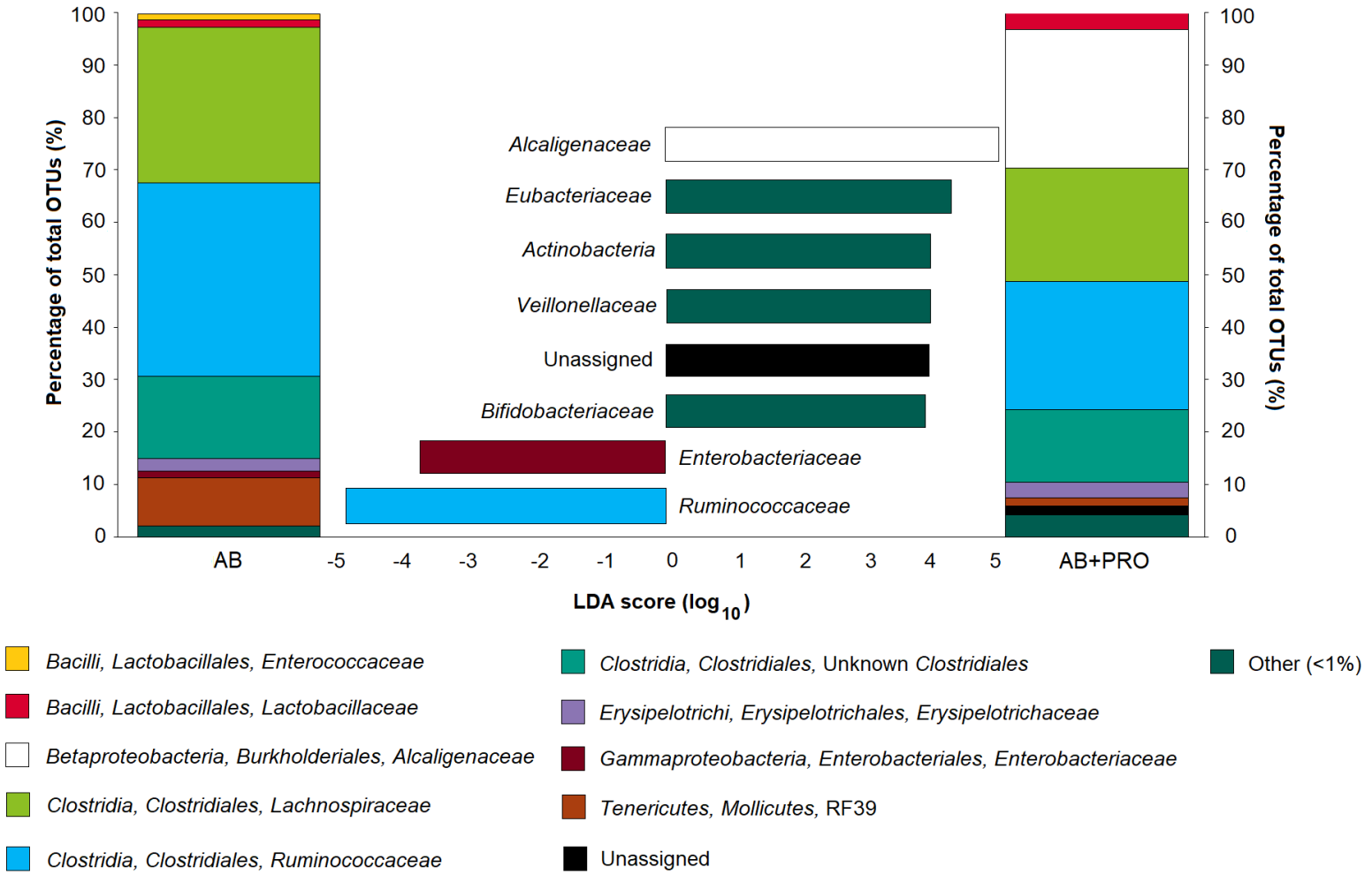


Figure 3.9 *NGS data comparing AB and AB+PRO at day 15.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

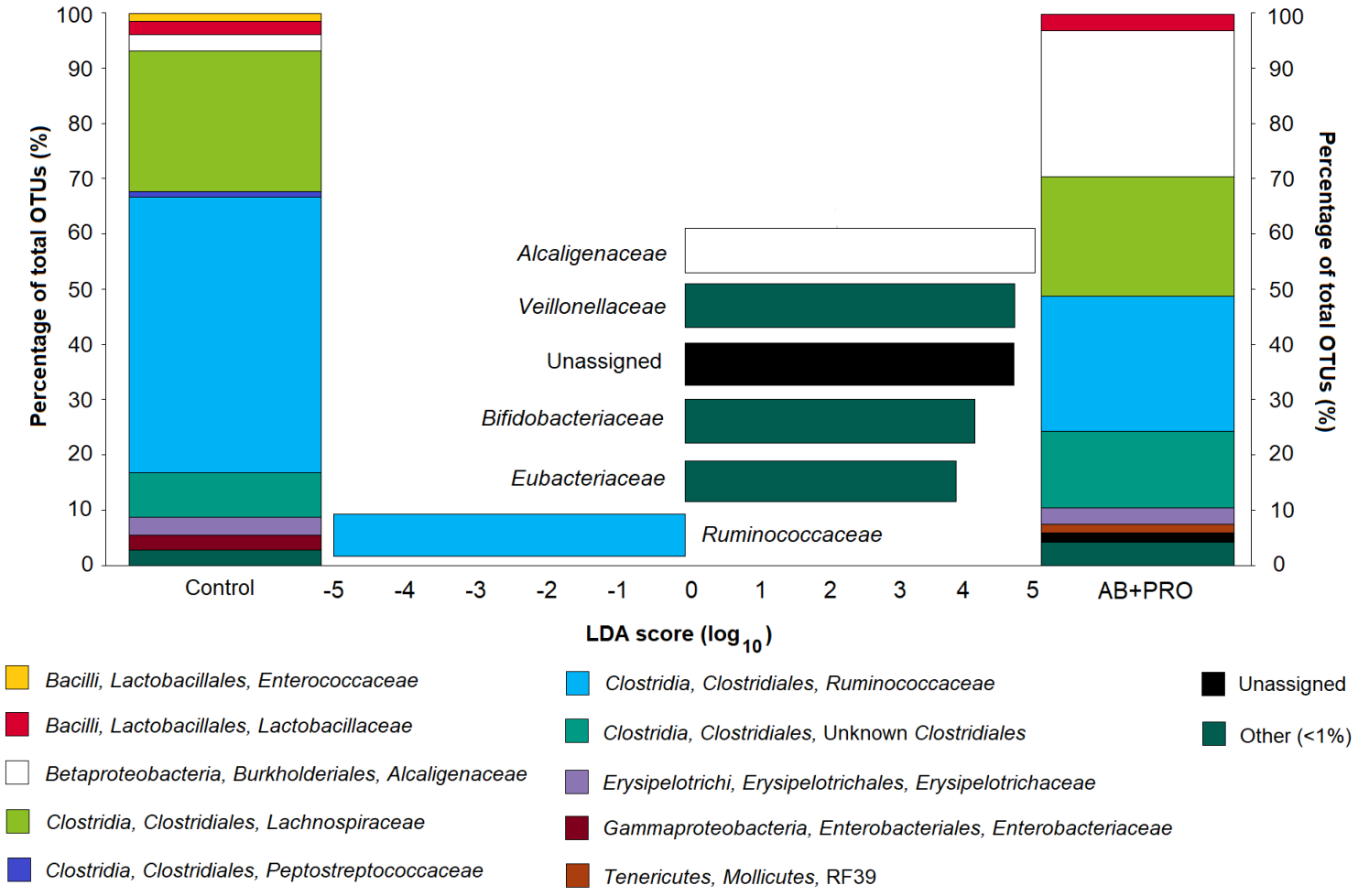


Figure 3.10 *NGS data comparing controls and AB+PRO at day 15.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

3.3.4.4. Day 29

Differences in *Lachnospiraceae* dissipated by day 29, as proportions were similar across all three treatment groups. Unassigned bacteria continued to be more abundant in AB+PRO than both controls and AB birds (**Figures 3.12 and 3.13**).

Levels of *Alcaligenaceae* were similar between AB and AB+PRO but were significantly higher in both groups compared with controls (P=0.011 and P=0.014, respectively), supporting the earlier hypothesis that colonisation depends upon antibiotic treatment. A number of families were identified in AB and AB+PRO samples only and were therefore found at significantly higher levels than controls, including *Eubacteriaceae* and *Veillonellaceae*. *P*-values for these differences are listed in **Appendix 1**.

Enterobacteriaceae continued to be biased towards controls compared with both AB (P=0.014; **Figure 3.11**) and AB+PRO (P=0.014); **Figure 3.13**), suggesting that the effects induced by the administration of LS in early life extend into later life. Since Enterobacteriaceae contains genera including *Salmonella* and *Escherichia* that are generally associated with human and avian disease, there is the possibility that LS treatment reduced the food-pathogen carrying capacity of birds, though the full identities of bacteria responsible for differences in Enterobacteriaceae could not be elucidated. Further work to investigate this effect is warranted.

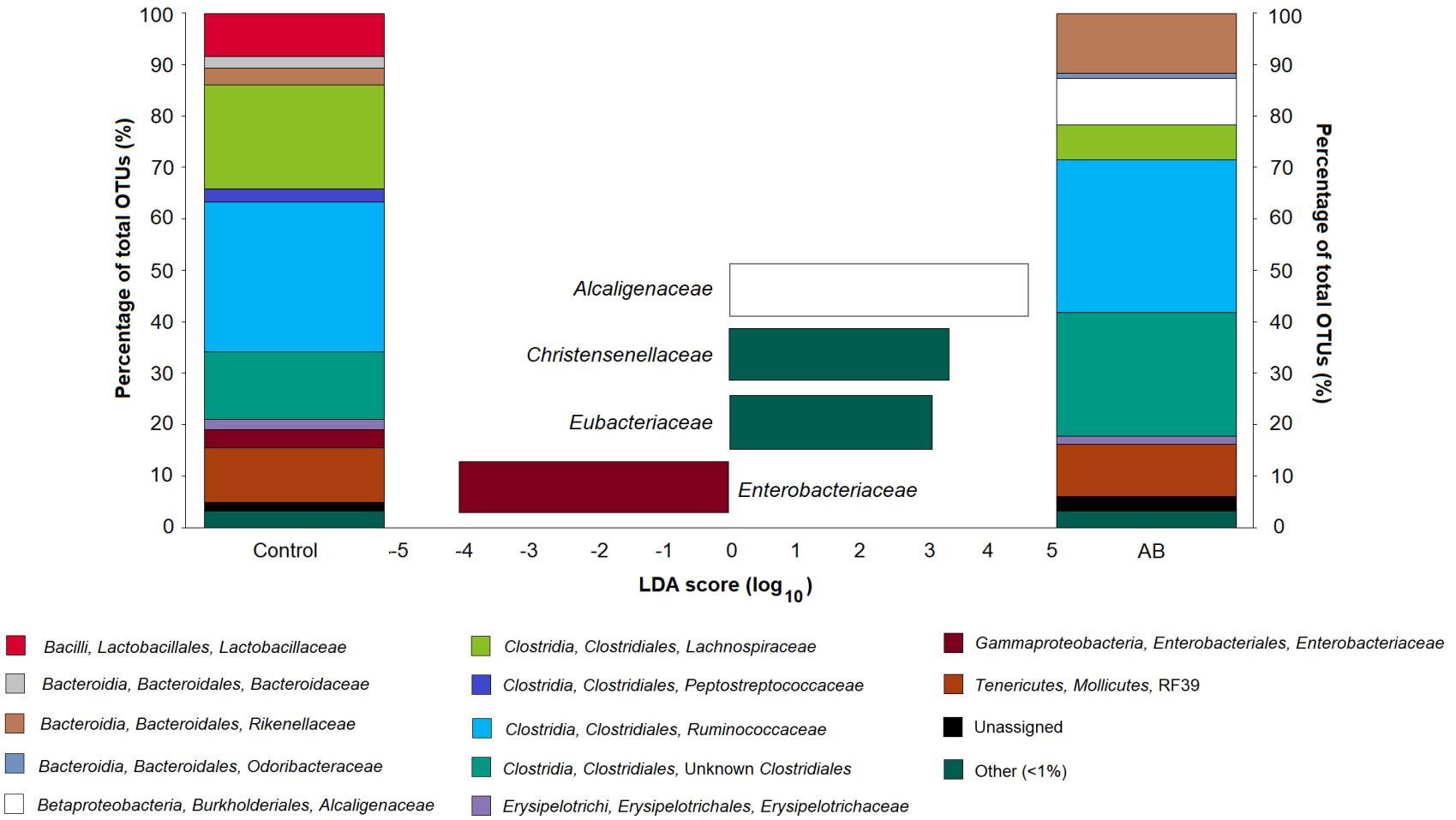


Figure 3.11 *NGS data comparing controls and AB at day 29.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

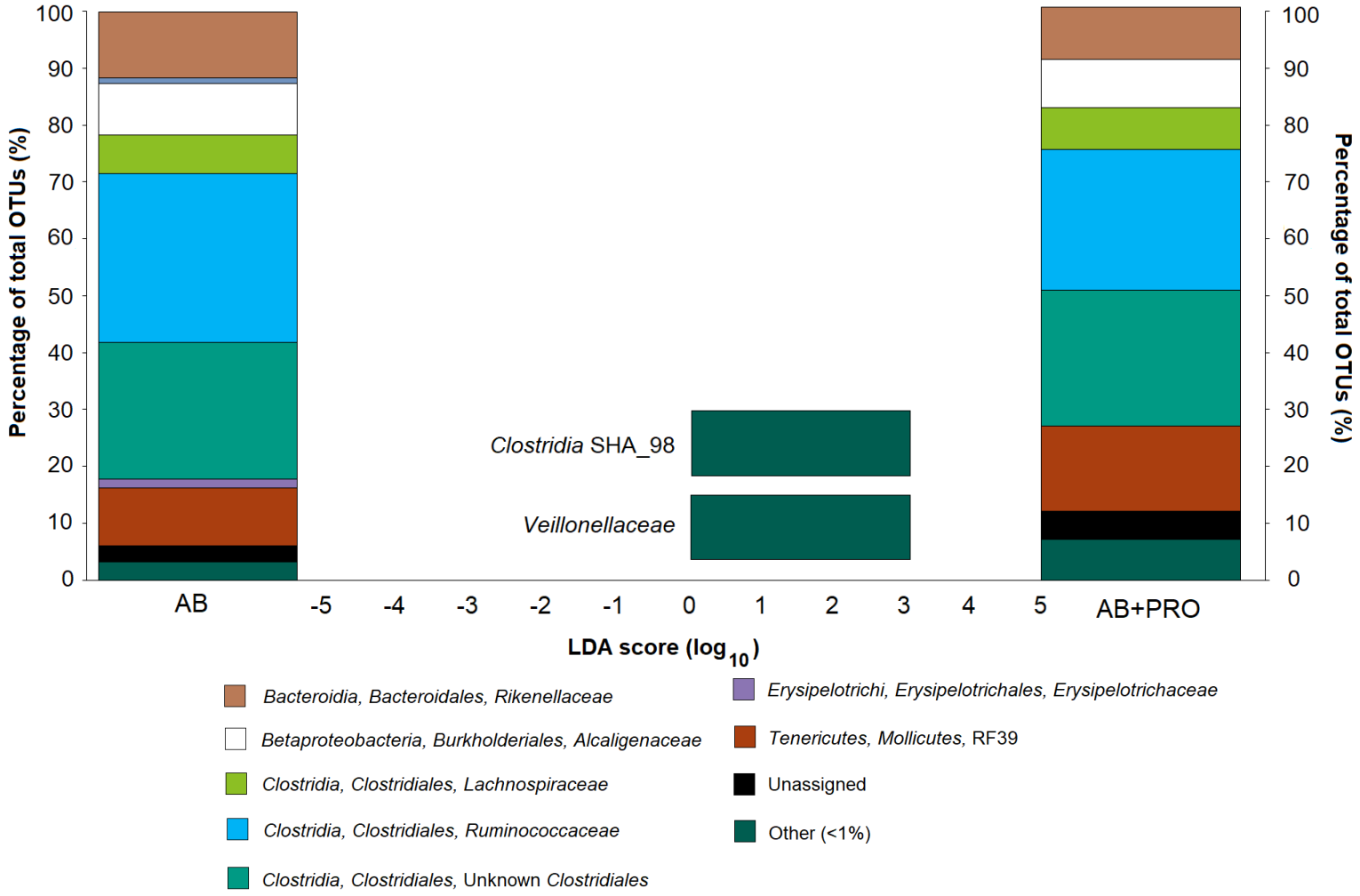


Figure 3.12 *NGS data comparing AB and AB+PRO at day 29.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

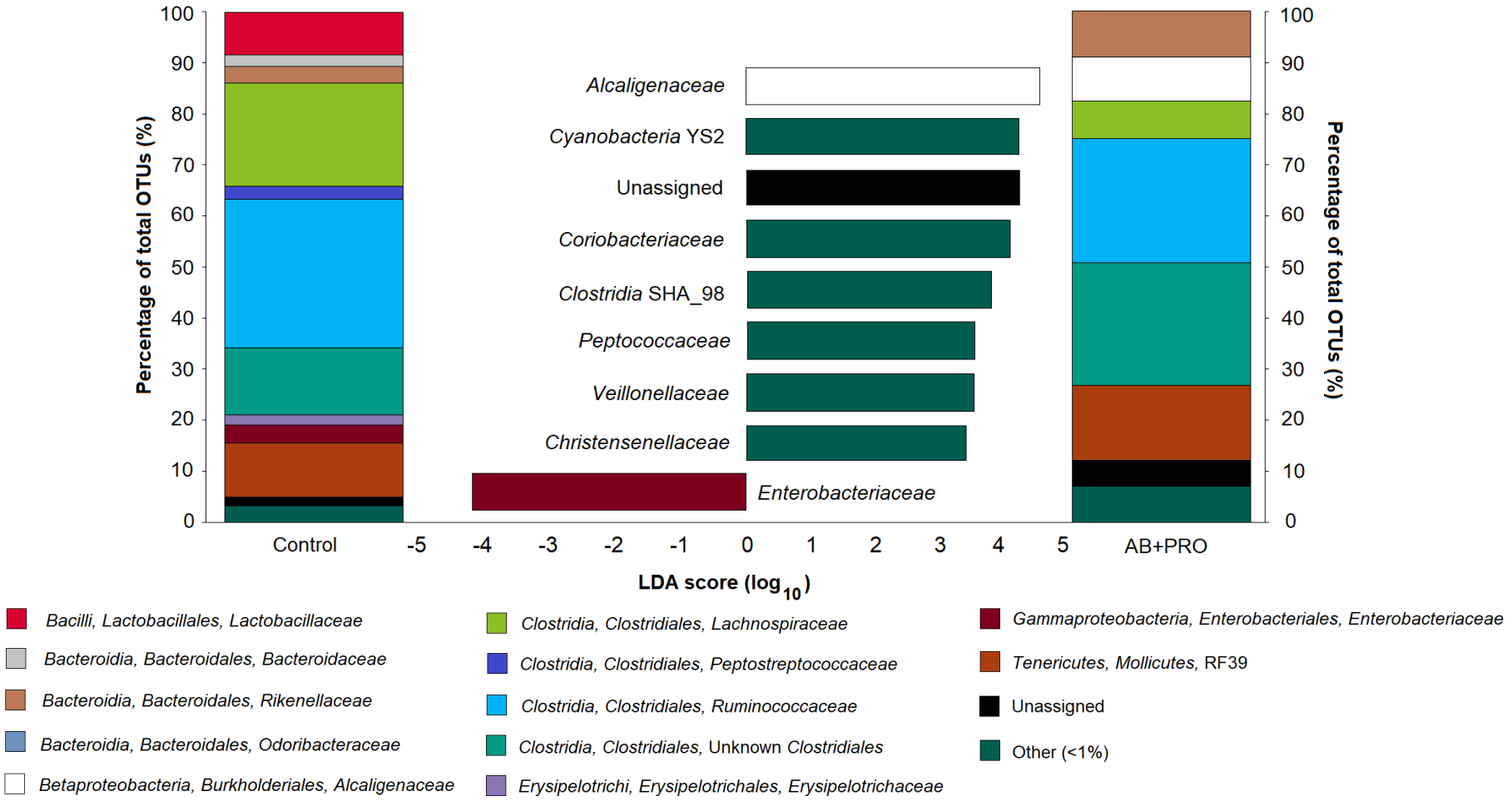


Figure 3.13 *NGS data comparing control and AB+PRO at day 29.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

3.3.5. NGS summary

Antibiotic prophylaxis significantly reduced the abundance of *Ruminococcaceae* at days 9 and 15 (*P*=0.046 and 0.045, respectively). These bacteria accounted for 37.9% of OTUs in Aviguard®, but levels were not altered in AB+PRO at day 9 compared with controls and AB. Both controls and AB exhibited a peak in levels of *Ruminococcaceae* at day 15 that was not observed in AB+PRO birds (**Figure 3.14**). Levels were fairly similar between treatment groups by day 29, suggesting that the administration of Aviguard® reduced fluctuations with age.

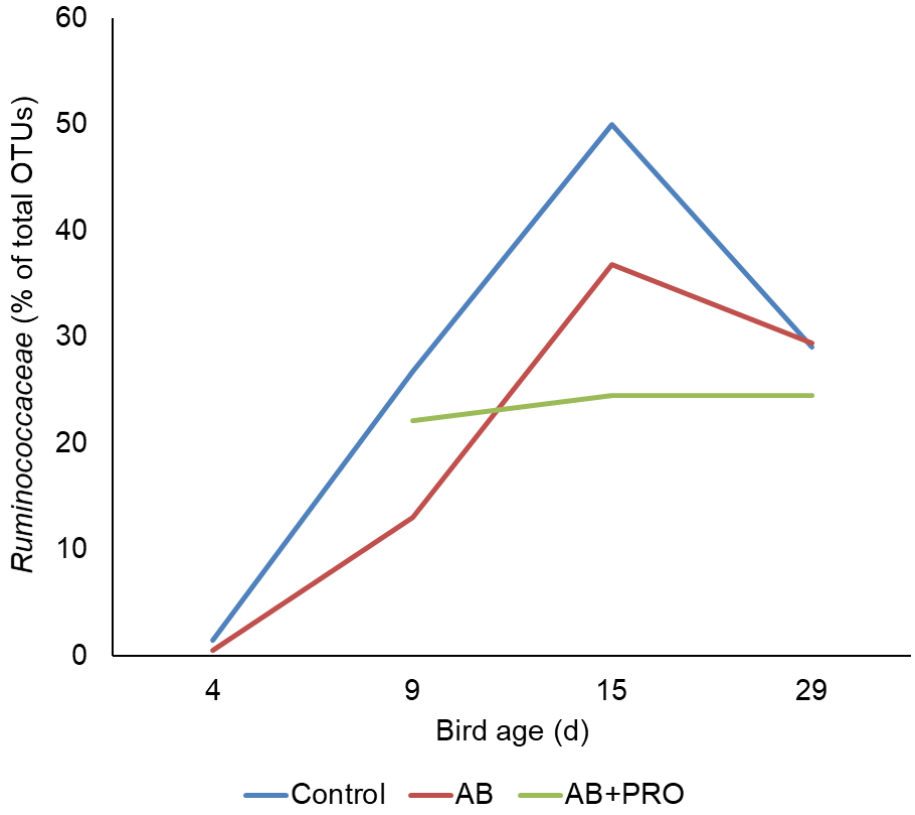


Figure 3.14 *Line plot illustrating the relative abundance of* Ruminococcaceae *within treatment groups.*

Alcaligenaceae were initially only present in AB+PRO birds but began to emerge in birds treated with LS as the study progressed. This suggests that LS acted as a predisposing factor for colonisation, an effect that was exacerbated by Aviguard®. It is hypothesised that LS treatment reduced the abundance of other taxa, providing a

niche in which *Alcaligenaceae* could colonise and proliferate. No records of the effects of these bacteria in poultry have been identified but links to gastrointestinal infections in humans have been observed (Wexler *et al.,* 1996), suggesting a potential issue in terms of food safety. Since *Alcaligenaceae* were present at much lower levels in controls, the effects of antibiotic and probiotic treatments on their growth should perhaps be investigated further.

Aviguard® is traditionally used as a method of reducing pathogenic bacterial load and has shown efficacy against *Clostridium perfringens* and *Salmonella spp.* (Hofacre *et al.,* 1998; Adudabos, 2013). Although NGS was unable to identify bacteria to genus level, bacteria from family *Clostridiaceae*, which includes *C. perfringens*, were increased at day 9 in AB samples with a subsequent reduction in AB+PRO to levels similar to that of controls. A similar effect was observed in Enterobacteriaceae, which includes *Salmonella*: LS initially increased abundance but it was subsequently reduced by Aviguard® at day 9. These findings were not statistically significant due to variation between samples, but these trends allow hypotheses to be drawn for future investigation. It is reasonable to suggest that LS disturbed the natural gut microflora, allowing *Clostridiaceae* and Enterobacteriaceae to colonise in the niches previously occupied by other autochthonous species such as *Lachnospiraceae*. The subsequent administration of Aviguard®, which contains mature microflora from healthy adult hens may have filled these niches with so-called ‘beneficial’ bacteria, preventing the colonisation of *Clostridiaceae*.

Evidence suggests that the caecal microbiome was not related to liveweight gain. Firstly, alpha diversity was calculated at intermediate levels in AB birds, where liveweight gain was significantly reduced from days 10-15, in comparison with controls and AB+PRO birds. Secondly, clustering of PCA datapoints from controls and AB birds at day 15 suggested a lack of relationship with beta diversity. Thirdly, no bacterial taxa were found at similar levels in controls and AB+PRO birds, but differing levels in AB

birds. This suggests that bacterial abundance may not have been intrinsically linked to liveweight gain.

It is thought that the observed difference in liveweight gain was a result of reduced feed intake, which leads to the hypothesis that measures of alpha and beta diversities and bacterial abundance in the caeca are not related to appetite. Appetite is depleted by glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) (Zhou *et al.,* 2008; Tolhurst *et al.,* 2012), the production of which is stimulated by SCFA which are often produced by gut bacteria. The mere presence of bacteria does not necessarily mean that such metabolites are being produced, and the relationship between said bacteria and metabolites may not be linear. Therefore, it would be beneficial to analyse concentrations of SCFA, anorexigenic and orexigenic molecules, irrespective of bacterial abundance to elucidate potential relationships with performance.

A large proportion of bacterial families were represented at levels below 1% - some of these were absent from some samples within a group, meaning that an average value was not truly representative. This may have been due to bias and differences in sensitivity in the PCR step of the sequencing process or misidentification of bacteria when comparing sequences to the Greengenes database

3.3.6. ^{1}H NMR: liver models

3.3.6.1. Treatment

OPLS-DA models of liver ^{1}H NMR were constructed to explore the effects of treatment on the hepatic metabolome (**Table 3.2**). These models suggest a lack of statistically significant differences.

Table 3.2 *Summary of OPLS-DA models of the hepatic metabolome, where R^2Y values indicate the predictive ability of the model and Q^2 values indicate the goodness of fit of the data (AB, Linco-spectin days 1-3; AB+PRO, Linco-spectin days 1-3 plus Aviguard® day 5).* P-*values are a result of 100 permutations.*

	OPLS model values		
	R^2Y	**Q^2**	***P*-value**
Day 4			
Control vs AB	0.8460	-0.4038	0.970
Day 9			
Control vs AB	0.6153	-0.2504	0.230
AB vs AB+PRO	0.6521	-0.0631	0.990
Control vs AB+PRO	0.6818	-0.1324	0.810
Day 15			
Control vs AB	0.7242	-0.2208	0.270
AB vs AB+PRO	0.7083	-0.2569	0.970
Control vs AB+PRO	0.8020	0.1389	0.120
Day 29			
Control vs AB	0.7158	0.2302	0.090
AB vs AB+PRO	0.7339	0.1724	0.990
Control vs AB+PRO	0.7089	0.3191	0.090

Correlation plots for all pairwise comparisons were constructed, irrespective of the predictive power of the models. No differences in peak intensities were observed, indicated by a lack of ‘warm’ colours and a plethora of ‘cool’ colours, confirming that the concentrations of hepatic metabolites were not altered by treatment. Pharmacokinetic studies suggest that negligible amounts of spectinomycin are absorbed from the gut and that its antimicrobial properties are exerted by altering the

microbial profile of the intestine. Although the exact mechanism of metabolism is unclear, there is evidence that the majority of orally administered spectinomycin can be recovered from urine and faeces, suggesting a lack of degradation (European Agency for the Evaluation of Medicinal Products [EMEA], 2000). It is therefore unsurprising that differences in the hepatic metabolome were not identified since it is unlikely that LS would have entered the circulatory system from the digestive tract for delivery to the liver.

Although the OPLS-DA models suggested a lack of difference, peaks assigned to acetate, butyrate, propionate and lactate were integrated and concentrations were calculated, initially to explore the effects of treatment on the ratios of SCFA. The means presented in **Table 3.3** were calculated from six birds per treatment group at each sampling point. It was found that differences in concentrations existed between treatments. LS significantly reduced acetate concentrations at day 9, but concentrations were restored by the subsequent administration of Aviguard®. Propionate was also affected at day 29, where concentrations were significantly lower in AB+PRO birds than controls.

Table 3.3 *ANOVA exploring the effects of treatment (AB, Linco-spectin days 1-3; AB+PRO, Linco-spectin days 1-3 plus Aviguard® day 5; n=6/treatment) on concentrations of hepatic SCFA. Statistical significance is denoted as* P*<0.05 and means were separated by Tukey's test.*

	Treatment				
	Control	**AB**	**AB+PRO**	**SEM**	***P*-value**
Day 4					
Acetate	0.04	0.03	-	0.005	0.586
Butyrate	0.52	0.49	-	0.036	0.772
Propionate	0.59	0.55	-	0.011	0.215
Lactate	0.48	0.48	-	0.031	0.983
Day 9					
Acetate	0.06^{ab}	0.05^{a}	0.07^{b}	0.002	0.018
Butyrate	0.48	0.39	0.46	0.021	0.267
Propionate	0.75	0.69	0.70	0.023	0.580
Lactate	0.64	0.64	0.82	0.034	0.064
Day 15					
Acetate	0.11	0.12	0.12	0.004	0.779
Butyrate	0.57	0.55	0.58	0.018	0.807
Propionate	0.96	0.87	0.87	0.349	0.554
Lactate	1.20	1.02	1.18	0.046	0.258
Day 29					
Acetate	0.10	0.11	0.10	0.006	0.785
Butyrate	0.87	0.82	0.86	0.042	0.875
Propionate	1.02^{a}	0.92^{ab}	0.86^{b}	0.022	0.031
Lactate	1.09	1.13	1.02	0.045	0.606

These results suggest that the use of LS did not immediately alter hepatic SCFA concentrations, though this is unsurprising since differences in the microbiome were also not observed. It would be beneficial to explore the microbiome of alternative locations within the gut – since the majority of digestion occurs in the small intestine, it is more likely that relationships would exist with these bacteria. SCFA concentrations have been linked to the development of intestinal architecture and the absorptive capacity of the gut, perhaps suggesting why AB birds gained significantly less weight whilst consuming a similar mass of feed. Despite this, the lack of concurrent

differences in performance with SCFA concentrations suggests that the observed reduction in the liveweights of AB birds was not related to concentrations of acetate, butyrate, propionate or lactate. This was confirmed through correlation analysis of average bird liveweight against SCFA concentrations (P>0.05).

3.3.6.2. Sex

Since differences in LW between male and female birds were observed, spectra were regrouped by bird sex irrespective of treatment group. OPLS-DA models suggested no differences between males and females, indicating that differences in LW between sexes were not reflected in the liver metabolome (**Table 3.4**).

Table 3.4 *Summary of OPLS-DA models investigating the effects of bird sex on the liver metabolome analysed through ^{1}H NMR.* P-*values are a result of 100 permutations.*

	OPLS model values		***P*-value**
	R^2Y	**Q^2**	
Day 9	0.7662	-0.6067	0.540
Day 15	0.7355	-0.5832	0.990
Day 29	0.4770	-0.1954	0.930

Investigating the effects of treatment on the hepatic metabolome and subsequent interactions with performance in chickens is novel. No statistically significant differences were observed between treatment groups, or between high and low performing birds, even though both lincomycin and spectinomycin are metabolised in the liver. Future work would perhaps aim to investigate other organs and tissues to explore effects on a systemic level.

3.4. Conclusion

The present study has provided an overview of the effects of LS and Aviguard® on the performance, gut microbiome and hepatic metabolome of growing broiler chicks. Although this experiment was primarily designed as a gut health study, results suggest that, whilst Linco-spectin reduced bird weight possibly because of reduced feed intake, Aviguard® appeared to reverse this effect. Aviguard® appeared to affect male birds more than females, highlighting the need for further work in sexed birds to remove the need to include sex as a covariate.

Both treatments affected the gut microbiome, indicating that endogenous bacteria were susceptible to the effects of medication, although repetition at higher levels of replication would enhance statistical power and enable more definite conclusions. It is thought that antibiotic prophylaxis altered the microbiome, creating niches in which bacteria could colonise. It appears that, if left to develop naturally, the microbiome of broilers dosed with an antibiotic eventually matches that of broilers subsequently dosed with Aviguard®. Aviguard® itself seems to accelerate the colonisation of the caeca, resulting in numerous differences compared with controls. Neither treatment consistently affected the hepatic metabolome of birds, suggesting a lack of interaction between performance, the gut microbiome and the metabolism of broilers.

Future work should consider larger sample sizes to increase statistical power, whilst carefully designing an experiment to reduce variability in data and allow more valid conclusions to be drawn. Similarly, it would be beneficial to explore the effects of a probiotic alone and compare this with birds previously dosed with an antibiotic.

4. Study 2: Effect of diet - protein source

4.1. Introduction

Fishmeal (FM) has long been included as a crude protein source in broiler diets with evidence of improvements to feed utilisation and growth in comparison with diets lacking FM (Shabani *et al.,* 2018). Through the provision of essential amino acids that are required for efficient growth, producers can reduce the volume of feed required to meet target weights, since FM provides highly concentrated nutrients that would otherwise require large volumes of plant matter to meet growth targets.

Protein is an expensive macronutrient (Skinner *et al.,* 1992) and it is therefore in the interest of producers to find cheap sources to maximise profits. Plant proteins are generally cheaper than animal proteins (Beski *et al.,* 2015) but have a tendency to be nutritionally unbalanced and therefore synthetic amino acids (SAA) are frequently added to diets to ensure that essential amino acid requirements are met. The use of animal proteins in animal diets is prohibited in some countries, meaning that the consideration of other sources is necessary. Zarate *et al.* (2003) report that formulating diets with SAA reduces crude protein requirements whilst improving performance efficiency in growing broilers. Since excess crude protein is metabolised and excreted as urea and nitrogenous compounds that are detrimental to both birds and the environment, reducing dietary crude protein levels and replacing with SAA may also reduce environmental impacts.

Broilers rely upon gut microbes for efficient digestion of feed, yielding nutritional, immunological and physical benefits (Forder *et al.,* 2007; Mountzouris *et al.,* 2009; Torok *et al.,* 2011). There a plethora of evidence that suggests that the diet significantly alters the gut microbiome (Torok *et al.,* 2008; Sun *et al.,* 2013; Wu *et al.,* 2014; Stanley *et al.,* 2014), since some bacteria are extremely reliant on certain amino acids for growth (Sebald and Costilow, 1975). Qu *et al.* (2008) report that 9% of genes in the

GIT microbiome are attributed to protein and amino acid metabolism, therefore it is expected that alterations in protein sources may alter the profile of the microbiome.

4.2. Aims

The present study aimed to:

- Investigate the influence of amino acid source, either FM or soya supplemented with SAA, upon the gut microbiome of growing Ross 308 broiler chicks.
- Profile the hepatic metabolome, since the hepatic metabolome since nutrient composition may alter the metabolic state.
- Explore relationships between hepatic metabolites and caecal bacteria altered by protein source.

4.3. Results and discussion

4.3.1. Performance

Performance data are summarised in **Table 4.1**. FCR, daily liveweight gain and daily feed intake were calculated on a cage-basis (n=8/treatment). Liveweight was calculated on an individual bird basis (n=16/treatment). All birds were a similar weight at arrival (day 1) and at the commencement of the experimental period (day 3), though feeding SAA over FM significantly reduced bird liveweight from 7 days of age ($P<0.05$). FM birds also consumed significantly more feed ($P=0.002$), though their overall FCR was improved ($P=0.003$).

Table 4.1 *Effects of protein source (FM, fishmeal; SAA, synthetic lysine/methionine) on bird liveweight, where individual birds were replicate units (n=16/treatment), and daily liveweight gain, daily feed intake and FCR, where cages were used as replicate units (n=8/treatment). Statistical significance was denoted as* P*<0.05 and means were separated by Tukey's test.*

	FM	SAA	SEM	*P*-value
Bird liveweight (g)				
Day 1	46.3	46.1	0.77	0.882
Day 3	63.8	65.1	1.09	0.549
Day 7	119	107	2.4	0.023
Day 14	364	243	11.3	<0.001
Day 17	456	308	15.1	<0.001
Day 3-17 performance				
Daily liveweight gain (g/bird/day)	28	17	1.2	<0.001
Daily feed intake (g/bird/day)	36	28	1.1	0.002
FCR (g/g)	1.29	1.66	0.052	0.003

Feeding soya supplemented with SAA reduced daily weight gain by 39% and daily feed intake by 22% compared with birds fed FM. FCR is a function of both of these parameters and was therefore significantly poorer in FM, though the larger difference in weight gain suggests that this was more influential than feed intake. Based upon average daily feed intake, nutrient intake was lower in birds fed soya supplemented with SAA. The reduction in feed intake observed in SAA-fed birds resulted in reduced metabolisable energy intake, at an average of 9.79 MJ/day compared with 6.89 MJ/day in SAA-fed birds, potentially explaining the large difference in liveweight gain.

Research exploring the effects of protein source on performance are inconclusive (Mikulec *et al.,* 2004; Karimi, 2006). Esmail (2002) reports that, so long as methionine and lysine requirements are met, a reduction in crude protein levels of 2-4% does not affect weight gain. There was a difference of 9.6% between diets in the present study, suggesting that exceeding the suggested 4% threshold negatively affects performance. When average daily feed intake per bird was calculated, it was found

that FM birds consumed around 9.79 g CP per day compared with 6.89 g by SAA birds. It is therefore likely that the excess difference in liveweight gain compared with feed intake was a result of differences in CP level, as documented by Farkhoy *et al.,* 2012, and that ensuring that methionine and lysine levels are sufficient is not enough to ensure maximum growth. It is suggested that cysteine levels may be influential in promoting efficient growth – Farkhoy *et al.* (2012) report an interaction between CP levels and methionine/cysteine levels that may influence performance.

Research concerning the relationship between dietary CP levels and feed intake is also inconsistent, with evidence that high CP can reduce feed intake (Ferguson *et al.,* 1988; Aletor *et al.,* 2000) or have no effect (Bregendahl *et al.,* 2002; Dean *et al.,* 2006). Dietary CP levels were higher in FM-fed birds, which also consumed more feed, contradicting the findings of Aletor *et al.* (2000). Rezaei *et al.* (2014) found that low dietary CP levels reduced feed intake and weight gain during the starter phase but had no lifetime effects. It would therefore be beneficial to extend the present study to include grower and finisher phases.

If CP level was the sole reason for differences in feed intake, and therefore weight gain, however, it is thought that effects would have been seen from day 1. Differences in liveweight were not observed until after the onset of the experimental period where birds were suddenly moved from large groups in brooding pens, where huddling was encouraged, to experimental pens in pairs, where huddling capacity was reduced. It is thought that this change may have acted as a stressor that birds fed the FM diet could cope with. There is the possibility, however, that SAA birds consumed more feed than FM birds from days 1-3 but still gained a similar amount of weight. Feed intake was not recorded during this period of time since this was an acclimatisation period before the onset of data collection. Future work should consider investigating performance from day 1 to explore whether diet affected birds from the onset.

4.3.2. NGS: alpha diversity

Alpha diversity, measured by Simpson's Diversity Index (*D*) and illustrated in **Figure 4.1**, was slightly higher in FM than SAA (0.8071 and 0.7669 respectively). This indicates that one would be 4% more likely to select different taxa from FM than SAA if bacteria were randomly sampled, though this difference was not significantly different due to variation between individual birds (*P*=0.309; SD=0.052). In general, birds fed the SAA diet consumed lower levels of crude protein in comparison with those on the FM diet (6.89 compared with 9.79 g/day). Level of protein inclusion has been shown to have no effect on colonic bacterial diversity in pigs Fan *et al.* (2017).

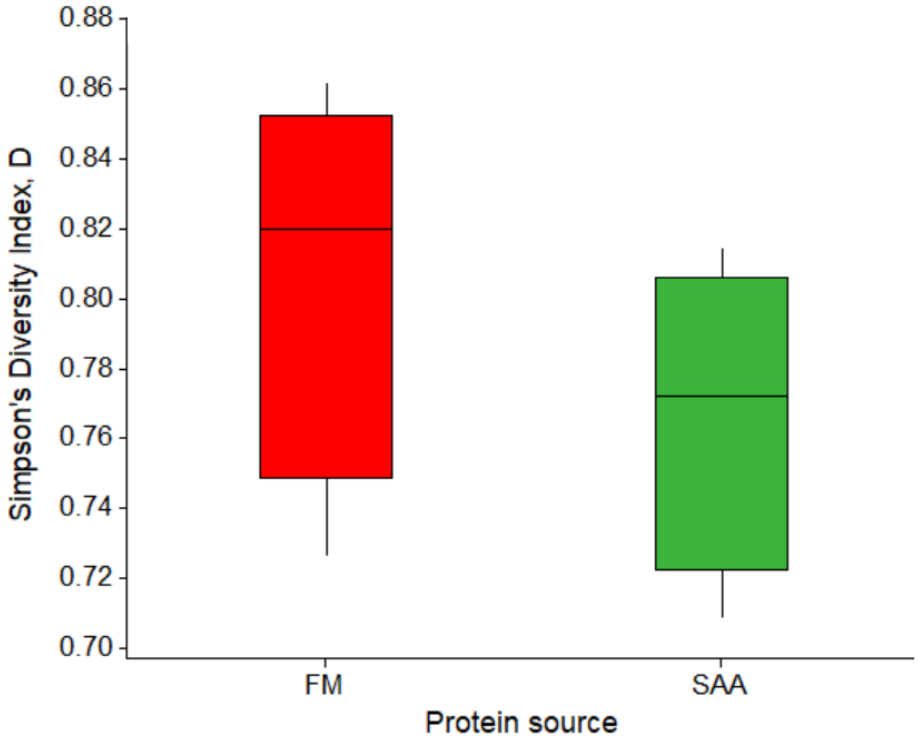


Figure 4.1 *Box plot illustrating the effects of protein source (FM, fishmeal; SAA, soya supplemented with synthetic lysine/methionine) on alpha diversity of caecal bacterial populations broilers at 17 days of age. Bacterial populations were profiled by next-generation sequencing, and alpha diversity was measured by Simpson's Diversity Index.*

4.3.3. NGS: beta diversity

Beta diversity was explored through principal component analysis. The score plot in **Figure 4.2** indicates clustering of data points with separation in the first principal component, suggesting that the profile of the caecal microbiome was altered by protein source: differences in taxa abundance are expected. The large spread of points across the second principal component suggests variation between individual birds within treatment groups. Points within a group appear to be equally scattered, suggesting that neither diet exerted more of a stabilising effect on the microbiome than the other.

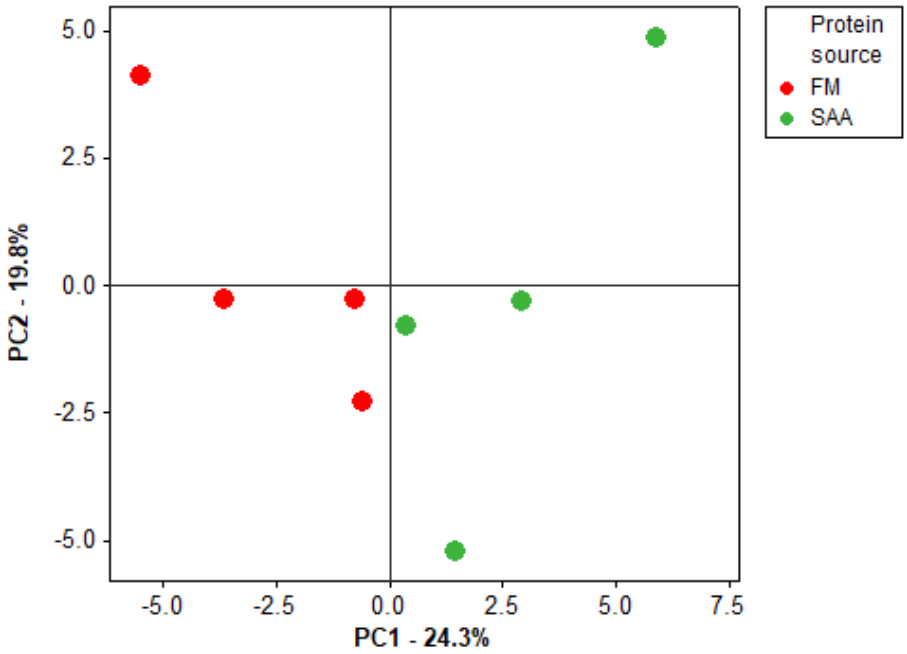


Figure 4.2 *PCA score plot illustrating the effects of protein source (FM, fishmeal; SAA, synthetic lysine/methionine) on beta diversity of caecal bacterial populations of broilers at 17 days of age, profiled through next-generation sequencing. Discrete clustering of samples in the first principal component (PC1) suggests differences between treatment groups.*

4.3.4. NGS: population analysis

The percentage abundances of bacteria were calculated from QIIME outputs, and differences were identified through LEfSe. The relative abundance of families and LDA scores of differentially abundant families are summarised in **Figure 4.3**. All discriminant features and *P*-values are listed in **Appendix 2**.

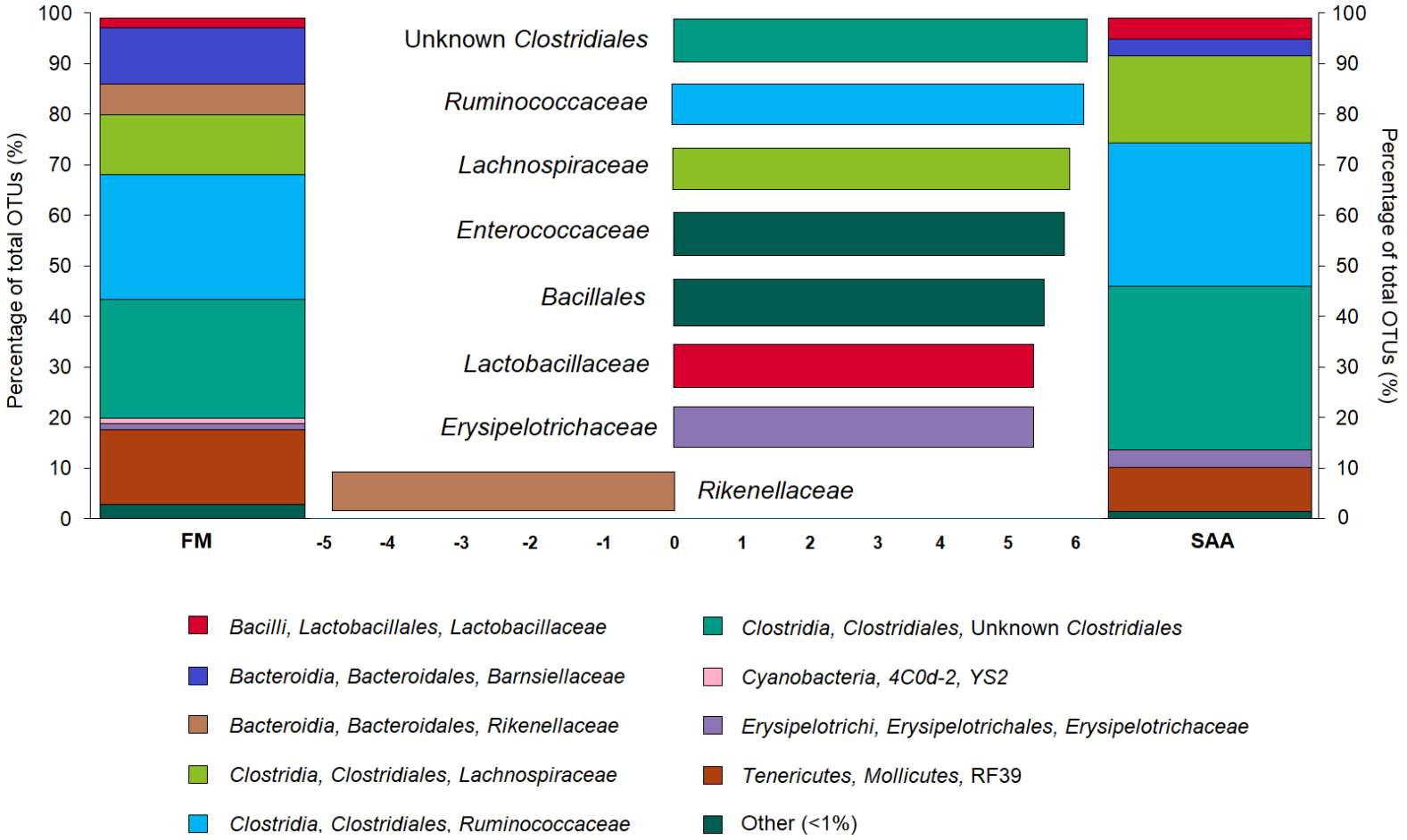


Figure 4.3 *LEfSe analysis of the effects of dietary protein source (FM, fishmeal; SAA, synthetic lysine/methionine; n=4 birds/treatment). Outer bars: stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'. Inner bars: log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

The proportion of *Firmicutes* was significantly higher in SAA-fed birds, at an average of 87.0% compared with 64.3%. Raw OTU data suggested that this difference was countered by a reduction in the abundance of phylum *Bacteroidetes* in SAA (3.4% compared with 17.4% in FM), though this was not identified as a statistically significant difference by LEfSe analysis due to variation caused by a single high SAA sample (13.4%) and single low FM sample (0.7%). Removing these samples compromised statistical power but reduced variation, and LEfSe analysis then suggested an effect of diet on *Bacteroidetes*. A strong negative correlation between *Firmicutes* and *Bacteroidetes* was observed (r=-0.925; P=0.001).

Numerous taxa from order *Clostridiales* were elevated in SAA-fed birds, including *Lachnospiraceae* (P=0.009) and *Ruminococcaceae* (P=0.047). These families contain key fermentative bacteria that produce SCFA by metabolising complex carbohydrates: Wu *et al.* (2014) report that feeding FM reduces the abundance of both families, potentially reducing the butyrate synthetic capacity of the bird. Butyrate is an important signalling molecule that promotes the formation of tight junctions and epithelial cell proliferation through protein and cytokine expression (Kasubuchi *et al.,* 2015). It is therefore suggested that feeding FM compromises the structural integrity of the gut by depleting the abundance of butyrate-producing bacteria, potentially explaining why FM has been identified as a predisposing factor for the development of necrotic enteritis.

Zhu *et al.* (2015) report that *Lachnospiraceae* were characteristic of mice fed plant-based protein over those fed animal-based protein, though the opposite was found for *Ruminococcaceae*. Since both of these families were biased towards SAA birds, where a plant-based protein was fed, it is unclear whether protein source truly affects bacterial abundance or whether CP intake is more influential.

Relative abundances may also have been affected by fat: Daniel *et al.* (2014) found a reduction in *Ruminococcaceae* in mice fed a high fat diet. It is hypothesised that, since

animal proteins tend to contain higher levels of fats than plant proteins, levels of fat would have been elevated in the FM diet. It is therefore reasonable to suggest that the bias of *Ruminococcaceae* towards the soya SAA diet in the present study may have been as a result of differences in dietary fat content. It would be beneficial to further explore the fat content of the diets used in this study to explore potential relationships with bacteria.

Both *Lachnospiraceae* and *Ruminococcaceae* utilise dietary starches for energy production: since the SAA diet would have contained higher levels of starches than the FM diet due to soya, it is hypothesised that their growth was partially fuelled by the provision of complex carbohydrates.

Also from phylum *Firmicutes*, *Erysipelotrichaceae* were found at levels in SAA birds that were thrice that of FM birds at an average of 3.6% and 1.2% respectively (P=0.009). Though Kar *et al.* (2017) also found that protein source affects levels of *Erysipelotrichaceae,* reasoning behind this effect is unclear.

Lactobacillaceae were also biased towards SAA-fed birds (P=0.047). These are key lactic acid-producing bacteria that have been linked to gut health and enhanced performance (Jin *et al.,* 1998). Zhu *et al.* (2015) report that *Lactobacillaceae* were found at higher levels in rats fed an animal protein-based diet than a plant protein-based diet, suggesting that it may not be the origin but the dietary levels of protein that affects bacterial abundance.

Unknown *Clostridiales* were also biased towards SAA birds (P=0.016) – these bacteria have been identified in the chicken gut by numerous authors (Wu *et al.,* 2014; Fasina *et al.,* 2016; Stanley *et al.,* 2015), though the lack of taxonomic assignment means that a potential biological role is yet to be explored.

Rikenellaceae, from phylum *Bacteroidetes*, was the only family to be biased towards FM birds (*P*=0.008). Ranjikar *et al.* (2016) found *Rikenellaceae* to be mainly focused in the caeca with evidence of an increase in abundance as birds age. Despite this, evidence of transfer between individuals is lacking – Polansky *et al.* (2016) inoculated young birds with caecal bacteria from adults and found that *Rikenellaceae* were detected less frequently in recipients than donors. Differences are likely to be due to environmental factors and diet, since birds were housed in small cages with few birds and so the potential for transfer between individuals was limited. Fan *et al.* (2017) found that relative proportions of *Rikenellaceae* in pigs were significantly decreased with reduced dietary protein levels and suggest that an adequate nitrogen source is important for proliferation. Crude protein levels were actually slightly higher in the FM diet in the present study, contradicting their findings. Proportions of *Rikenellaceae* have been found at lower levels in patients with non-alcoholic fatty liver disease (Jiang *et al.,* 2014) and a positive relationship between *Rikenellaceae* and a high-fat diet in mice has been identified (Kim *et al.,* 2012; Daniel *et al.,* 2014). It would be beneficial, therefore, to further analyse the diets used in the present study to explore fat content and potential relationships with *Rikenellaceae*.

4.3.5. ^{1}H NMR: liver models

^{1}H NMR spectra were measured from the methanolic extract of livers, from which an OPLS-DA model was constructed to explore differences in metabolic profiles. An R^2Y value of 0.8964 was indicative of a good model and a Q^2 value of 0.5849 accompanied by a *P* value of <0.001 suggested that the model would perform well in sample classification and therefore differences between treatment groups were expected. Correlation plots were constructed to highlight peaks that were responsible for the suggested differences in metabolic profiles (**Figure 4.4**). Differences in peaks assigned to α and β glucose were indicated by warm colours (red and orange).

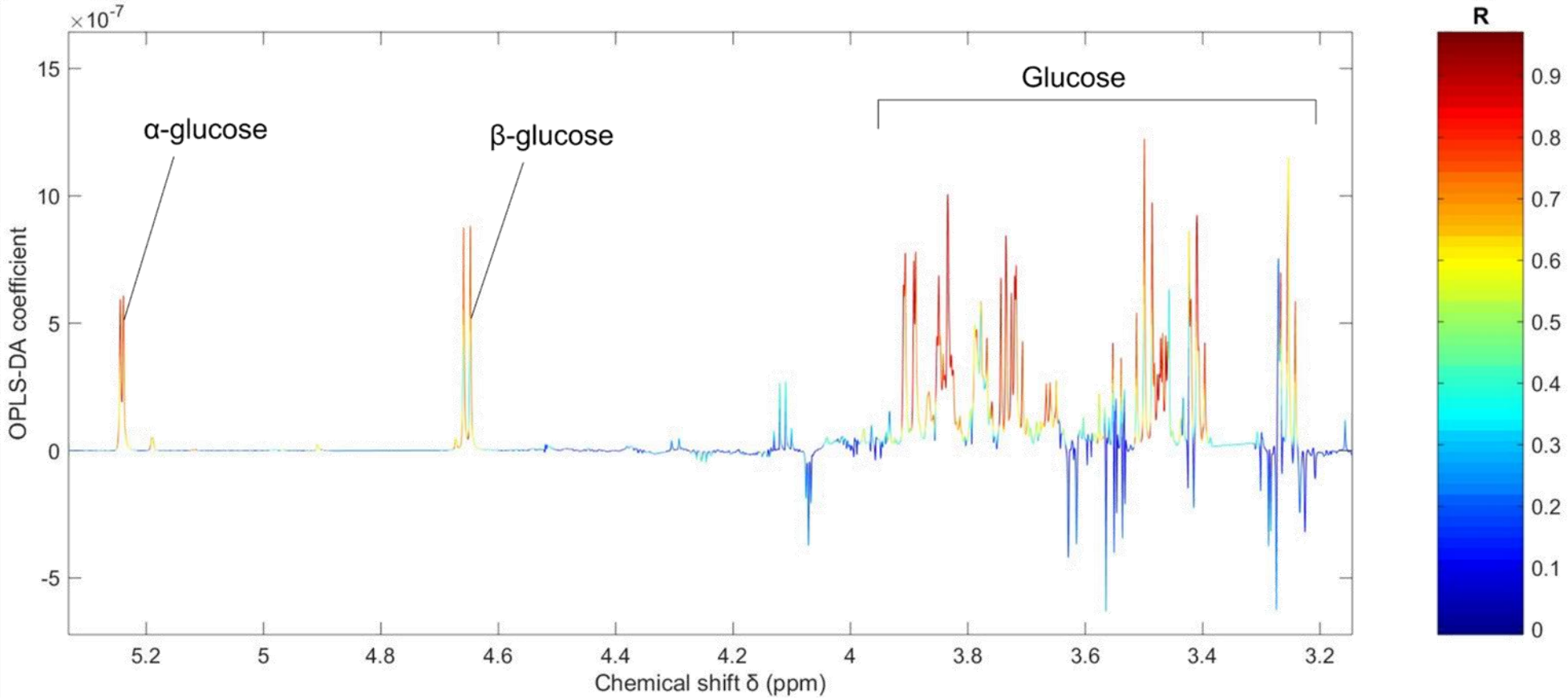
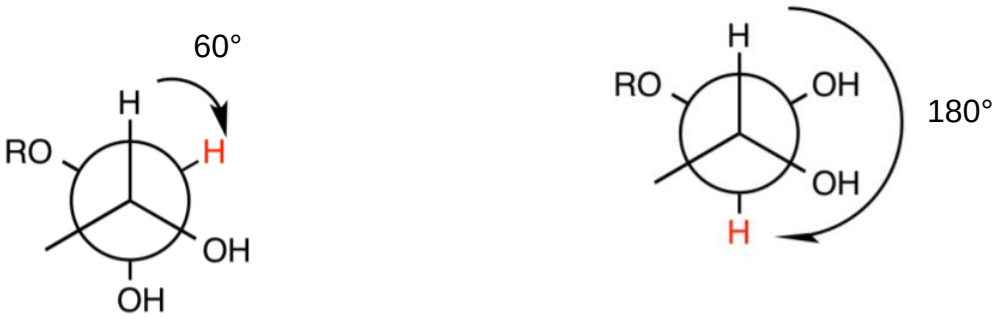


Figure 4.4 *OPLS-DA correlation plot illustrating differentially represented peaks in the 3.2 < δ < 5.2 ppm region. Warmer colours (red/orange) indicate a greater peak height in one treatment group (fishmeal or synthetic lysine/methionine; n=8 birds/treatment) over another. Cooler colours (blue/green) indicate no difference. Peak assignments are based upon the Human Metabolome Database (Wishart* et al., *2018).*

The stereoisomeric configuration of α and β glucose, illustrated in **Figure 4.5** was confirmed by exploring the coupling constants (*J*) of the doublet peaks at δ=5.24 and δ=4.65. *J* constants differ depending upon the dihedral angles between two atoms and is calculated by multiplying the difference between maximum peak positions (ppm) by the frequency of the spectrometer in MHz. Since β glucose exhibits a dihedral angle of 180°, unlike the 60° angle in the α anomeric configuration, the difference in *J* can help in the confident assignment of molecular identities. In this case, the doublet at δ=5.24 ppm gave a *J* constant of 3.5 Hz whereas the doublet at δ=4.65 ppm gave a *J* constant of 8.4 Hz, closely matching the published data in the Human Metabolome Database (HMDB; Wishart *et al.,* 2008) and consolidating identity assignment.



α glucose
J = 3.5 Hz

β glucose
J = 8.4 Hz

Figure 4.5 *Anomeric configurations of glucose illustrating differences in dihedral angles that give rise to differences in coupling constants (J) in the present NMR spectra.*

Peaks were integrated and compared against an internal standard of a known concentration for estimation of metabolite concentration. ANOVA revealed that glucose concentrations were numerically different between treatment groups but differed between individual birds, reducing statistical significance (**Table 4.2**). Despite this, the numerical difference suggests a general physiological effect. The α:β isomer ratio was also investigated – on average, the β configuration was 1.34 times more abundant than the α configuration though no difference between treatment groups was identified, with an average of 1.32 and 1.36 in FM and SAA respectively (*P*=0.143).

Table 4.2 *Assignments and average concentrations of hepatic metabolites affected by protein source (FM, fishmeal; SAA, synthetic lysine/methionine; n=8 birds/treatment) per gram of starting material calculated through peak integration of ^{1}H NMR spectra ± standard deviation. Peaks in bold were integrated for concentration calculation.* P*-values have been obtained from ANOVA where significance was denoted as* P*<0.05 and means were separated by Tukey's test. (s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet).*

Metabolite	**Assignment**			**Average concentration (mM/g)**		***P*-value**
	Peak position (ppm)	**Splitting pattern**	**Coupling constant (Hz)**	**FM**	**SAA**	
α-glucose	3.42	m	-	2.473 ± 1.047	1.362 ± 0.408	0.095
	3.72	m	-			
	3.74	m	-			
	3.78	m	-			
	5.24	**d**	**3.5**			
β-glucose	3.25	m	-	3.237 ± 1.355	1.848 ± 0.549	0.106
	3.48	m	-			
	3.51	m	-			
	3.90	m	-			
	4.65	**d**	**8.4**			

The observed numerical reduction in hepatic glucose concentrations in SAA birds is likely attributed to the aforementioned reduction in feed intake. Roseborough *et al*. (1990) report that feeding a diet low in CP and lysine significantly reduces net glucose production. Average available CP and lysine intakes were estimated to be 9.79 and 0.46 g/bird/day for FM-fed birds, and 6.89 and 0.36 g/bird/day for SAA-fed birds, respectively. This reduced bioavailability of lysine may therefore have affected hepatic glucose concentrations. It was also reported that pyruvate for glucose production was higher in birds fed a low CP diet - reduced CP intake may therefore have altered the extent to which hepatic gluconeogenesis occurred.

Tinker *et al.* (1986) report that starvation increases hepatic gluconeogenesis and that the high metabolic rate of the broiler allows the liver to maintain glucose homeostasis. This was clearly not the case in the present study, since hepatic glucose levels differed between treatment groups, and it is therefore hypothesised that the gluconeogenic capacity of the liver is capped by substrate availability deriving from feed intake. The metabolisable energy content of the FM diet was lower than that of the SAA diet, and differences in feed intake resulted in an average energy intake of 0.45 and 0.35 MJ/bird/day respectively. Roseborough *et al*. (2007) found that the activities of aspartate aminotransferase, isocitrate dehydrogenase and malic enzyme were reduced in birds fed a low CP diet – all three of these enzymes are involved in the citric acid cycle, suggesting that energy production may be directly affected by CP level. This work would benefit from proteomic analysis to analyse enzymatic production and further explore this hypothesis.

Bollard *et al.* (2009) found that liver glucose concentrations were reduced in birds that had undergone a partial hepatectomy. It is suggested that this is due to augmented glycolysis to satisfy increased energy requirements for the repair of liver tissue. Though liver health and circulating blood glucose levels were not analysed in the

present study, they may have been altered by protein source and CP levels and therefore warrant exploration in future work.

Peaks assigned to SCFA (acetate, butyrate and propionate) and lactate were integrated to explore the effects of protein source on concentrations: ANOVA found no significant effects (**Table 4.3**).

Table 4.3 *ANOVA exploring the effects of protein source (FM, fishmeal; SAA, synthetic lysine/methionine; n=8 birds/treatment) on the concentrations of hepatic SCFA (mM/g liver), where statistical significance was denoted as* P*<0.05 and means were separated by Tukey's test.*

	Protein source			
Concentration (mM/g liver)	**FM**	**SAA**	**SEM**	***P*-value**
Acetate	0.25	0.31	0.012	0.077
Butyrate	1.01	1.36	0.097	0.186
Propionate	0.31	0.41	0.035	0.194
Lactate	2.90	2.54	0.187	0.383

The lack of differences are somewhat surprising since feed intake, and therefore the level of carbohydrates available for bacterial fermentation, was lower in SAA-fed birds. It would therefore be expected for SCFA concentrations to be lower in these birds. The results presented suggest that feed intake may not directly affect SCFA production – it is hypothesised that microbial populations are more influential on SCFA concentrations, and that the abundances of certain species may override dietary effects.

4.3.6. Correlation analysis

Correlation analysis highlighted significant relationships between the relative abundances of bacteria and concentrations of hepatic SCFA and lactate (**Table 4.4**).

Table 4.4 *Pearson correlation analysis exploring relationships between abundances of caecal bacteria (%) and concentrations of hepatic SCFA/lactate (mM/g liver) that were significantly affected by dietary protein source (fishmeal or synthetic lysine/methionine; n=16 birds).*

Bacteria	SCFA	R	*P*-value
Rikenellaceae	Acetate	-0.841	0.009
Bacillaceae	Acetate	-0.852	0.007
Unknown *Clostridiales*	Acetate	-0.904	0.002
Enterococcaceae	Butyrate	0.854	0.007
	Lactate	-0.870	0.005
Lactobacillaceae	Butyrate	0.743	0.035
	Lactate	-0.737	0.037
Mollicutes	Butyrate	-0.737	0.037
	Propionate	-0.768	0.026
	Lactate	0.773	0.024

It is hypothesised that bacteria in SAA-fed birds may have metabolised complex carbohydrates to a greater extent than those in FM-fed birds to account for differences in nutrient availability and prevent a difference in SCFA concentrations. If this were the case, then it is hypothesised that bacteria that were elevated in SAA birds would be positively correlated with SCFA concentrations, whereas those that were elevated in FM birds would be negatively correlated with SCFA concentrations. Of taxa that were affected by protein source, unknown *Clostridiales, Enterococcaceae* and *Lactobacillaceae* were elevated in SAA birds, and *Rikenellaceae* were elevated in FM birds. Only butyrate and acetate were positively correlated with any of these taxa. The hypothesis that caecal bacteria may have accounted for differences in feed intake by metabolising dietary components to a greater extent may therefore only be true for butyrate and acetate. Exploring relationships with bacteria further up the GIT may help

to expand upon these hypotheses and explain the lack of differences in SCFA production.

Though levels were not significantly affected by diet, populations of caecal Enterobacteriaceae were positively correlated with concentrations of hepatic glucose. Enterobacteriaceae ferment glucose, but since bacterial levels were not affected by diet, it is thought that this relationship is a coincidental result of feed intake rather than experimental treatment. Circulating blood glucose levels were not directly tested and should be considered for future work.

Concentrations of SCFA and relative abundances of bacteria were also correlated against FCR for any indication of a cause for the difference in performance between dietary treatments. No relationships were observed, suggesting that neither the caecal microbiome or hepatic SCFA concentrations are indicative of bird performance. It should be noted, however, that FCR was calculated on a pen basis: although there were only two birds per pen, it is merely assumed that each bird within a replicate unit performed equally. In reality it is likely that FCR per bird would have differed, making the apparent lack of association with the microbiome and metabolome a mere suggestion that can be built on in future work.

4.4. Conclusion

This study aimed to explore the effects of protein source on the performance, caecal microbiome and hepatic metabolome of young broilers. Feeding soya supplemented with synthetic lysine and methionine significantly reduced feed intake, weight gain and FCR in comparison with birds fed FM. It is thought that feed intake was reduced as a product of stress or through satiation, though this warrants further work. Differences in crude protein levels may have negatively affected liveweight gain, and therefore

growth efficiency. It is thought that feeding appropriate levels of lysine and methionine is insufficient to support growth, and that crude protein levels must be adequate.

Protein source significantly altered the profile of the caecal microbiome, either through the provision of differing nutrients with each diet, or through differing microbial profiles of the diets themselves. Feeding soya supplemented with SAA also reduced hepatic glucose concentrations: it is likely that this was a result of reduced feed intake, though effects on the rate of glycolysis should be explored in further work.

In conclusion, this study showed that protein source significantly alters the performance of broilers in the starter phase. Further work should consider investigating birds from days 1-3 and into the grower and finisher phases to explore whether these effects continue into adulthood.

5. Study 3: Effects of diet - cereal base

5.1. Introduction

By mid-2017, the UK attributed 1,792 thousand and 197 thousand hectares to wheat and maize production respectively (DEFRA, 2017). Both of these grains are utilised in animal feed, though research suggests that each cereal varies in nutritive value. Dietary components that are not digested by the animal are available as substrates for bacterial growth: the composition of said nutrients deriving from the host's diet will therefore naturally affect the composition of microbial populations. Evidence suggests that feed composition significantly affects the gut microbiota of numerous species including sheep (Ellison *et al.,* 2014), cows (Kong *et al.,* 2010) and humans (Singh *et al.,* 2017), though less attention has been paid to the effects of cereal base in broilers.

There is evidence that cereal base can also alter the cellular architecture of the gut. Wheat-fed birds exhibit larger caeca and jejunal villus height:crypt depth ratio than those on a maize-based diet, increasing the absorptive capacity of the digestive tract (Masey-O'Neill *et al.,* 2014; Ghayour-Najafabadi *et al.,* 2017). This difference may be due to the increased passage of resistant carbohydrates to the caeca where they are fermented by resident microbes to short chain fatty acids (SCFA) including acetate, propionate and butyrate, the preferred fuel for enterocyte proliferation (Rehman *et al.,* 2007).

Evidence from human trials suggests that differences in diet can also be reflected in modulations of metabolic profiles (O'Gormon *et al.,* 2013; Playdon *et al.,* 2017). This allows investigations into nutrient utilisation and may inform hypotheses concerning bacterial metabolism if the gut microbiome-metabolome axis is investigated. A recent caprine model suggests relationships between diet, the gut microbiome and the metabolome (Tao *et al.,* 2017).

5.2. Aims

The present study aimed to:

- Explore the effects of a wheat-based diet *vs* a maize-based diet on bird performance in terms of liveweight, liveweight gain, feed intake and FCR.
- Explore the effects of diet base on caecal bacterial populations and diversities.
- Explore the effects of diet base on the hepatic metabolome.
- Suggest relationships between hepatic metabolites and caecal bacteria that were affected by diet base.

The effects of diet on performance cannot be appropriately hypothesised due to inconsistencies in existing literature. It is anticipated that the results presented here will expand existing knowledge to improve what is known about the effects of cereal grains on broiler performance.

5.3. Results and discussion

5.3.1. Performance

Performance data are summarised in **Table 5.1**, where FCR was calculated on a pen basis (n=6/treatment) and liveweight, feed intake, and weight gain were calculated on an individual bird basis.

There was no effect of diet base on liveweight, but a trend in FCR was evident during the grower/finisher phase (days 15-35; P=0.062), where maize-fed birds were slightly less productive (higher FCR). This difference may have been the result of a numerical, but statistically insignificant, reduction in average daily liveweight gain of 4 g between days 22-35.

Table 5.1 *Effects of diet base (wheat/maize) on bird liveweight, where individual birds were replicate units, and daily liveweight gain (g/bird/day), feed intake (g/bird/day) and FCR, where pens were replicate units (n=6/treatment). Daily feed intake and FCR for days 1-15 have not been statistically analysed due to a lack of pen replicates during the starter phase. Statistical significance was denoted as* P*<0.05 and means were separated by Tukey's test.*

	Diet base			
Parameter	**Wheat**	**Maize**	**SEM**	***P*-value**
Liveweight (g)				
Day 1	42.1	41.9	0.47	0.844
Day 15	439	445	4.8	0.694
Day 22	876	884	9.7	0.697
Day 35	2255	2201	29.1	0.365
Daily feed intake (g/bird/day)				
Days 15-22	91	95	1.8	0.269
Days 22-35	159	161	2.9	0.901
Days 15-35	132	134	2.3	0.602
Daily liveweight gain (g/bird/day)				
Days 1-15	26	26	0.4	0.816
Days 15-22	61	62	1.3	0.658
Days 22-35	97	93	1.6	0.222
Days 15-35	80	82	1.5	0.693
FCR (g/g)				
Days 15-22	1.492	1.532	0.0146	0.209
Days 22-35	1.639	1.731	0.0209	0.066
Days 15-35	1.602	1.673	0.0169	0.062

Existing literature presents varying conclusions, with some authors suggesting that maize-fed birds tend to perform better in terms of FCR (Jia *et al.,* 2009; Rodriguez *et al.,* 2012) and others suggesting enhanced performance in wheat-fed birds (Mathlouthi *et al.,* 2002; Kiarie *et al.,* 2014; Masouri *et al.,* 2017). Conversely, others found no difference in growth efficiency (Shakouri *et al.,* 2009; Ghayour-Najafbadi and Khosravinia, 2017). These observations show discrepancies between experiments, perhaps due differences between cultivars, since the nutritional values of cereals been shown to be rather varied (Cowieson, 2005). The methods by which cereals are

processed can also alter their digestibilities and nutritional values, with suggestions that exogenous enzyme activity is influenced by processing temperatures (Amerah *et al.,* 2011. These inconsistencies suggest that the named cereal base alone does not influence bird growth.

There are suggestions that differences in the relative nutritive values of each cereal are because of digesta viscosity, influenced by levels of complex carbohydrates including non-starch polysaccharides (NSP) that cannot be digested by broilers. Concentrations of NSP are much higher in wheat than maize (147 g/kg and 37 g/kg, respectively; Amerah, 2015), which can be linked to heightened digesta viscosity and a decrease in the apparent digestibilities of lipids, proteins and starches in wheat-fed birds (Maisonnier *et al.,* 2001; Shakouri *et al.,* 2009). This may cause a reduction in nutrient availability since a smaller feed bolus surface area is exposed for digestion. Birds are therefore forced to consume more feed to meet nutritional requirements, particularly since there is evidence that protein digestibility is compromised in birds fed a wheat-based diet (Romero *et al.,* 2014; Munyaka *et al.,* 2016). The extent to which this affects bird growth is unclear perhaps arising from the aforementioned inconsistences in literature and variation between cereal cultivars. Results may be affected by experimental variables, potentially explaining the discrepancies between studies and lack of statistical significance in performance data in the present study.

5.3.2. NGS: alpha diversity

Alpha diversity was explored through Simpson's Diversity Index (*D*) and the effects of bird age and dietary treatment were analysed by ANOVA. Average values with standard deviation with respect to diet type at each sampling point are illustrated in **Figure 5.1**.

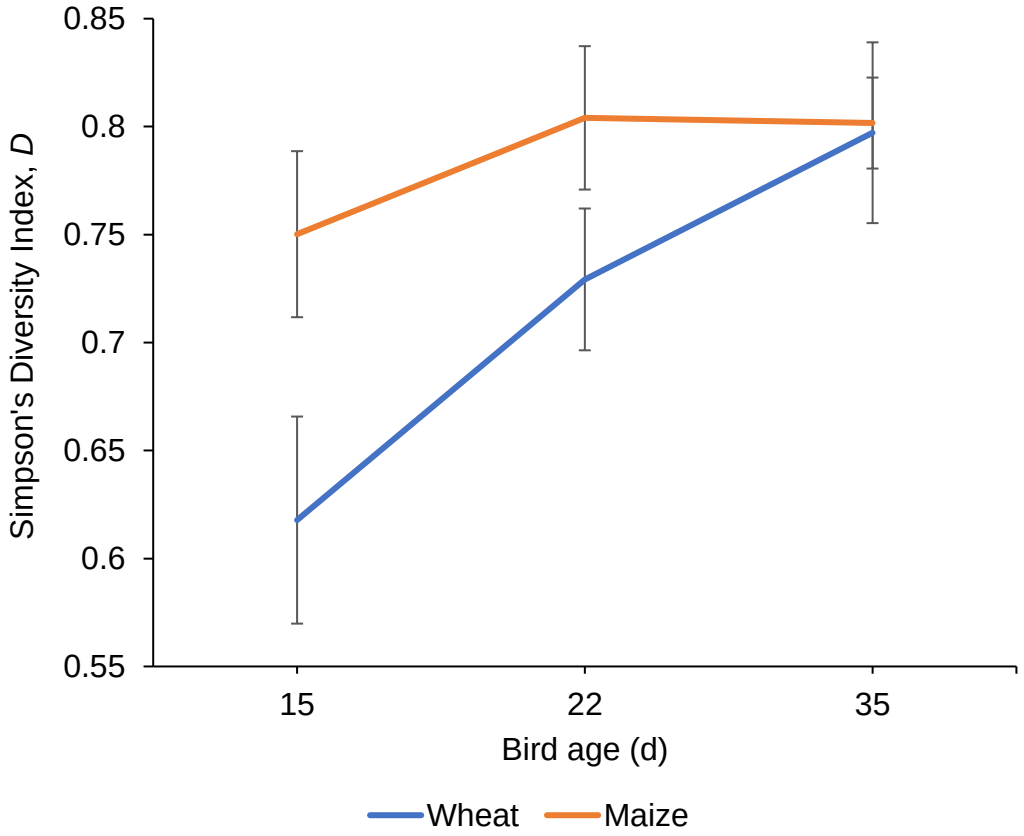


Figure 5.1 *Line plot illustrating alpha diversity of caecal bacterial populations in broilers fed either a wheat- or maize-based diet (n=6/treatment), profiled by next-generation sequencing and measured by Simpson's Diversity Index,* D. *Error bars illustrate standard deviation with respect to diet type at each sampling point.*

Alpha diversity developed with age irrespective of feed base, with a significant increase between days 15 and 22 in both treatment groups (*P*=0.003 and *P*=0.036 in wheat and maize, respectively). In addition to this, a significant increase from days 22 to 35 was also observed in wheat-fed birds (*P*=0.026), though this was not observed in maize-fed birds where diversity peaked by day 22.

Alpha diversity was significantly higher in maize-fed birds than wheat-fed birds at the end of the starter phase (day 15; *P*<0.001) and half way through the grower/finisher phase (day 22; *P*=0.012). Differences diminished by day 35 where *D* reached comparable levels (*P*=0.820).

It is widely accepted that the complexity of the gut microbiota develops sequentially with age. Bacterial complexity appears to be more susceptible to the effects of age in wheat-fed birds. Since diversity was increased by feeding a maize-based diet at day 15, is it thought that younger birds were primed for a more diverse microbiome and the capacity for further development with age was therefore reduced. Conversely, Munkaya *et al.* (2002) and Pan (2014) found that diet base did not affect bacterial diversity, which may suggest that other environmental factors may influence the development of the caecal microbiome aside from cereal base.

5.3.3. NGS: beta diversity

Beta diversity was explored through principal component analysis (PCA) with respect to both bird age and dietary treatment. Age appeared to alter the profile of the microbiome irrespective of dietary treatment (**Figure 5.2**), suggesting sequential development of the microbial profile towards an adult population. Overlapping of data points from days 15 and 22 in wheat-fed birds suggests a gradual shift towards such microbial populations, whereas the beta diversity of maize-fed birds appeared to be more sensitive to the effects of age, expressed through more discrete clustering of data points at each sampling point.

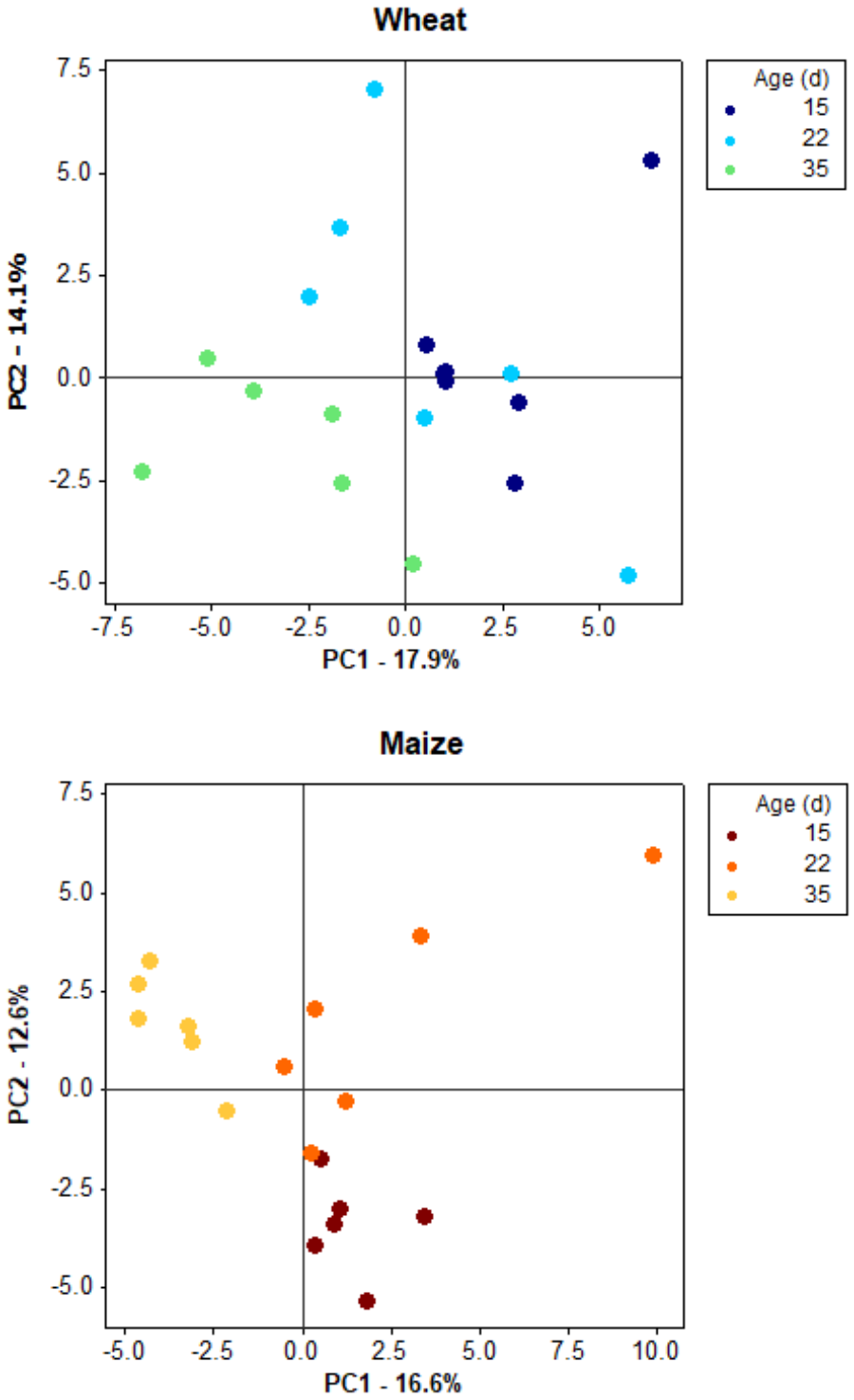


Figure 5.2 *Score plots from unsupervised PCA of caecal bacterial populations profiled by next-generation sequencing, illustrating the effects of bird age (days 15, 22 and 35, n=6/age) within dietary treatment groups (wheat- or maize-fed birds). Discrete clustering of datapoints suggests an effect of age.*

Diet base appeared to alter the profile of the caecal microbiome at the end of the starter phase (day 15), illustrated by discrete clustering of data points in **Figure 5.3**.

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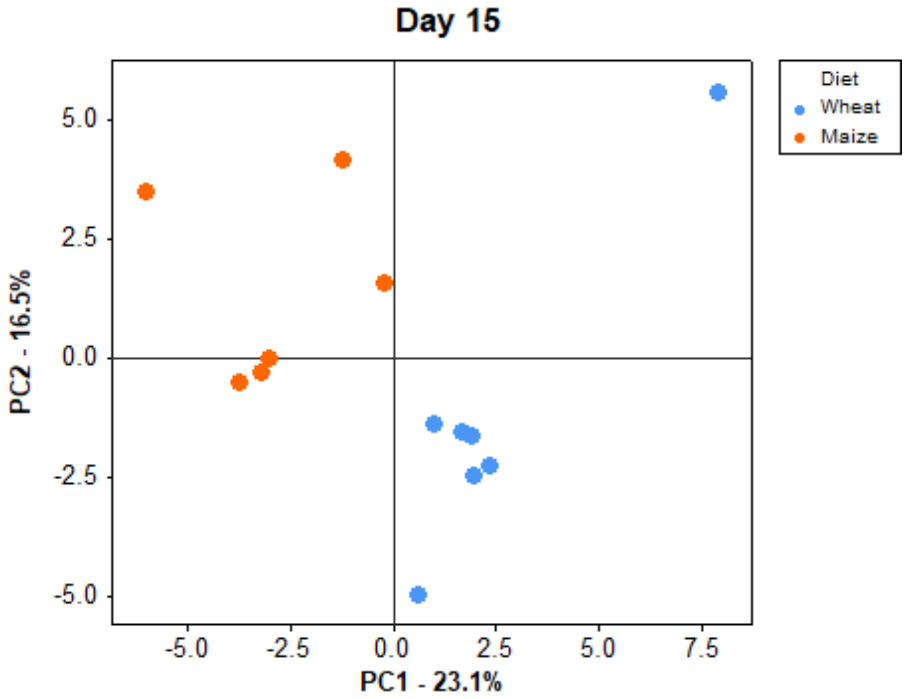

Figure 5.3 *Score plot from unsupervised PCA of caecal bacterial populations profiled by next-generation sequencing, illustrating the effects of treatment (wheat-fed or maize-fed birds, n=6/treatment) at day 15. Discrete clustering of datapoints suggests an effect of age.*

The extent to which data points clustered was diminished at day 22 compared with day 15, and further diminished by day 35, suggesting that diet had less of an effect on diversity with bird age (**Figure 5.4**). Despite this, differences between treatment groups were still apparent by day 35, with four out of six maize samples clustering differently to wheat samples. It is thought that dietary treatment had more of an effect on the caecal microbiome of younger birds, followed by a gradual shift towards a more common 'core' microbiome as birds aged. It is hypothesised that the overlapping of datapoints would have continued to increase, had sampling continued beyond day 35.

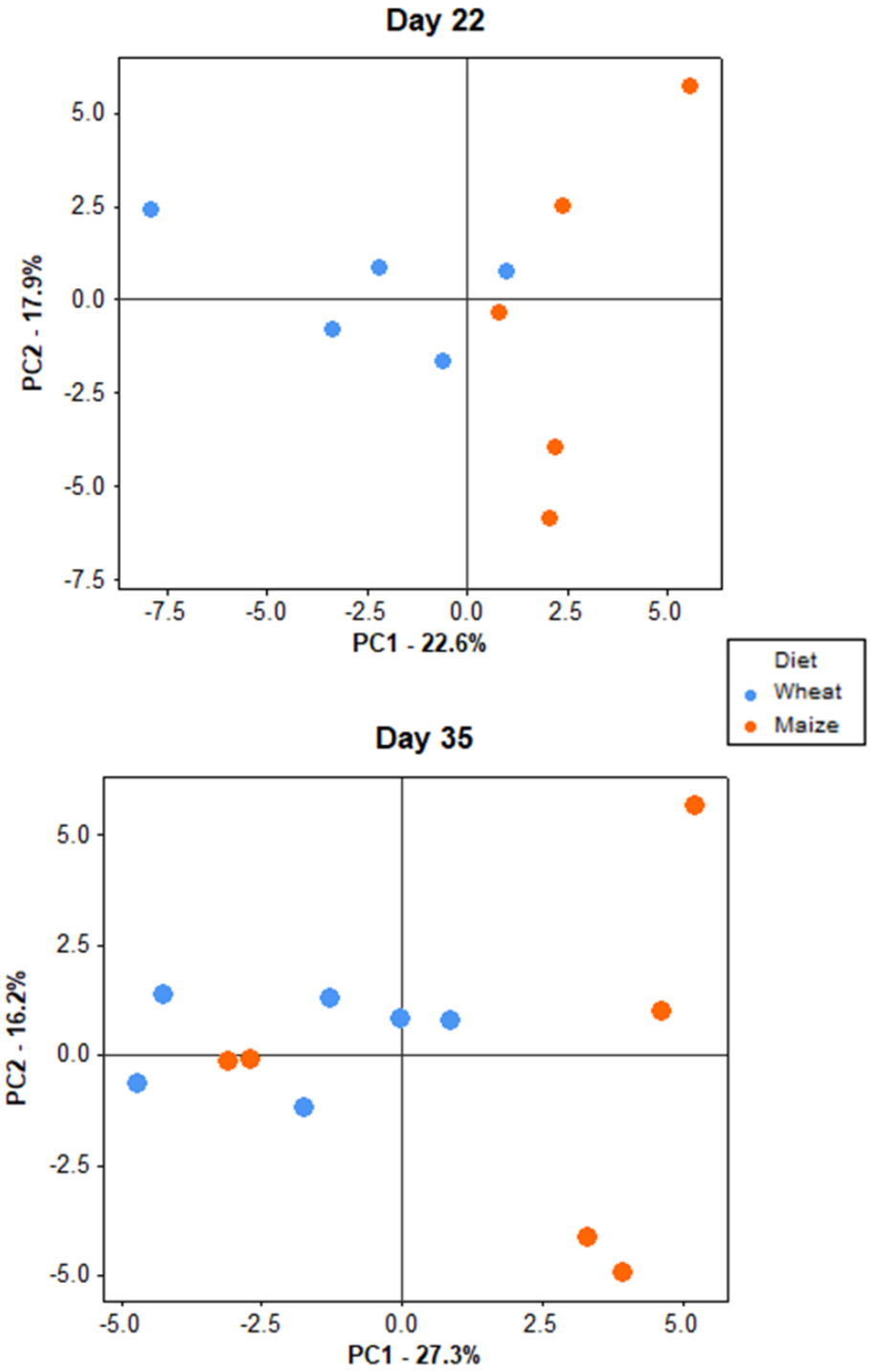

Figure 5.4 *Score plots from unsupervised PCA of caecal bacterial populations profiled by next-generation sequencing, illustrating the effects of treatment (wheat-fed or maize-fed birds, n=6/treatment) at days 22 and 35. A reduction in the discrete clustering of data points suggests a lack of treatment effect.*

5.3.4. NGS: population analysis

Percentage abundances of bacterial taxa were calculated by dividing the number of OTUs assigned to each taxon by the total number of reads. Percentages were analysed through LEfSe to identify discriminative factors between treatment groups – a list of all discriminant features and their *P*-values are listed in **Appendix 3**.

5.3.4.1. Day 15

Firmicutes was the dominant phylum in both treatment groups but was found at significantly higher proportions in birds fed wheat than maize, at an average of 97.6% and 70.2% respectively (*P*=0.027; **Figure 5.5**). Conversely, *Tenericutes* were elevated in maize-fed birds at an average of 27.0% compared with 0.2% (*P*=0.003). This suggests a negative relationship between phyla, confirmed through regression analysis (*r*=–0.997; *P*<0.001). Variation between individual birds was also higher in maize-fed birds, suggesting a stabilising effect of a wheat-based diet.

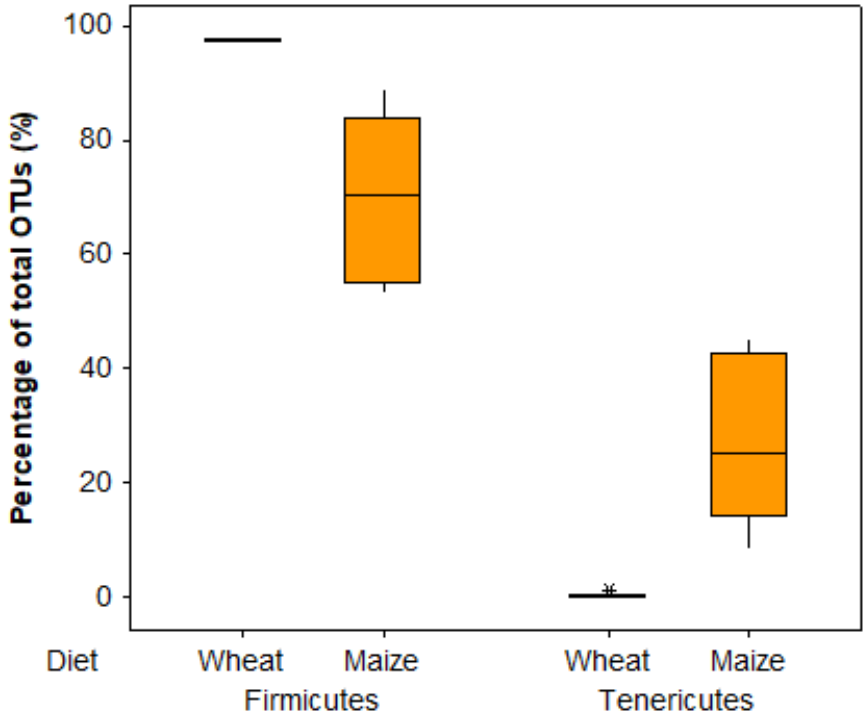

Figure 5.5 *Box plot illustrating the % abundances of phyla* Firmicutes *and* Tenericutes *in the caecal microbiome of birds fed either a wheat- or maize-based diet at day 15 (n=6/treatment). % abundances were calculated from the number of OTUs assigned to each taxa following next-generation sequencing.*

The relative abundances of bacterial families and associated LDA scores of differing taxa are illustrated in **Figure 5.6** – a summary of all discriminative features is listed in **Appendix 3**. The entirety of phylum *Tenericutes* was attributed to an unclassified order denoted as 'RF39' (class *Mollicutes; P*=0.003). Umu *et al.* (2015) found that the relative abundance of *Mollicutes* was significantly reduced in pigs fed a diet high in resistant starches – birds fed a wheat-based diet, hypothesised to contain higher concentrations of NSPs, exhibited lower levels of *Mollicutes* in the present study, supporting their conclusions.

Clostridia were elevated in wheat-fed birds (88.9% compared with 62.5%; *P*=0.003), resulting in a downstream effect on bacteria of lower levels of taxonomy. A large proportion of this difference was accounted for by the families *Lachnospiraceae* (53.5% in wheat *vs* 18.9% in maize; *P*=0.003) and *Ruminococcaceae* (28.4% in wheat *vs* 21.9% in maize; *P*=0.003). These taxa are important in the degradation of complex polysaccharides, resulting in the production of SCFA. The present results perhaps suggest that the bioavailabilities of such resistant starches were varied between diets, affecting the capacity of *Lachnospiraceae* and *Ruminococcaceae* to proliferate in the gut. Though both families are involved in similar metabolic pathways (Biddle *et al.,* 2013), there was no evidence of a relationship in their relative abundances (*r*=0.246; *P*=0.418).

Erysipelotrichaceae were also elevated in wheat-fed birds (*P*=0.003). These bacteria are highly coated in IgA in comparison with other taxa and may be linked to levels of TNF-α (Palm *et al.,* 2014; Dinh *et al.,* 2015), potentially heightening the inflammatory state of the bird. Evidence suggests positive relationships between inflammation and the lipid content of the host diet (Nahidi *et al.,* 2013; Harris *et al.,* 2014). This is potentially due to the loss of the fatty acid biosynthesis capability of some taxa within *Erysipelotrichaceae* (Kwok *et al.,* 2014), suggesting that bacterial growth is reliant on lipids. In support of this, a summary by Kaakoush (2015) suggests a link between a

high fat diet, abundance of *Erysipelotrichaceae,* and the inflammatory status of the gut. Oil, and therefore lipid, levels were very similar between diets in the present study and is therefore unlikely that the overall abundance of lipids has affected bacterial abundance. However, the distribution of individual lipid species (*e.g.* ratio of saturated *vs* unsaturated fatty acids) could still be linked to the microbiome.

Lactobacillaceae were biased towards wheat-fed birds (P=0.003). Not all published findings corroborate this, suggesting that other factors may affect bacterial abundance (Mathlouthi *et al.,* 2002; Shakouri *et al.,* 2009; Masouri *et al.,* 2017). Cereal base has been shown to alter *Lactobacillus* down to strain level (Hammons *et al.,* 2010) with evidence of a relationship with β-glucans in the diet (Jonsson and Hemmingsson, 1991). Knudsen (2014) reports that the β-glucan composition of wheat and maize are similar, suggesting that this was not the reason for differences in *Lactobacillaceae* in the present study. Yan *et al.* (2017) report high proportions of *Lactobacillus* in the caecum of high-performing broilers though this relationship was not observed in the present study since performance was not affected by diet. At averages of 5.0% (wheat) and 2.2% (maize), relative abundance of *Lactobacillaceae* was much higher in the present study than has been identified in the literature, where reports of levels at around 1% are common (Witzig *et al.,* 2015; Torok *et al.,* 2011; Gong *et al.,*2007).

Enterobacteriaceae were found at significantly higher levels in wheat-fed birds at an average of 1.7% compared with 0.9% in maize-fed birds (P=0.003). Enterobacteriaceae is a diverse family of bacteria that encompasses both pathogenic and autochthonous species including *E. coli, Salmonella* and *Campylobacter* (Mellen *et al.* 2014). The vast majority of OTUs from Enterobacteriaceae were attributed to an unclassified genus and therefore further exploration is warranted.

Fewer taxa were biased towards maize-fed birds. *Rikenellaceae* (order *Bacteroidales*) were found in maize-fed birds alone at an average of 0.04% (P=0.001) and play a role

in anaerobic fermentation of carbohydrates (Graf, 2014). It is thought that these bacteria were perhaps present in the feed and were ingested by the birds leading to subsequent colonisation. Alternatively these bacteria may have been ubiquitously present but were only sustained in maize-fed birds after the provision of alternative substrates for growth that were not available in the wheat-based diet.

Several other taxa found at levels below 1% were also affected by diet base: *Leuconostocaceae* (*P*=0.002), *Mogibacteriaceae* (*P*=0.003), *Coriobacteriaceae* (*P*=0.003), *Staphylococcaceae* (*P*=0.003)and *Enterococcaceae* (*P*=0.010) were all biased towards wheat-fed birds. Differences in *Bacillaceae* (*P*=0.024), *Christensenellaceae* (*P*=0.009), *Odoribacteriaceae* (*P*=0.004), *Aerococcaceae* (*P*=0.042) and *Peptococcaceae* (*P*=0.004) were observed in maize-fed birds. The low relative abundances of these taxa make their biological relevance hard to determine, though there were no observed differences in gross disease occurrence and therefore their differences are thought to not have been at any detriment to the health of the birds.

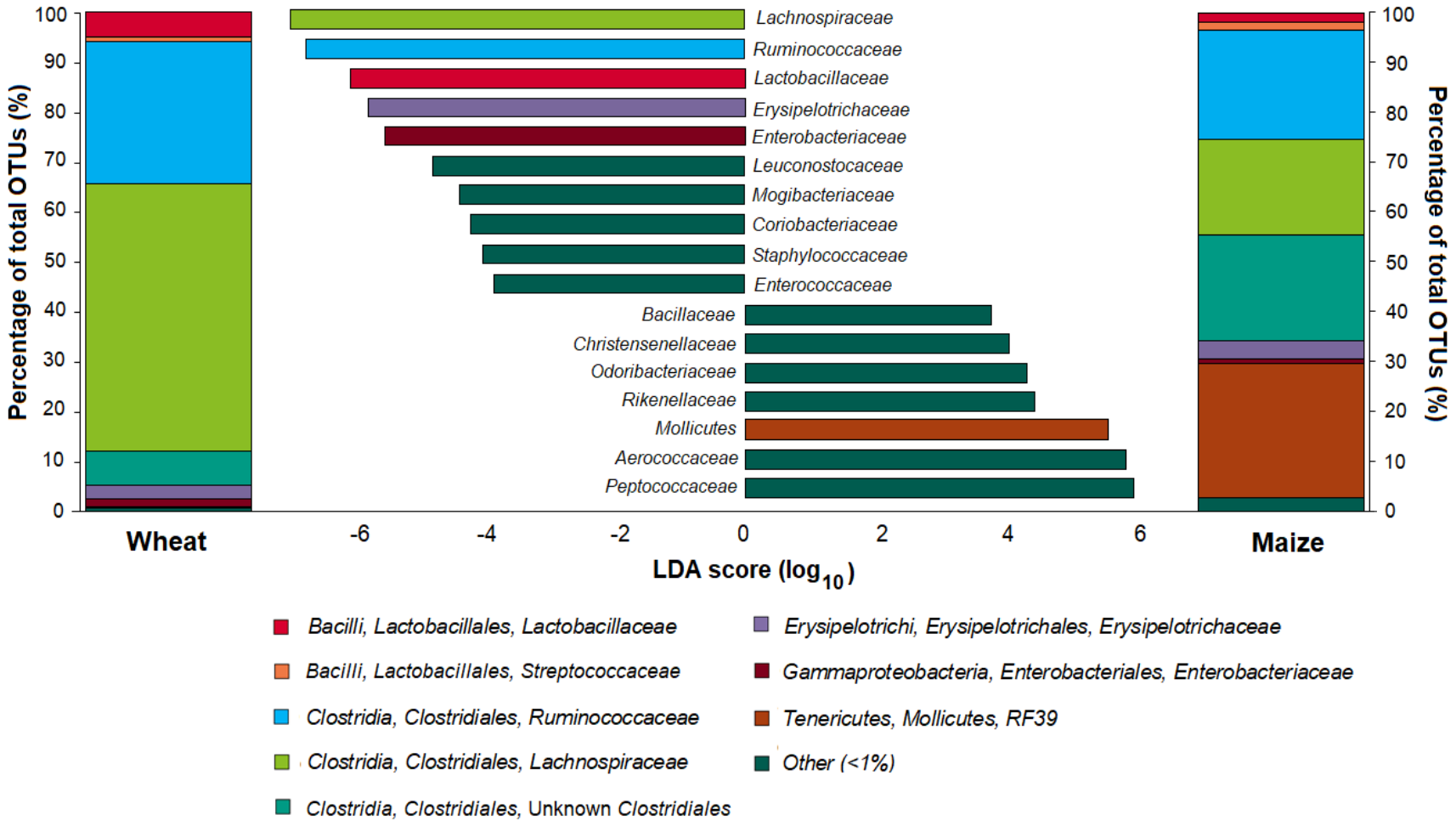

Figure 5.6 *NGS data from day 15.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar (wheat/maize diet; n=6/treatment) illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

5.3.4.2. Day 22

Firmicutes were elevated in wheat-fed birds at day 22, at an average of 90.0% *vs* 66.5% in maize-fed birds (*P*=0.047; **Figure 5.7**). As at day 15, this was offset by *Tenericutes,* found at an average of 20.1% in maize-fed birds compared with 7.5% (*P*=0.009), resulting in a strong negative correlation between these phyla (r=0.81; *P*<0.001).

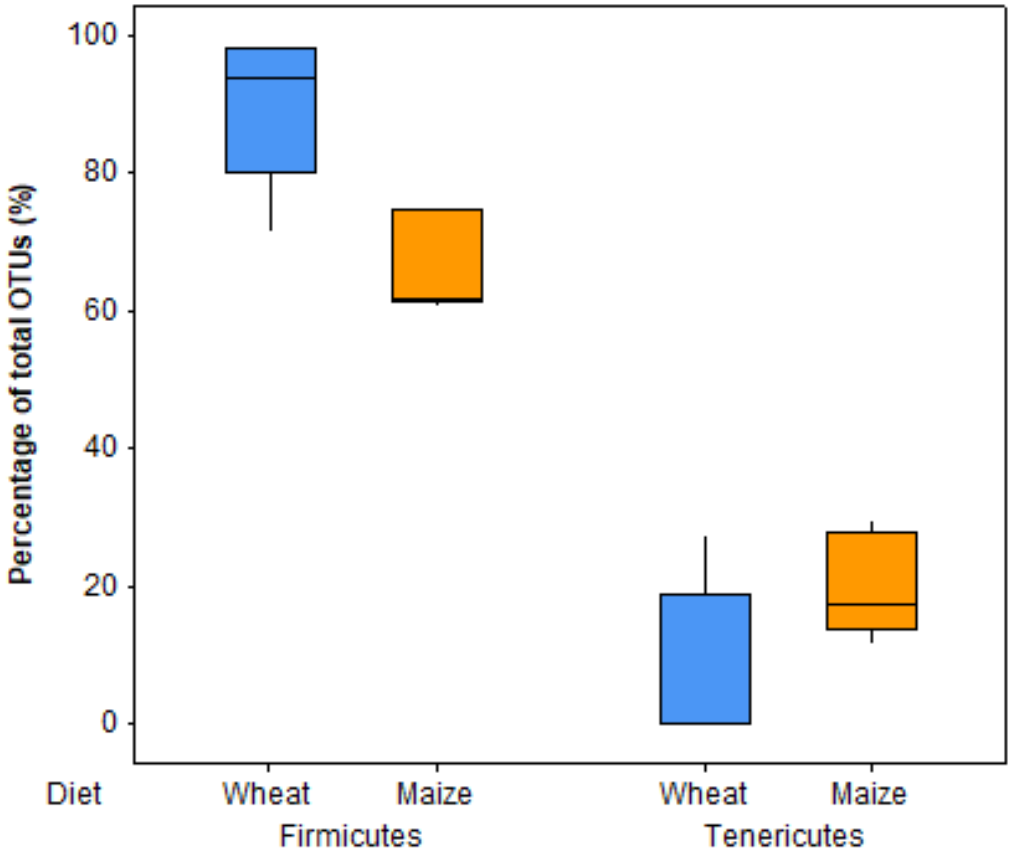

Figure 5.7 *Box plot illustrating the % abundances of phyla* Firmicutes *and* Tenericutes *in the caecal microbiome of birds fed either a wheat- or maize-based diet (n=6/treatment) at day 22. % abundances were calculated from the number of OTUs assigned to each taxa following next-generation sequencing. The abundances of* Firmicutes *and* Tenericutes *were significantly higher and lower in wheat-fed birds, respectively (*P*<0.001)*.

The relative abundance of families and LDA scores of differentially abundant families are summarised in **Figure 5.8**.

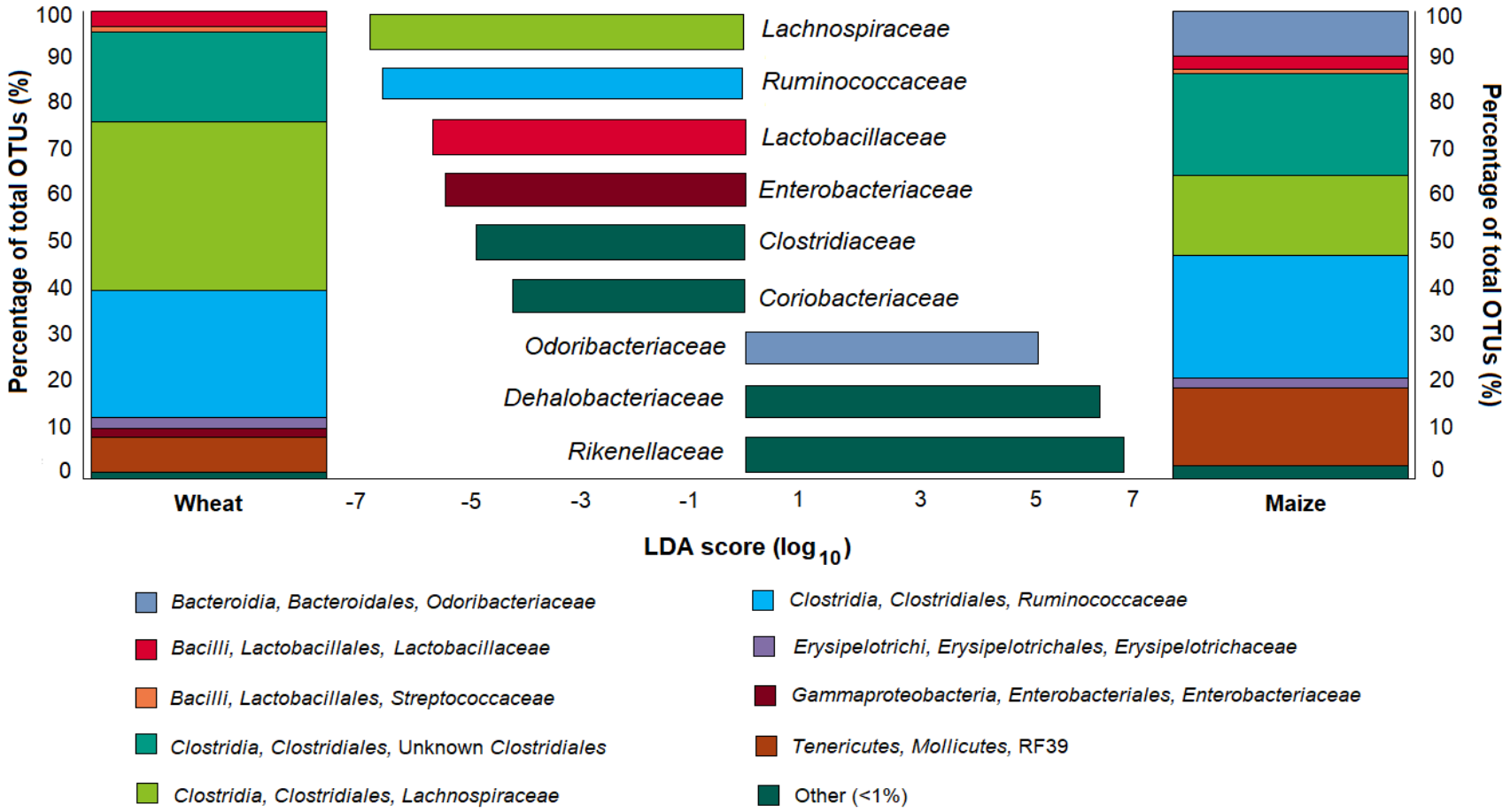

Figure 5.8 *NGS data from day 22.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar (wheat/maize diet; n=6/treatment) illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

As at day 15, *Ruminococcaceae* and *Lachnospiraceae* were biased towards wheat-fed birds (*P*=0.016 for each family), though there was no association between their relative abundances (r=0.43; *P*=0.219).

Enterobacteriaceae were elevated in wheat-fed birds (1.9% *vs* 0.002%; *P*=0.009). Correlation analysis identified an overall positive relationship between Enterobacteriaceae and *Lachnospiraceae* (*r*=0.93; *P*<0.001), though a strong positive correlation was found in wheat-fed birds alone (r=0.92; *P*=0.030), whereas a weak negative correlation was found in maize-fed birds (r=0.72; *P*=0.170; **Figure 5.9**). This suggests that the presence or absence of *Lachnospiraceae* alone is not enough to predict levels of Enterobacteriaceae. It is more likely that differences in nutrient availability differentially fuel the growth of each taxa, rather than a relationship between bacteria.

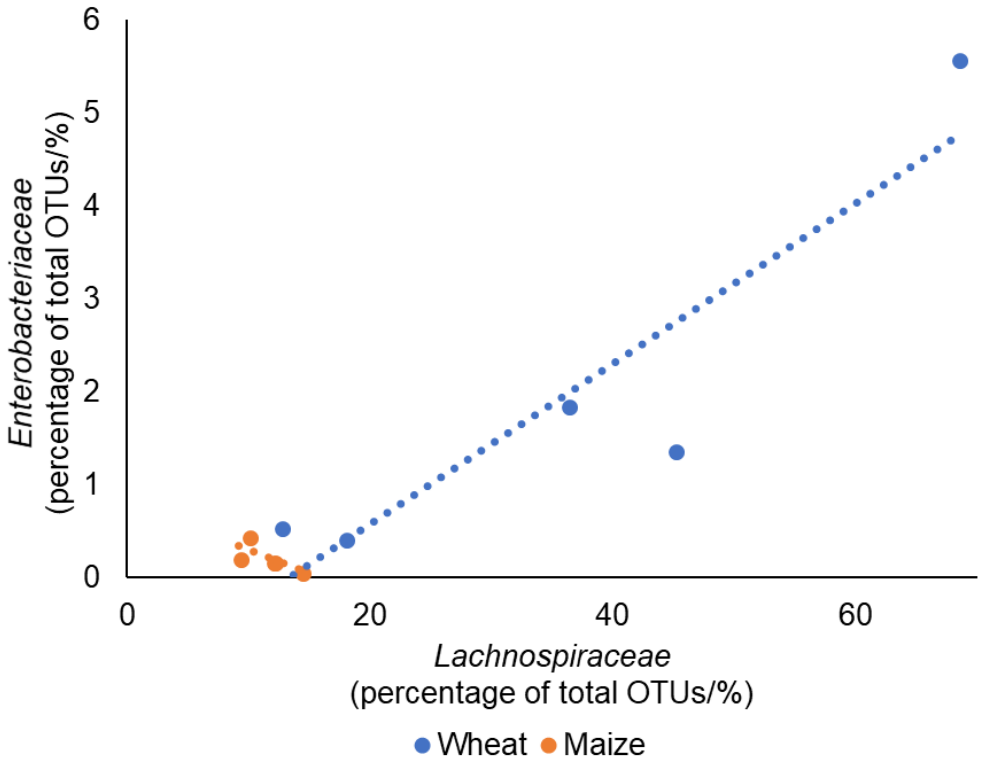

Figure 5.9 *Line plot illustrating the relationship between the relative abundances of* Lachnospiraceae *and Enterobacteriaceae within treatment groups (wheat- or maize-based diets; n=6/treatment) at day 22. Percentage abundances were calculated from the number of OTUs assigned to each taxa following next-generation sequencing. Correlation analysis suggested a strong positive relationship in wheat-fed birds alone (*r*=0.92;* P*=0.030).*

Also from phylum *Firmicutes*, *Clostridiaceae* were biased towards wheat-fed birds at an average of 0.5% compared with 0.2% (*P*=0.016). A large component of cereals is comprised of NSPs, a group of complex molecules found in plant cell walls that increase digesta viscosity (Vincken *et al.,* 2003). Digesta viscosity is a predisposing factor for the development of necrotic enteritis, a condition attributed to *Clostridium perfringens*, a species from family *Clostridiaceae,* that can seriously compromise bird health and welfare (Timbermont *et al.,* 2011; Tellez *et al.,* 2015). These observations suggest that wheat-fed birds may be more challenged than maize-fed birds in terms of pathological challenge. Though not identified to genus level, *Clostridiaceae* abundance was elevated in wheat-fed birds, where NSP levels are generally higher. Digesta viscosity was not analysed, but it is hypothesised to have been higher in wheat-fed birds, potentially explaining the increase in *Clostridiaceae.*

Lactobacillaceae continued to be biased towards wheat-fed birds (*P*=0.047), though relative abundances within treatment groups did not differ greatly between days 15 and 22. Since a dietary change occurred between these sampling points, it is hypothesised that differences in nutrient composition between the grower and starter/finisher diets did not affect the abundance of *Lactobacillaceae* in the caeca. It appears that these bacteria were resistant to changes such as a reduction in soybean meal inclusion.

Fewer taxa were biased towards maize-fed birds, the largest difference being in the abundance of *Odoribacteriaceae,* found at an average of 8.3% compared with 0.0007% in wheat-fed birds (*P*=0.008). Little is known about the role and importance of this family, though it has been isolated from healthy humans (Singh *et al.,* 2015) and ostriches (Videvall *et al.,* 2018). Though carried out in humans, work by Lang *et al.* (2014) found relationships between *Odoribacteraceae* and dietary nutrient compositions, with positive correlations with fibre and protein, and negative correlations with sugars and fatty acids. This may explain differences in bacterial

abundance between treatment groups in the present study, though correlations with feed, and therefore nutrient, intake could not be assessed since feed intake was monitored on a pen basis. It was therefore impossible to record the amount of feed eaten per bird and it is assumed that all birds ate a similar amount. Similarly, *Odoribacteriaceae* may have been present in maize-based but not wheat-based feed, resulting in ingestion and colonisation by one group of birds but not the other.

Rikenellaceae continued to be identified in maize-fed birds alone at an average of just 0.02% (P=0.009). It is hypothesised that the shift in dietary phase following sampling at day 15 caused relative abundance to halve by day 22. This may be due to differences in nutrient composition and bioavailability to fuel growth, or competition from other species that arose from an increase in bacterial diversity with age. The abundance of *Dehalobacteriaceae* was also elevated in maize-fed birds (P=0.005) but was found at levels below 1% and are therefore grouped as 'other' in **Figure 5.8**.

5.3.4.3. Day 35

The relative abundance of families and LDA scores of differentially abundant families from day 35 are summarised in **Figure 5.10**. Unlike days 15 and 22, no differences in phyla were identified. Despite this, the strong negative correlation between *Firmicutes* and *Tenericutes* persisted (r=0.91; P<0.001). Fewer differences at family level were observed in comparison with the previous two sampling points: Enterobacteriaceae alone were biased towards wheat-fed birds (2.5% *vs* 0.2%; P=0.004). Four taxa were elevated in maize-fed birds, exceeding the number of taxa biased towards wheat-fed birds for the first time. An unclassified group of *Cyanobacteria,* denoted as YS2, were found at an average of 1.2% compared with 0.04% (P=0.025). The origin of these bacteria is unclear since the same diet was offered at day 35 as at day 22 where *Cyanobacteria* were not identified. The remaining three families (*Dehalobacteriaceae* [P=0.025], *Gracilibacteriaceae* [P=0.022] and *Christensenellaceae* [P=0.025]) were all found at levels below 1% and thus their biological significance is unclear.

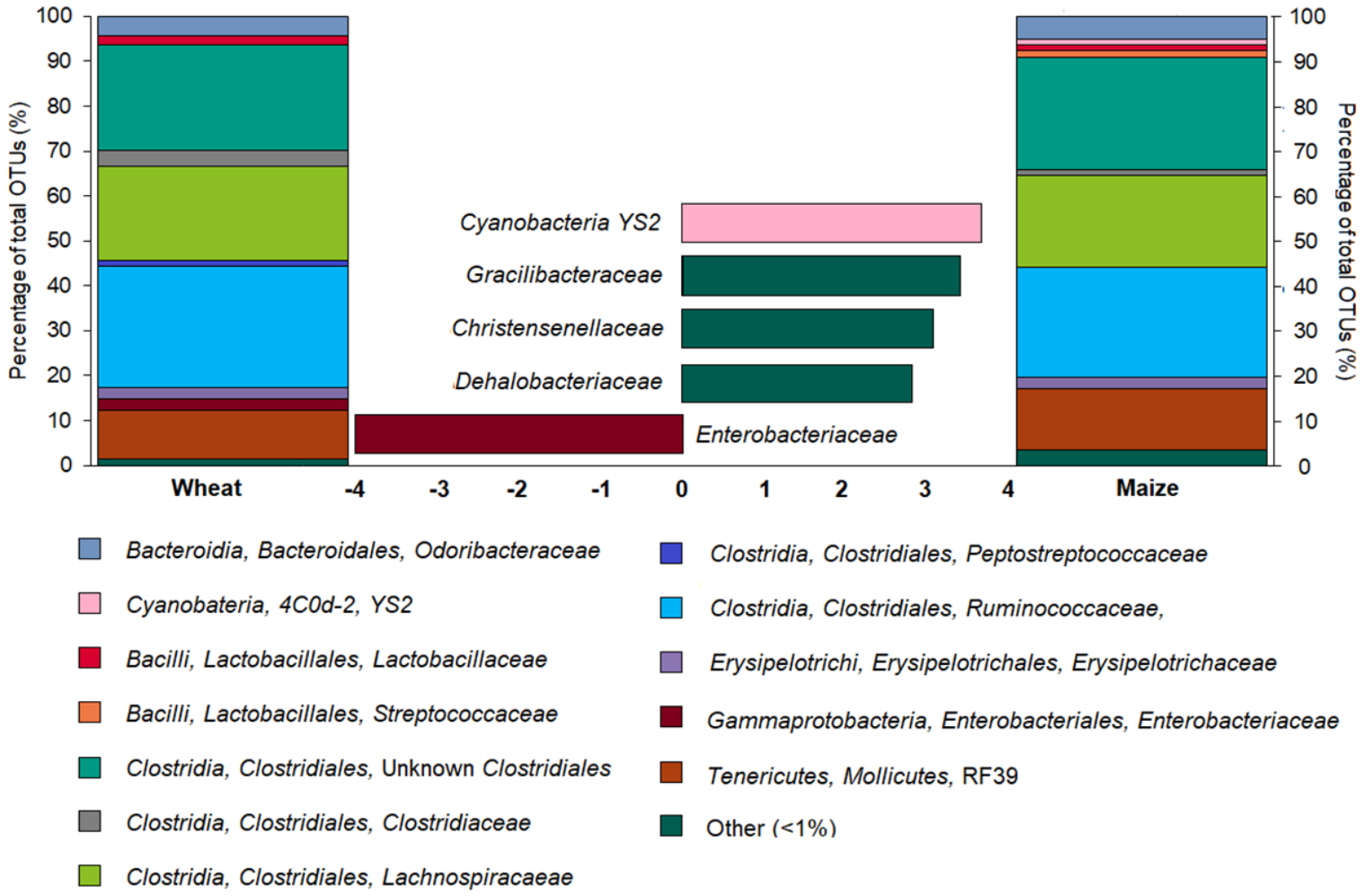

Figure 5.10 *NGS data from day 35.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where α < 0.05 and LDA > 2.0. The direction of the bar (wheat/maize diet; n=6/treatment) illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

5.3.5. NGS summary

The observed effects of diet and age on bacterial diversity were consolidated by a reduction in the number of differentially abundant taxa with age, suggesting that differences induced by diet base in young birds became redundant with maturity. All birds, irrespective of diet base, reached a relatively common end-point by day 35. This suggests that the caecal microbiome of young birds is highly susceptible to change but becomes less sensitive to environmental factors with age. A consistent negative relationship between *Firmicutes* and *Tenericutes* suggested competition for colonisation in the caeca.

Stanley *et al.* (2016) found negative correlations between FCR and abundances of *Lachnospiraceae, Ruminococcaceae* and *Erysipelotrichaceae*. Since these taxa were attributed to the largest differences between treatment groups in the present study, one may expect to have seen lower FCR values in wheat-fed birds. Such a relationship was not observed since all birds performed equally as well, suggesting that the abundances of these taxa may not be indicative of bird performance.

5.3.6. ^{1}H NMR: liver models

OPLS-DA models of spectra obtained from profiling of liver metabolites were constructed (**Table 5.2**). High R^2Y and Q^2 values at day 22 suggest an effect of diet (P<0.001), though large differences at days 15 and 35 suggest a lack of effect.

Table 5.2 *Summary of OPLS-DA models of the metabolic profile of liver taken at days 15, 22 and 35. High R^2Y and Q^2 values are suggestive of an effect of diet. P-values were obtained from 100 permutations, except for day 22 where 1000 permutations were analysed.*

	R^2Y	**Q^2**	***P*-value**
Day 15	0.8425	-0.6293	0.690
Day 22	0.9405	0.4104	<0.001
Day 35	0.8029	0.0956	0.170

Correlation plots were constructed for all sampling points, irrespective of OPLS model values, to visualise any differences in peak size between treatment groups (**Figures 5.11 and 5.12**). Differences in peak sizes assigned to trimethylamine (TMA), trimethylamine-*N*-oxide (TMAO), creatine and acetate were indicated by warm colours. The areas beneath red peaks were integrated and compared against a TSP standard to estimate concentration per gram of starting material and analysed through ANOVA (**Table 5.3**). TMA and TMAO were found at significantly higher concentrations in wheat-fed birds at day 15. TMAO was also elevated in wheat-fed birds at day 22, alongside creatine, and a trend in acetate concentration was also observed. No metabolites were found at elevated concentration in maize-fed birds.

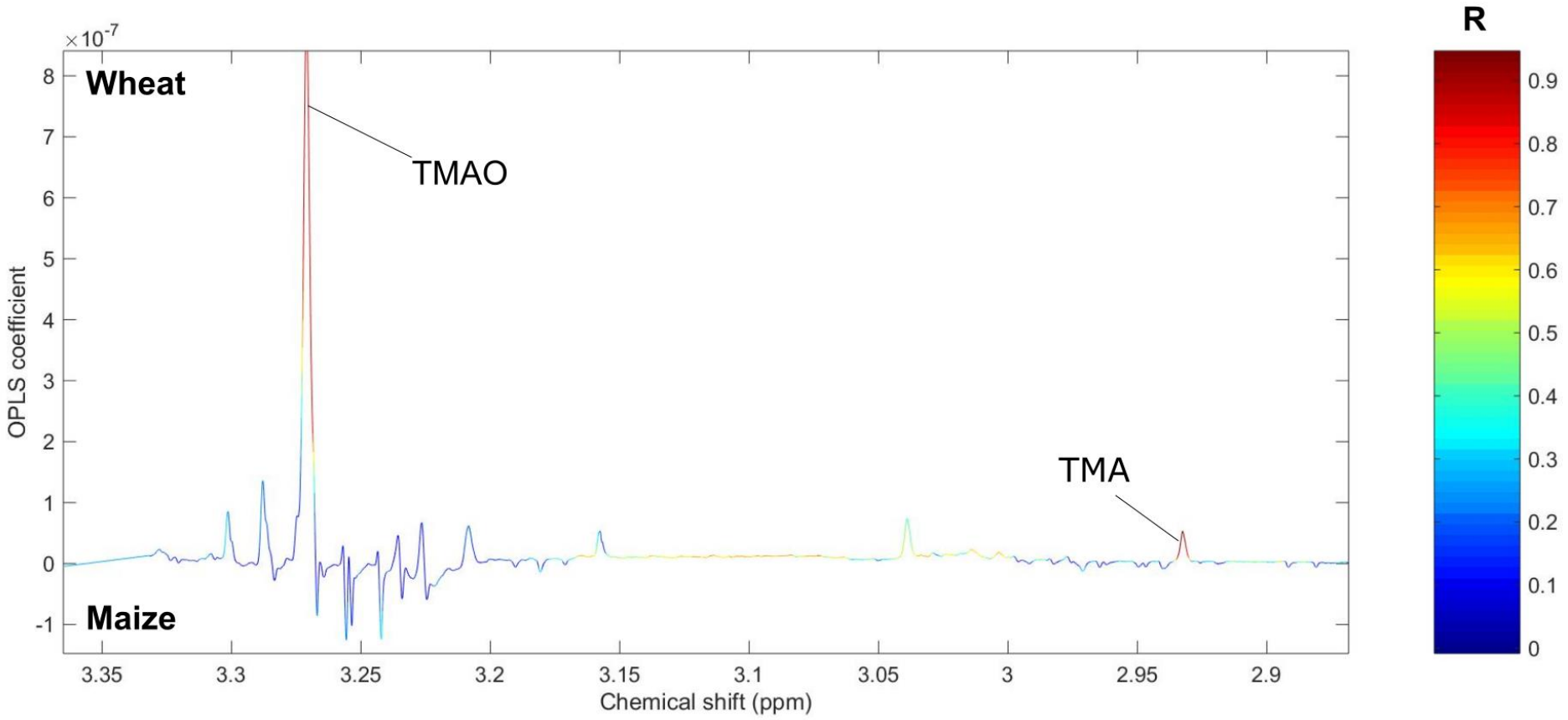

Figure 5.11 *OPLS-DA correlation plot of ^{1}H NMR spectra of liver metabolic extracts from day 15, indicating peaks at differing intensities depending upon diet type (wheat/maize diet; n=6/treatment). Warmer colours such as reds indicate a difference in peak intensity, and therefore suggest a difference in metabolite concentration between treatment groups. Suggested identities of peaks are based upon existing literature and data from the Human Metabolome Database (Wishart et al., 2018).*

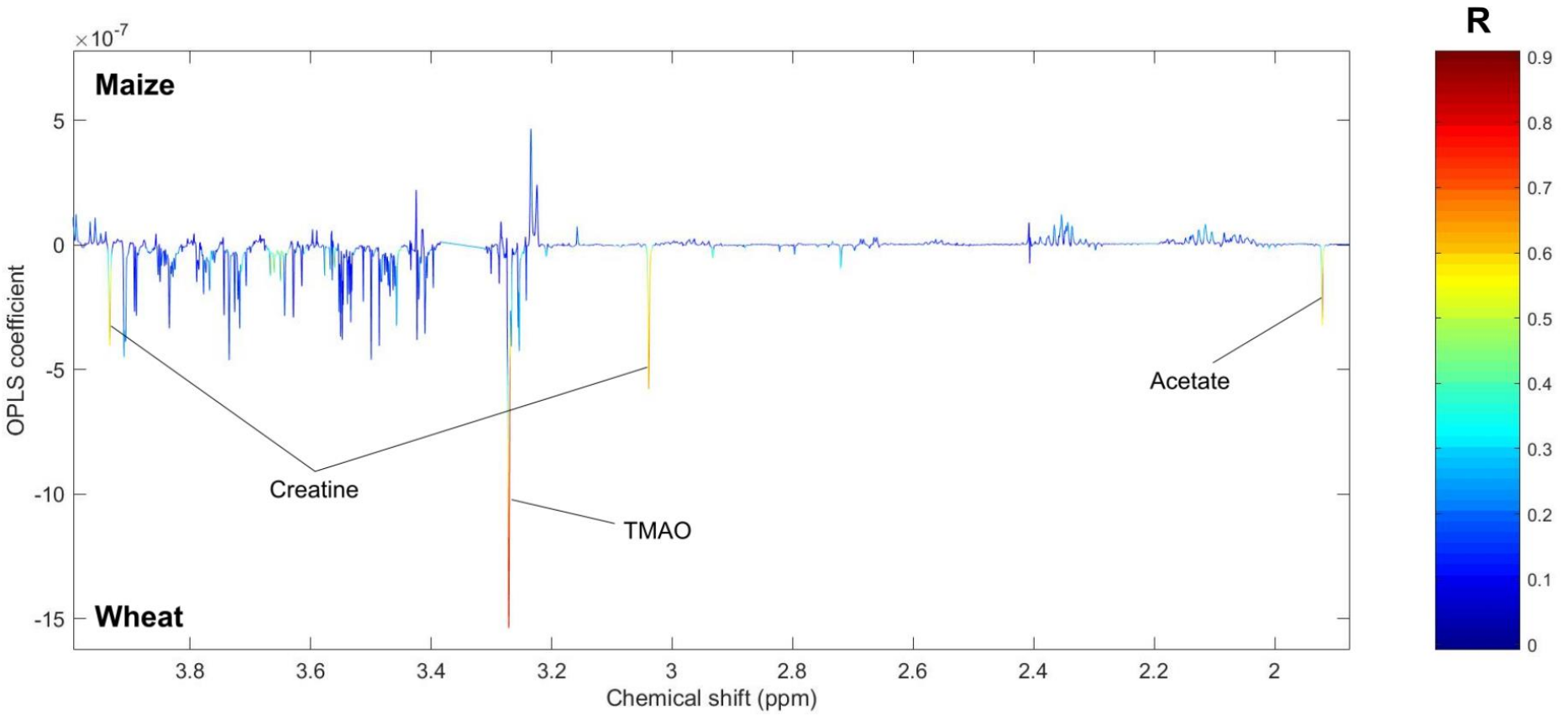

Figure 5.12 *OPLS-DA correlation plot of ^{1}H NMR spectra of liver metabolic extracts from day 22, indicating peaks at differing intensities depending upon diet type (wheat/maize diet; n=6/treatment). Warmer colours such as reds indicate a difference in peak intensity, and therefore suggest a difference in metabolite concentration between treatment groups. Suggested identities of peaks are based upon existing literature and data from the Human Metabolome Database (Wishart* et al., *2018).*

Table 5.3 *Assignments and concentrations of differentially abundant hepatic metabolites affected by dietary cereal base (wheat/maize; n=6/treatment) per gram of starting material calculated by peak integration of ^{1}H NMR spectra ± standard deviation. Peaks in bold were integrated for concentration calculation.* P*-values have been obtained from ANOVA where significance was denoted as* P*<0.05 and means were separated by Tukey's test. (s, singlet).*

		Peak assignment		Concentration (mmol/g)			*P*-value
Metabolite	**Bird age (d)**	**Position (ppm)**	**Splitting pattern**	**Wheat**		**Maize**	
Acetate	22	1.92	s	0.236	>	0.176	0.073
Creatine	22	3.03	s	0.191	>	0.098	0.001
		3.94	s				
TMA	15	2.93	s	0.012	>	0.006	<0.001
TMAO	15	3.27	s	0.103	>	0.057	<0.001
	22			0.237	>	0.138	<0.001

5.3.6.1. Choline metabolism

Concentrations of trimethylamine (TMA) were elevated in wheat-fed birds at day 15 (P<0.001). TMA is produced through the cleavage of dietary choline by a bacterial enzyme in the small intestine and is subsequently absorbed into the bloodstream – for this reason, choline was not found in liver spectra since it had already undergone first-pass metabolism. TMA is further metabolised enzymatically by flavin-containing monooxygenase-3 to trimethylamine-*N*-oxide (TMAO) in the liver, concentrations of which were also elevated at days 15 and 22 in wheat-fed birds (P<0.001). A schematic cartoon of this metabolic process is illustrated in **Figure 5.13**.

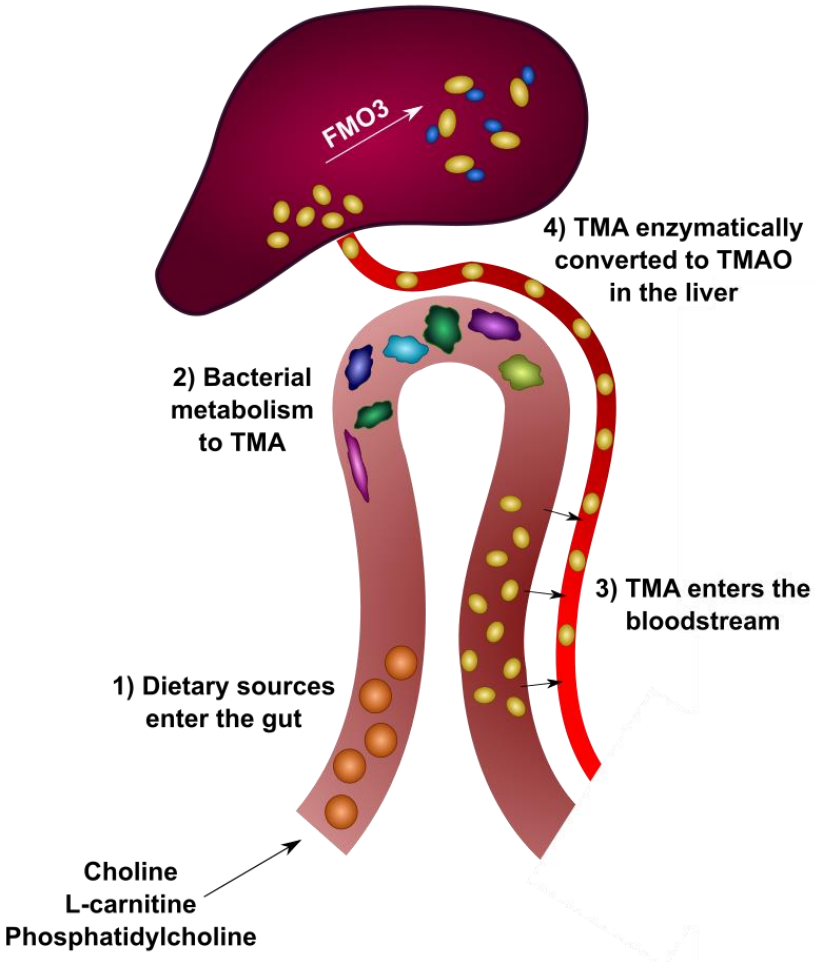

Figure 5.13 *Schematic cartoon illustrating the metabolism of TMA derivatives such as choline, L-carnitine and phosphatidylcholine to TMAO in the intestines and liver. (FMO3, flavin-containing monooxygenase-3. [Own image]).*

Choline is generally found at higher concentrations in wheat than maize (Menten *et al.,* 1998), suggesting why concentrations of TMA and TMAO were elevated in wheat-fed birds. Evidence suggests that TMAO concentrations are elevated when a high starch and high fat diet is fed (Berganon *et al.,* 2016; Sanguinetti *et al.,* 2018). Metabolomic studies have suggested that concentrations of TMAO are positively related to dietary protein levels (Rasmussen *et al.,* 2012). The crude protein content of the wheat-based diets was slightly higher than that in the maize-based diets, though no significant correlations between feed intake in bird days and TMAO concentrations at day 22 were made. Correlations at day 15 could not be explored due to a lack of replicate pen units, since birds were housed in one of two brooding pens until day 15. These observations led to the hypothesis that it is the composition and metabolic capacity of the intestinal bacteria, not the intake of dietary precursors, that affect TMA/TMAO concentrations.

Negative correlations between TMA/TMAO and *Bacteroidales, Peptococcaceae* and *Lachnospiraceae* have been suggested (Bergeron *et al.,* 2016; Sanguinetti *et al.,* 2018), with evidence of positive correlations with *Ruminococcaceae* and *Mollicutes* (O'Connor *et al.,* 2014). These associations were not made in the present study, though a negative correlation between TMAO and *Firmicutes* was identified in wheat-fed birds at day 22 (r=0.89; P=0.041.) This relationship was not noted in maize-fed birds (r=-0.14; P=0.785) or in either treatment group at day 15, suggesting correlation without causation. TMA concentrations were negatively correlated to *Christensenellaceae* and *Staphylococcaceae* in wheat-fed birds at day 15 (r=0.85 and 0.83 respectively). Previous report of these relationships could not be found in existing literature, and there is no evidence that these families express the genes required for TMA synthesis. It is therefore hypothesised that *Christensenellaceae* and *Staphylococcaceae* levels are not causative of an increase in TMA concentrations, despite an apparent correlation.

Romano *et al.* (2015) report that TMA/TMAO production is not affected by bacterial abundance but merely by the presence of certain species, suggesting that interactions with other factors may influence choline metabolism. Despite evidence of relationships between TMA/TMAO production and bacteria in humans and mice, the lack of strong and consistent relationships in the present study suggests that caecal bacteria may not affect this metabolic pathway in broiler chickens. Though first-pass metabolism of dietary precursors mainly takes place in the small intestine, Koeth *et al.* (2015) identified that the caecum exhibited the highest TMA synthetic capacity in mice. Despite this, the observations made in the present study and comparisons with existing literature consolidate the need for further exploration into the microbial profile of proximal gut. This may be due to physiological differences in the digestive system, and the difference in the capacity of the host to metabolise dietary components such as resistant starches.

5.3.6.2. Creatine metabolism

An increase in creatine, an organic acid that facilitates the recycling of ATP by donating phosphate groups to ADP, was found in wheat-fed birds at day 22 (P=0.001). Creatine is primarily synthesised from glycine, arginine and methionine in the liver, passes into the circulatory system and is actively transported into muscles for use as an energy source. Since crude protein levels were slightly higher in the wheat-based diet, it was hypothesised that wheat-fed birds had a heightened biosynthetic capacity of creatine due to elevated substrate availability. No correlations with average feed intake were found (P=0.223), suggesting that dietary precursors may not be indicative of the potential for hepatic biosynthesis of creatine. Since creatine is synthesised enzymatically by the host, it is unsurprising that there were no correlations with caecal bacterial abundance.

Dietary creatine has been shown to improve weight gain (Halle *et al.,* 2006; Carvalho *et al.,* 2013; Zhao *et al.,* 2017) and has also been linked to a reduction in the detrimental effects of stress on carcass quality by reducing oxidative stress (Min and Ahn, 2005; Deminice and Jordao, 2012; Zhang *et al.,* 2014; Wang *et al.,* 2015). Since creatine was found at levels twice as high in wheat-fed birds than maize-fed birds, it is hypothesised that the meat quality of the former group may have exceeded that of the latter, a parameter that may be explored in future work.

5.3.6.3. SCFA

The OPLS-DA model for day 22 suggests an elevation in the concentration of acetate in wheat-fed birds (*P*=0.073). Further analysis of other SCFA suggest heightened concentrations of lactate, butyrate and propionate at day 15 and acetate at day 35 in wheat-fed birds (**Table 5.4**).

Table 5.4 *ANOVA exploring the effects of diet base (wheat/maize; n=6/treatment) on the concentrations of hepatic SCFA (mM/g liver) where statistical significance was denoted as* P*<0.05 and means were separated by Tukey's test.*

	Diet base			
	Wheat	**Maize**	**SEM**	***P*-value**
Day 15				
Acetate	0.15	0.16	0.005	0.247
Butyrate	2.05	0.92	0.162	0.006
Propionate	0.75	1.06	0.049	0.012
Lactate	1.20	3.26	0.086	<0.001
Day 22				
Acetate	0.27	0.21	0.017	0.093
Butyrate	1.13	1.22	0.065	0.501
Propionate	0.99	1.01	0.027	0.785
Lactate	0.94	1.06	0.054	0.292
Day 35				
Acetate	0.22	0.26	0.009	0.039
Butyrate	1.85	2.02	0.102	0.428
Propionate	0.98	0.87	0.039	0.182
Lactate	1.43	1.57	0.075	0.385

5.3.7. Correlation analysis

Kiarie *et al.* (2014), though they did not directly explore the caecal microbiome, found differences in concentrations of SCFA in the caecum of broilers fed either a wheat or maize-based diet. Since SCFA are microbial metabolites, a statistical difference in their prevalence suggests a shift in microbial profiles. *Ruminococcaceae* and *Lachnospiraceae* are two of the main SCFA-producing bacteria in the broiler gut (O'Keefe, 2008) and the relative abundances of both of these families were elevated in wheat-fed birds at days 15 and 22. No relationships between hepatic SCFA concentration and abundance of caceal *Ruminococcaceae* were observed, but *Lachnospiraceae* were positively correlated with concentrations of butyrate and negatively correlated with lactate at day 22. Despite these apparent associations, it must be remembered that SCFA are produced from the fermentation of otherwise indigestible carbohydrates, such as NSP, by endogenous bacteria in the gut of the host. SCFA concentrations were elevated in wheat-fed birds, where NSP as a percentage of total dry matter is generally higher than in maize-based diets (Boros and Fraś, 2015). It is therefore hypothesised that this increase in hepatic acetate concentrations was a result of increased availability, and therefore fermentation, of complex carbohydrates.

Authors have previously identified a bacteriostatic effect of SCFA on pathogenic bacterial species. Van der Wielen *et al.* (2000) found a strong negative correlation between concentrations of caecal SCFA and numbers of Enterobacteriaceae, concluding a bacteriostatic effect on pathogenic *Salmonella enterica* (subsp. *Typhimurium*). Though taxa could not be accurately assigned to genus level, and therefore relationships with *Salmonella* could not be explored, a lack of correlation between acetate and Enterobacteriaceae (r=0.14; *P*=0.823) suggests no downstream effect on bacteria of lower taxonomy, such as *Salmonella*. Guyard-Nicodeme *et al.* (2015) also found negative relationships between SCFA concentrations and levels of *Campylobacter jejuni* (family *Campylobacteraceae*) in the caecum. *Campylobacter*

was not found the in caecal content of any birds in the present study and so this relationship could not be explored. These observations may indicate that there are no relationships between the caecal microbiome and hepatic SCFA concentrations, particularly if metabolites are absorbed by enterocytes or metabolised by portal vein blood cells before reaching the liver. There may, however, have been effects on other microbial populations: the gut-liver axis has been highlighted as a potential metabolic mechanism in the prevention of subclinical necrotic enteritis by facilitating lipid metabolism in broiler chicks (Qing *et al.,* 2017). The lipid fraction of the hepatic metabolome was not assessed in the present study, so these suggested relationships could not be explored.

5.4. Conclusion

This study aimed to explore the effects of wheat and maize-based diets on the performance, caecal bacterial populations and the hepatic metabolome of growing broiler chicks. The presented results suggest that diet base induces many differences in bacterial abundance during early life, though these modulations diminish with age. Despite this, differences in performance were not evident and it can therefore be concluded that the observed differences in the caecal microbiome and hepatic metabolome were not related to bird growth. Concentrations of choline-derived metabolites, creatine and acetate were altered by diet base, though these were not found to be related to the caecal microbiome. Differences in feed components will, naturally, affect the metabolome since the microbiome and host can only use the chemical pool that is available. Future work should explore relationships with the microbiome of the small intestine, since factors such as nutrient availability, digesta pH, and oxygen levels select for different bacterial species. This may assist the elucidation of the role of bacterial taxa on TMA/TMAO metabolism.

These observations suggest that differences in the microbiome and metabolome may not influence the functional phenotype of broilers, allowing birds to utilise their feed differently, but just as effectively. The work presented in this study suggests that wheat and maize-based diets can be fed to growing broilers without any effect on performance and that the microbiome and hepatic metabolome are not sole predictors of bird growth.

6. Study 4: Effects of bedding – supplementation with excreta

6.1. Introduction

An estimated 1.25 tons of litter is produced per 1000 placed broiler chicks (Foy *et al.,* 2014). Given that an average of 971.5 million broilers have been placed in the UK per year between 2012 and 2017 (DEFRA, 2018), this suggests that just over 1.2 million tons of litter is produced annually in the UK alone. This huge volume undoubtedly carries heavy financial and environmental burdens for producers, which may be lessened through management practices.

Bedding is traditionally discarded at the end of each batch of birds and sheds are disinfected before the next chick placement. However, the reuse of bedding is a management technique that is widely used in the USA but is less common in other countries. Reusing bedding reduces material cost, labour time, and improves the environment by, for example, lowering greenhouse gas emissions arising from delivery vehicles. Litter can be either fully or partially reused following shed disinfection with the most widely-accepted technique being partial reuse, where litter is heaped following shed clear-out with subsequent spread on the 'grower' section of the freshly disinfected shed for the next placement of chicks.

Numerous perceived barriers against the reuse of bedding exist although research suggests that the majority of these concerns are misguided. Odour emissions are not affected by the reuse of bedding (Dunlop *et al.,* 2010) though dust levels can be higher in a partial reuse system (Modini *et al.,* 2010). There may also be concerns over ammonia levels, which can be detrimental to bird health if exceeding 25 ppm. An Australian Government Report (Walkden-Brown, 2010) found that ammonia levels, though still well below this threshold, were around twice as high in sheds with reused litter than those with fresh litter. Evidence suggests that ammonium build-up is manageable by increasing ventilation and the use of urease inhibitors

(Walkden-Brown, 2010). Importantly, there appears to be a lack of an effect on bird live-weight (Torok *et al.,* 2009).

Another concern for bedding reuse is the potential for pathogens in the bedding to transfer to newly placed chicks. For example, Kassem *et al.* (2010) found that *Campylobacter jejuni* and *Campylobacter coli* can survive for at least 20 days in reused litter, creating a reservoir that may predispose new flocks to infection. It is therefore general practice to heap and compost bedding before reuse, since this causes the temperature rise to a level that is sufficient to kill potential animal and human pathogens including *Salmonella, E. coli, Campylobacter, Clostridium perfringens*, chicken infectious anaemia virus and infectious bursal disease virus (Macklin *et al.,* 2006; Chinivasagam, 2009; Walkden-Brown, 2010; Roll *et al.,* 2011; Wei *et al.,* 2013a).

Since chicks are hatched into a microbiologically clean, but not sterile environment in industry, the development of their intestinal microbiome is influenced by exogenous factors at the hatchery (Garrido *et al.,* 2004; Torok *et al.,* 2009). The gut microbiome develops rapidly when exposed to environmental bacteria; exposure to a complex 'soup' of microbes from mature birds in reused litter most probably influences the development of the microbial population in the chick (Torok *et al.,* 2009; Cressman et al, 2010, Wang *et al.,* 2016). Litter is a combination of bedding material and excreta which contains a plethora of bacteria which can be transferred between batches of birds if reused. It is therefore likely that it is this excreta, rather than purely the bedding material itself, that influences the microbiome of the growing broiler.

6.2. Aims

Since the gut microbiome influences numerous factors including bird growth (Torok *et al.,* 2011), the immune system (Oakley and Kogut, 2016) and feed utilisation (Rinttila and Apajalahti., 2013), the effects of bedding may influence bird physiology and

metabolism. Despite this, extensive research into the longitudinal effects of exposure to excreta from mature birds is lacking.

This study aimed to:

- Investigate the effects of supplementing bedding with excreta from adult hens on the performance of growing broilers.
- Explore the effects of bedding on caecal bacterial populations.
- Explore the effects of bedding on the profile of the hepatic metabolome.
- Suggest relationships between populations of caecal bacteria and concentrations of hepatic metabolites.

6.3. Results and discussion

6.3.1. Performance

Performance was explored through ANOVA (**Table 6.1**). FCR, daily liveweight gain and daily feed intake were calculated on a pen-basis (n=6 pens/treatment) whereas bird liveweight was calculated on an individual bird basis. The number of birds per pen varied between time points, since birds were sacrificed for biological sampling at days 15, 22 and 35.

All birds weighed a similar amount upon arrival (day 1; P=0.830). The subsequent liveweights and daily liveweight gain of birds at sampling points (days 15, 22 and 35), where individual birds were the replicate units, were not altered by bedding (P>0.05). Daily feed intake and FCR were analysed with pen as the replicate unit – since birds were housed in two brooding pens from day 1-15, statistical analysis for this period of time could not be performed due to a lack of unit replicates. Bedding had no effect on either daily feed intake or FCR from days 15 onwards (P>0.05), meaning that bird performance was not altered by bedding.

Table 6.1 *Effects of bedding (clean shavings/excreta supplementation) on bird liveweight, where individual birds were replicate units, and daily liveweight gain (g/bird/day), feed intake (g/bird/day) and FCR, where pens were replicate units (n=6/treatment). Daily feed intake and FCR for days 1-15 have not been statistically analysed due to a lack of pen replicates during the starter phase. Statistical significance was denoted as* P*<0.05 and means were separated by Tukey's test.*

	Bedding type			
	Control	**Supplemented**	**SEM**	***P*-value**
Bird liveweight (g)				
Day 1	42.1	42.2	0.35	0.830
Day 15	439	427	6.1	0.351
Day 22	876	874	10.3	0.929
Day 35	2255	2168	35.1	0.222
Daily feed intake (g/bird/day)				
Days 1-15	37	37	N/A	N/A
Days 15-22	91	96	3.6	0.544
Days 22-25	159	163	4.1	0.694
Days 15-35	132	130	3.1	0.772
Daily liveweight gain (g/bird/day)				
Days 1-15	29	28	0.9	0.645
Days 15-22	61	63	1.3	0.575
Days 22-35	97	98	2.0	0.537
Days 15-35	82	80	1.3	0.463
FCR (g/g)				
Days 1-15	1.396	1.438	N/A	N/A
Days 15-22	1.492	1.519	0.0330	0.699
Days 22-35	1.651	1.663	0.0173	0.761
Days 15-35	1.609	1.618	0.0157	0.630

6.3.2. NGS: alpha diversity

A total of 1,400,870 sequences were obtained from DNA isolated from caecal digesta, giving an average of 116,739 per caecal sample. Alpha diversity, measured by Simpson's Diversity Index, *D*, was significantly elevated in birds on supplemented bedding at day 15 (*P*=0.009; **Figure 6.1**). Since *D* is the probability that two sequences picked at random are from different taxa, one would be 8.9% more likely to isolate

different bacteria from birds on supplemented bedding than controls (0.7294 and 0.6403, respectively). No differences were observed at day 22 (*P*=0.404) or 35 (*P*=0.973), where both treatment groups exhibited similar levels of alpha diversity.

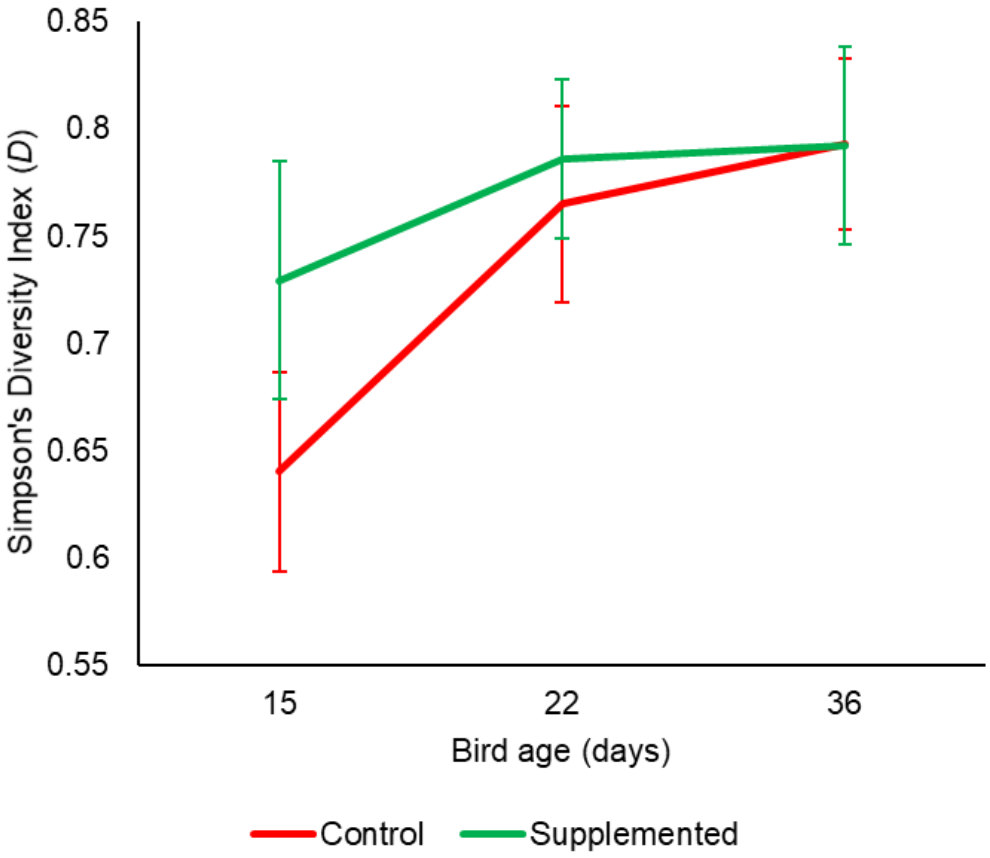

Figure 6.1 *Line plot illustrating the effects of bedding (clean shavings or excreta supplementation; n=6/treatment) on alpha diversity of caecal populations, profiled by next-generation sequencing and measured by Simpson's Diversity Index* (D)*. Error bars represent standard error with respect to treatment at each sampling point.*

Further analysis indicated that age significantly affected alpha diversity in controls, where *D* increased by 12.5% between days 15 and 22 (*P*=0.003). This difference was less pronounced in birds on supplemented bedding where *D* increased by just 5.6% (*P*=0.080). It is hypothesised that age had less of an effect in birds on supplemented bedding, since young birds were exposed to 'adult' bacterial populations earlier in life.

6.3.3. NGS: beta diversity

Beta diversity was explored through principal component analysis, results from which were visualised through PCA score plots. Bedding appeared to alter the profile of the caecal microbiome at day 15, illustrated by clustering of data points in **Figure 6.2**.

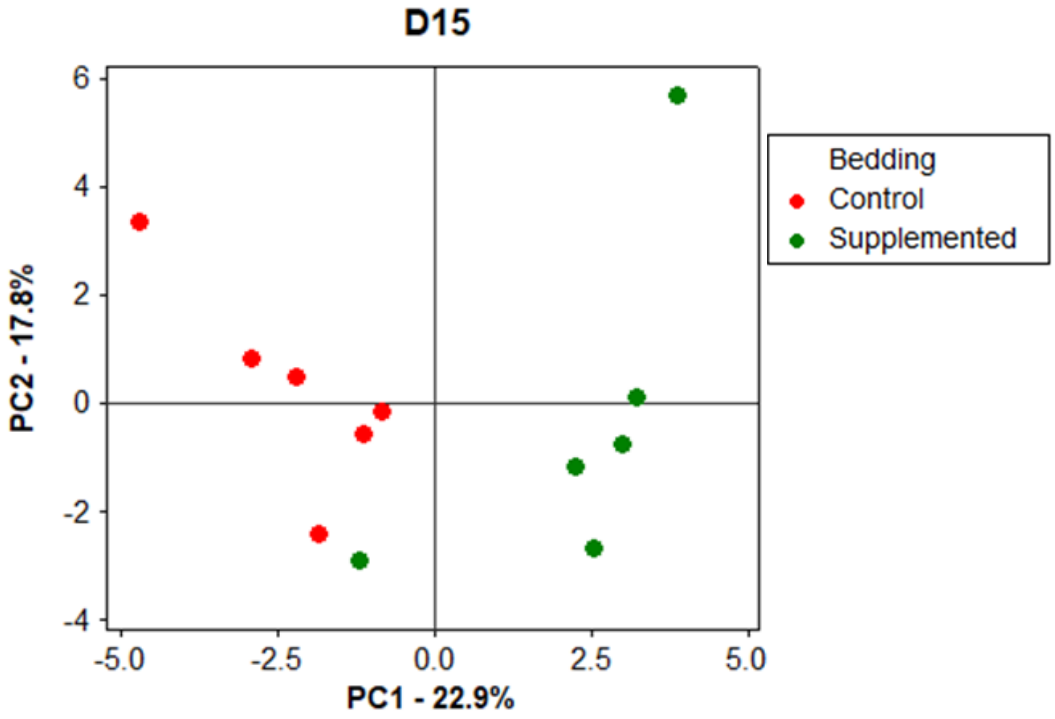

Figure 6.2 *PCA score plot illustrating the effects of bedding (clean shavings/excreta supplementation; n=6/treatment) on beta diversity of caecal bacterial populations taken from broilers at day 15 and profiled through next-generation sequencing.*

Clustering was less pronounced at days 22 and absent at day 35 (**Figure 6.3**). These results suggest that bedding initially altered the profile of the caecal microbiome, but differences dissipated as the study progressed.

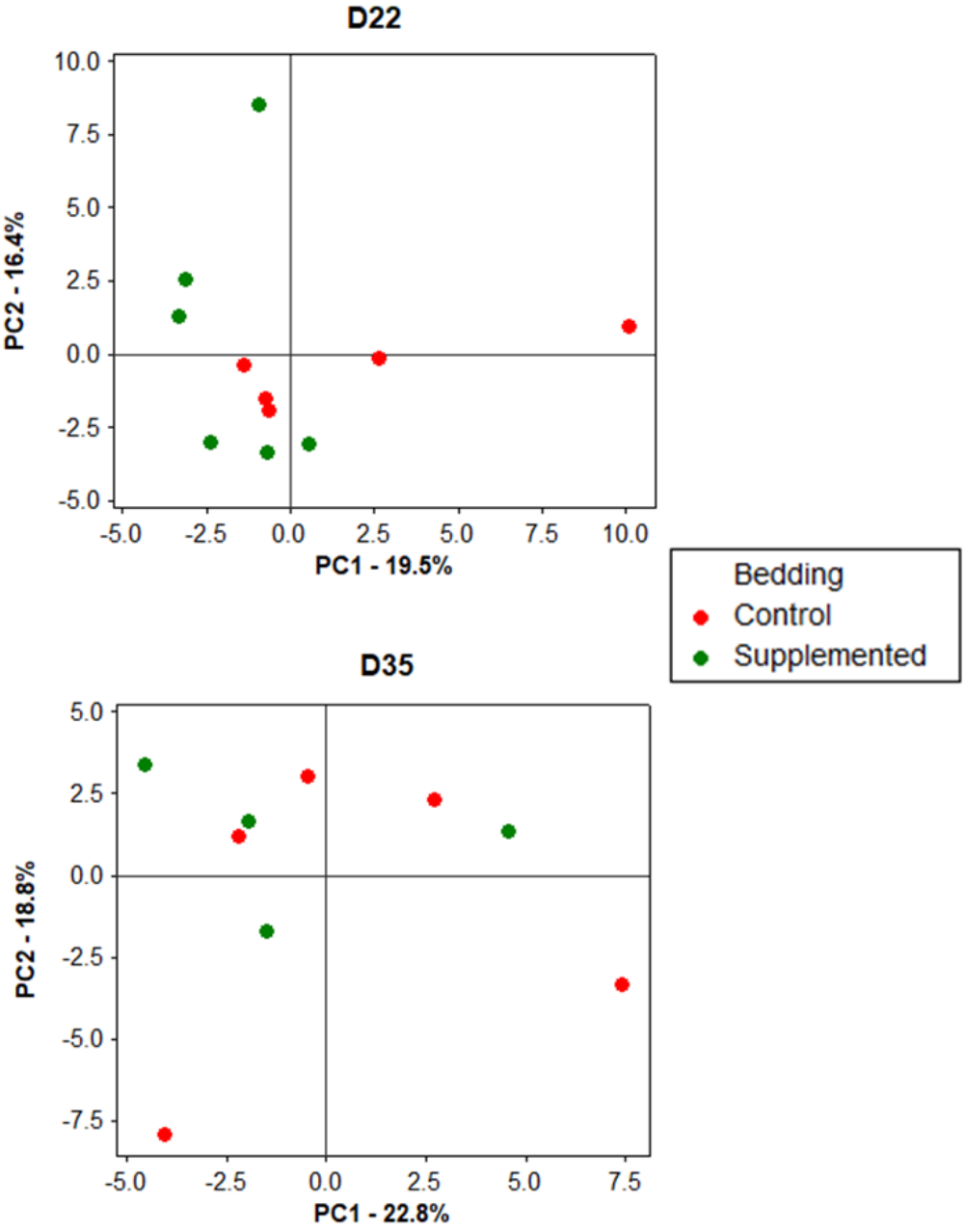

Figure 6.3 *PCA score plot illustrating the effects of bedding (clean shavings/excreta supplementation; n=6/treatment) on beta diversity of caecal bacterial populations taken from broilers at days 22 and 35 and profiled through next-generation sequencing.*

Similar variation within treatment groups was observed at all ages, suggesting that bedding type did not affect the individual variation in the caecal microbiome. These data prompted the hypothesis that bedding supplementation accelerated the development of alpha diversity, by exposure of a relatively naïve bird to an adult bacterial population from which its own microbiome could develop more rapidly. A PCA

model comparing day 15 supplemented samples and day 22 controls illustrated a lack of discrete clustering of data points (**Figure 6.4**) suggesting bedding supplementation accelerated the development of a mature microbiome.

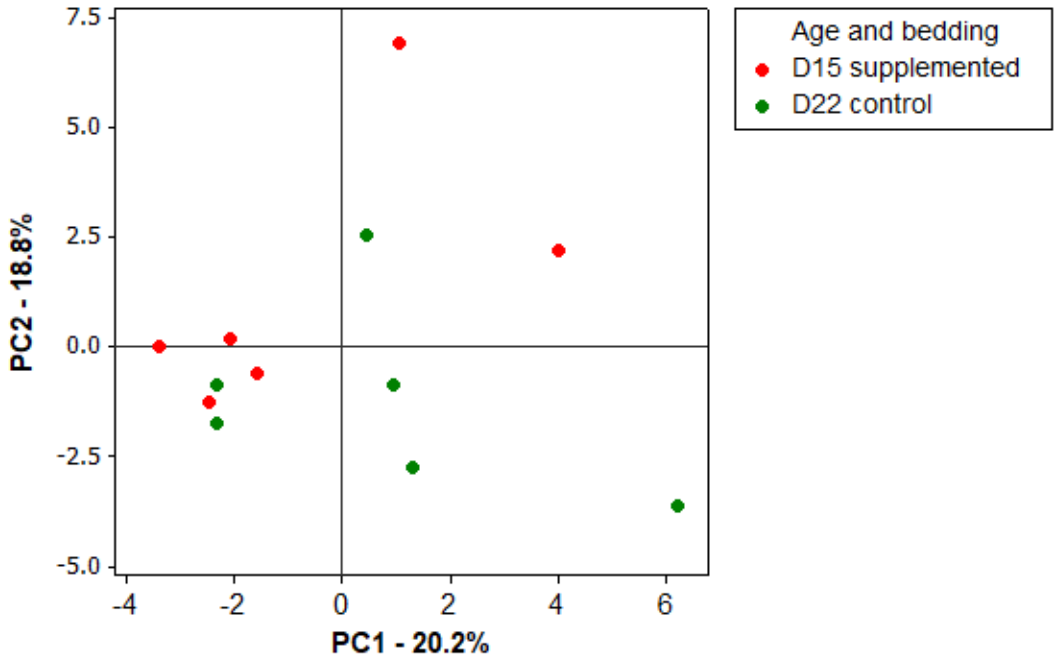

Figure 6.4 *PCA score plot illustrating differences in the profile of the caecal microbiome of birds on clean shavings at day 15, and shavings supplemented with excreta from adult birds at day 22 (n=6/treatment). Bacterial populations were profiled through next-generation sequencing.*

6.3.4. NGS: population analysis

The effects of bedding on the relative abundances of bacterial taxa were explored though LEfSe – all discriminative features and their associated *P* values is listed in **Appendix 4**.

6.3.4.1. Day 15

Firmicutes was the dominant phylum irrespective of treatment (**Figure 6.5**), but relative abundance was significantly reduced in birds on supplemented bedding (84.1% compared with 97.9% in controls; *P*=0.004). *Lachnospiraceae*, a family from order *Clostridiales*, was also elevated in controls at an average of 54.7% compared

with 24.4% in supplemented samples (*P*=0.010). Conversely, unknown bacteria from the order *Clostridiales* were elevated in supplemented samples at 24.5% compared with 7.2% in controls (*P*=0.016). Cressman *et al.* (2010) found that *Lachnospiraceae* dominated the caecal digesta of birds on fresh litter at day 7, whereas those on reused litter were dominated by an unknown class of *Clostridiales.* Though birds were not sampled until day 15 in the present study, the same observations were made. These results suggest that the caecal microbiome may not change dramatically between day 7 and 15, and that supplementation of bedding seems to favour the growth of unclassified groups of *Clostridiales* in the caeca.

The difference in *Firmicutes* was primarily accounted for by phylum *Tenericutes*, where an unknown clade of *Mollicutes* (denoted as RF39), were significantly elevated in supplemented samples at an average of 12.4% compared with 0.2% in controls (*P*=0.004). A number of taxa represented at levels below 1% were also affected by bedding type: *Coriobacteriaceae* (*P*=0.006), *Enterococcaceae* (*P*=0.037), *Staphylococcaceae* (*P*=0.025) and *Leuconostocaceae* (*P*=0.016) were elevated in control birds, whereas unclassified *Firmicutes* (*P*=0.037), unclassified *Bacillales* (*P*=0.034) and bacteria from the family *Bacillaceae* (*P*=0.020) were elevated in supplemented birds.

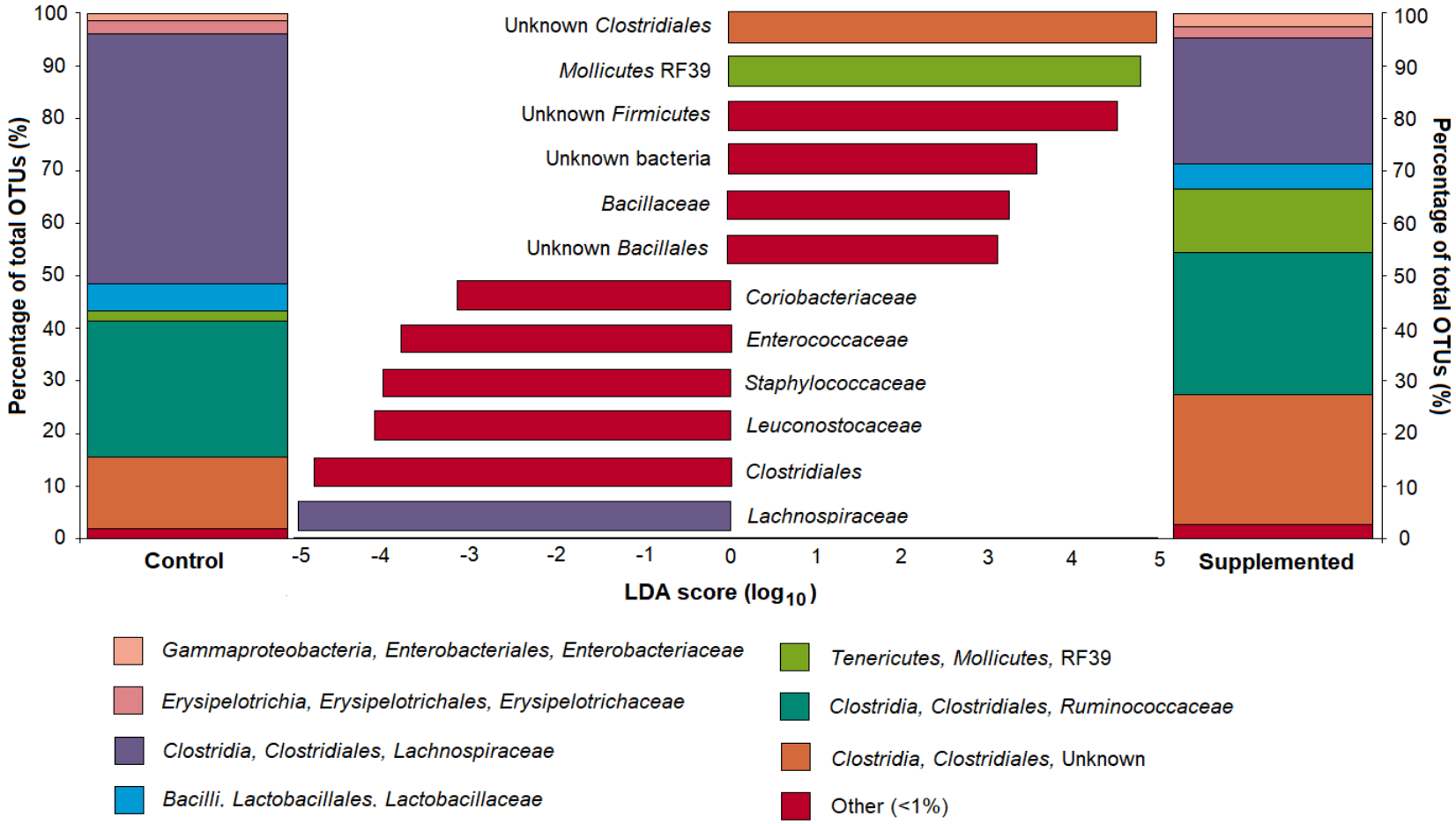

Figure 6.5 *NGS data from day 15.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar (clean/supplemented bedding; n=6/treatment) illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

6.3.4.2. Day 22

Statistically significant differences were not observed at day 22 (**Figure 6.6**). *Ruminococcaceae*, *Lachnospiraceae* and unknown *Clostridiales* were the dominant taxa in all birds.

6.3.4.3. Day 35

A few differences were noted at day 35 (**Figure 6.7**). *Odoribacteraceae* were significantly elevated in supplemented birds, at an average of 7.8% compared with 3.3% in controls (*P*=0.016). These bacteria represented the entirety of phylum *Bacteroidetes*. Also elevated in supplemented birds were *Eubacteriaceae* (*P*=0.016)*, Dehalobacteriaceae* (*P*=0.010)*, Christensenellaceae* (*P*=0.004) and *Cyanobacteria* YS2 (*P*=0.010). These taxa were consistently found at levels below 1% and therefore biological significance may be questionable. Conversely, *Peptostreptococcaceae* were five times more abundant in controls than supplemented birds at an average of 2.6% and 0.5% respectively (*P*=0.010).

6.3.4.4. Day 15 supplemented vs day 22 controls

Since PCA models suggested that bedding supplementation appeared to have accelerated the development of the caecal microbiome, LEfSe analyses of day 15 supplemented *vs* day 22 control samples were carried out, but no differences were identified. Bedding supplementation significantly altered the caecal microbiome of broilers at day 15 but these effects were absent by day 22, suggesting that the microbiome of control birds shifted to match that of those on supplemented bedding between sampling points, supporting observations made by Cressman *et al.* (2010). Since the excreta added to supplemented pens was taken from mature adult birds, it is thought that the exposure to this complex ‘soup’ of bacteria exerted something of a probiotic effect on young birds.

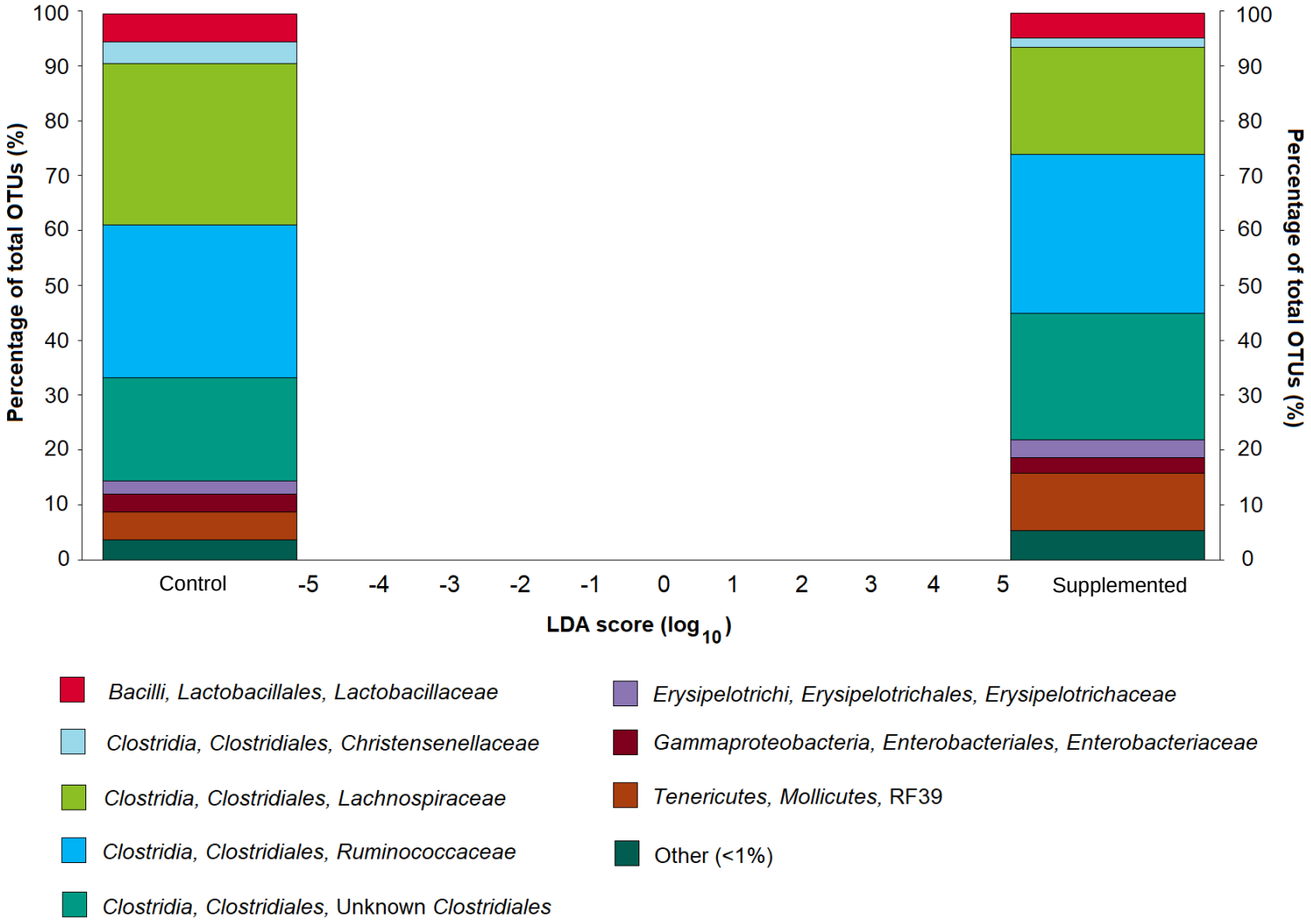

Figure 6.6 *NGS data from day 22.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'. The lack of inner bars illustrates that no bacterial taxa were affected by bedding.*

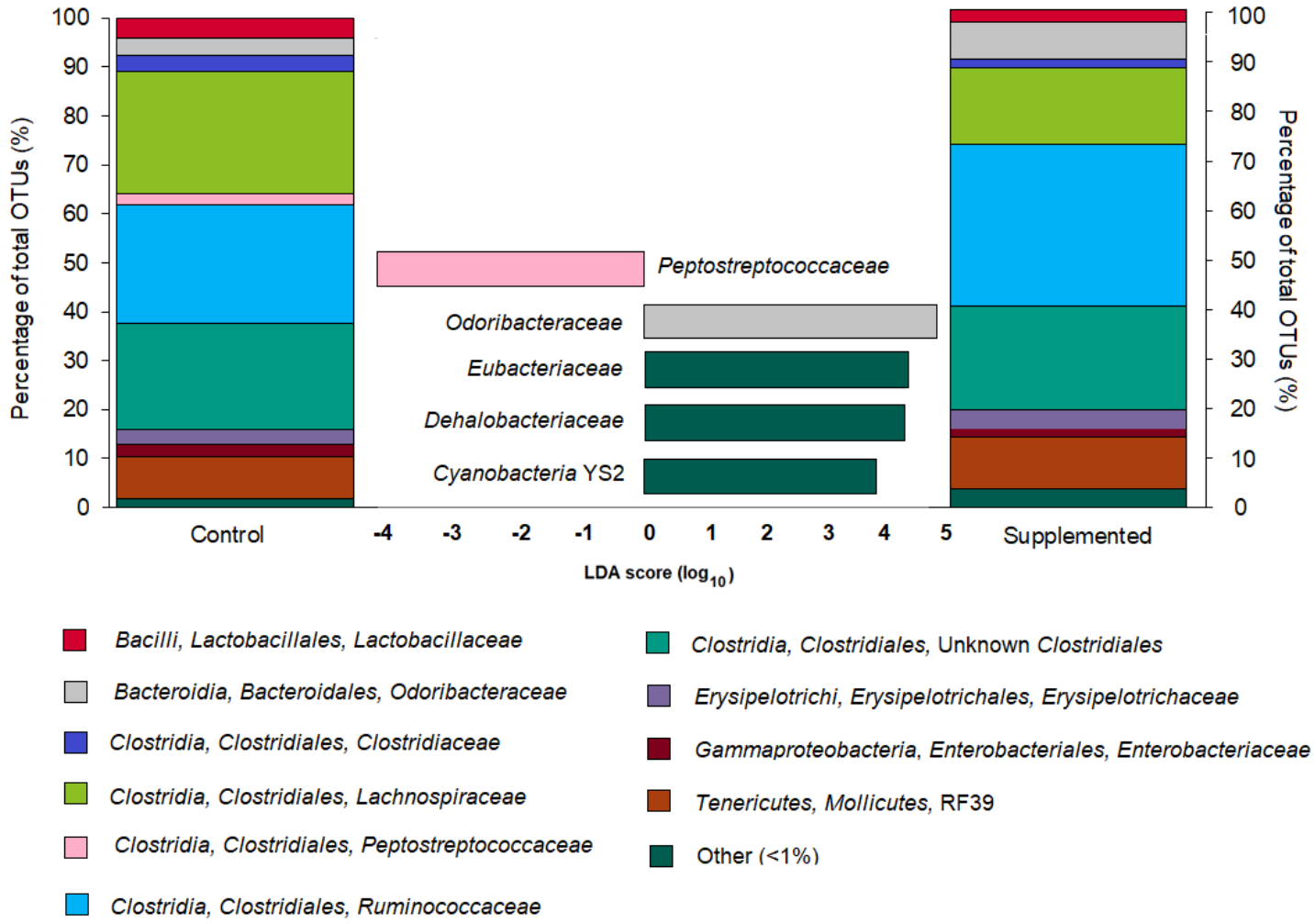

Figure 6.7 *NGS data from day 35.* ***Outer bars:*** *stacked bar chart illustrating the average percentage abundance of the most common bacterial families. Any taxa found at levels <1% are grouped as 'other'.* ***Inner bars:*** *log_{10} LDA scores illustrating differentially abundant bacterial taxa, identified through LEfSe analysis where $\alpha < 0.05$ and LDA > 2.0. The direction of the bar (clean/supplemented bedding; n=6/treatment) illustrates towards which treatment group taxa are biased, where larger bars contribute more to overall variation between treatment groups.*

6.3.5. ^{1}H NMR: liver models

Metabolic profiles were used to construct OPLS-DA models to explore the effects of treatment on the overall structure of the liver metabolome (**Table 6.2**). The large differences between R^2Y and Q^2 values in models from days 15 and 35 suggest that data were overfitted and that the models were not robust. The lack of statistical significance at 100 permutations (P>0.05) supports these observations. Models constructed from day 22 suggested differences between treatments due to high R^2Y and Q^2 OPLS-DA values with a P-value of 0.013 at 1000 permutations.

Table 6.2 *Summary of OPLS-DA models of liver metabolic profiles. P-values are a result of 100 permutations except for day 22 where 1000 permutations were analysed.*

	R^2Y	**Q^2**	***P*-value**
Day 15	0.4255	0.0790	0.190
Day 22	0.7732	0.6058	0.013
Day 35	0.7748	0.2188	0.250

Correlation plots of all datasets were constructed to identify any molecules that were differentially represented between treatment groups, where red peaks indicated differences in average intensity and blue peaks indicated no difference. Spectra from each treatment group were similar at days 15 and 35, as was expected based upon the outputs of OPLS-DA models. Numerous peaks appeared to be differentially represented in spectra taken from day 22 samples (**Figures 6.8-6.12**).

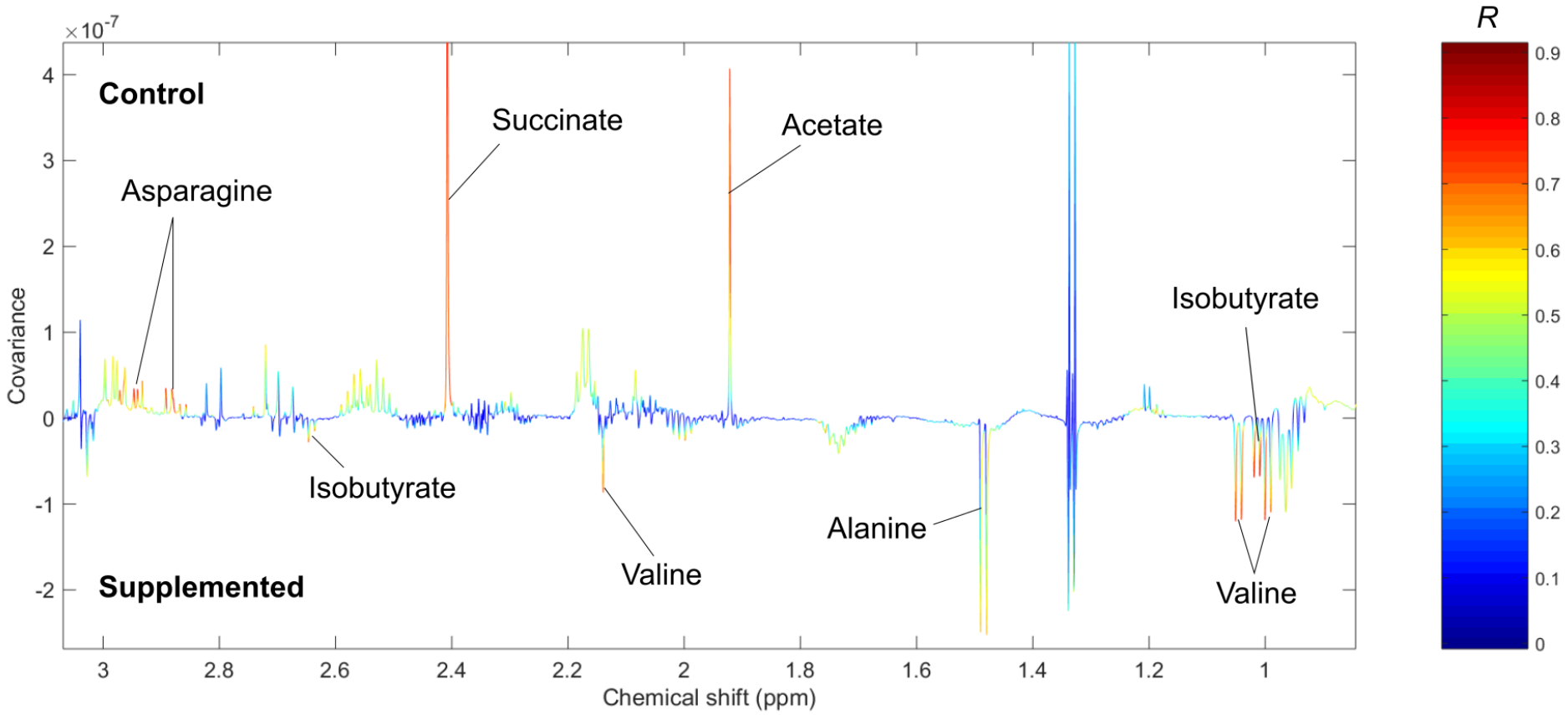

Figure 6.8 *OPLS-DA correlation plot of ^{1}H NMR spectra of liver metabolic extracts from day 22, indicating peaks at differing intensities depending upon bedding type (clean/supplemented bedding; n=6/treatment). Warmer colours such as reds indicate a difference in peak intensity, and therefore suggest a difference in metabolite concentration between treatment groups. Suggested identities of peaks are based upon existing literature and data from the Human Metabolome Database (Wishart* et al., *2018).*

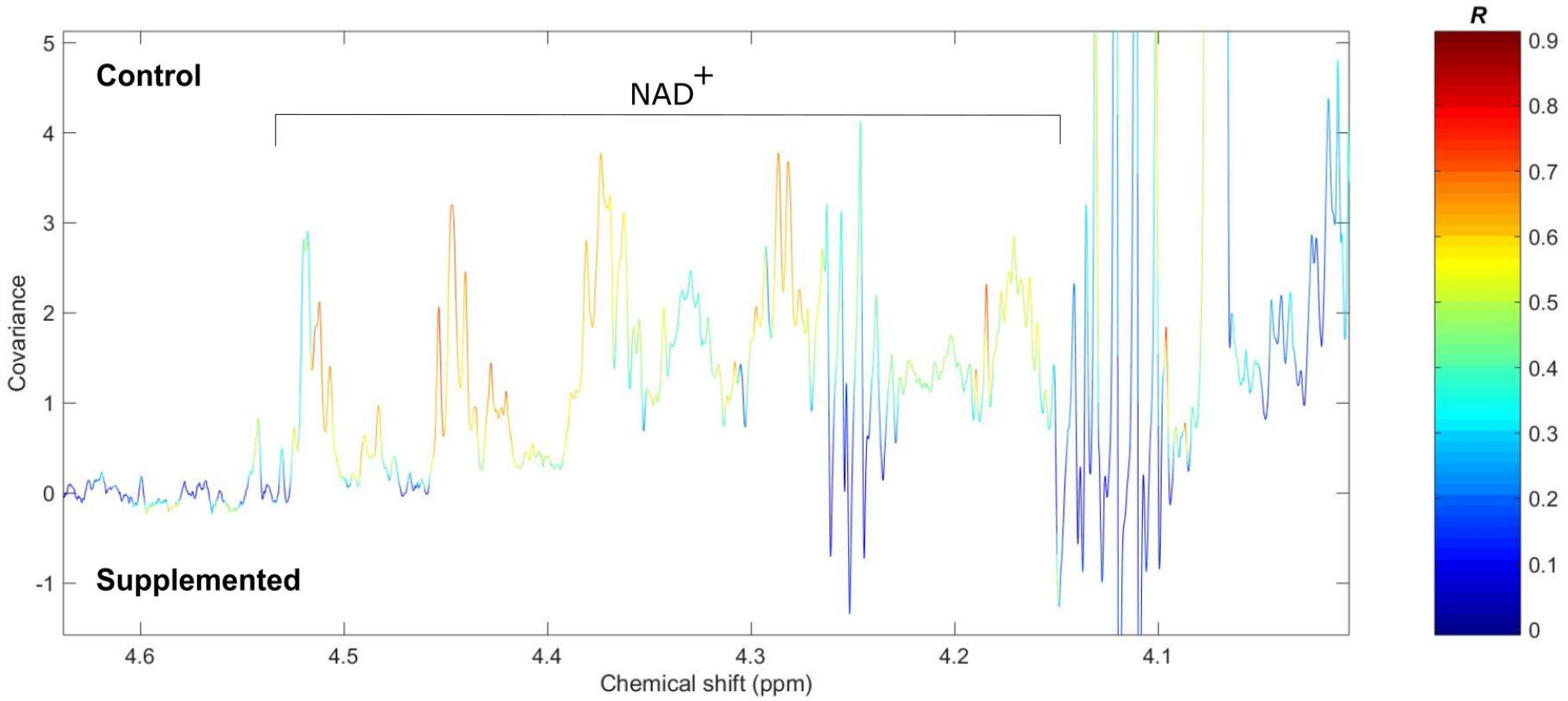

Figure 6.9 *OPLS-DA correlation plot of ^{1}H NMR spectra of liver metabolic extracts from day 22, indicating peaks at differing intensities depending upon bedding type (clean/supplemented bedding; n=6/treatment). Warmer colours such as reds indicate a difference in peak intensity, and therefore suggest a difference in metabolite concentration between treatment groups. Suggested identities of peaks are based upon existing literature and data from the Human Metabolome Database (Wishart* et al., *2018).*

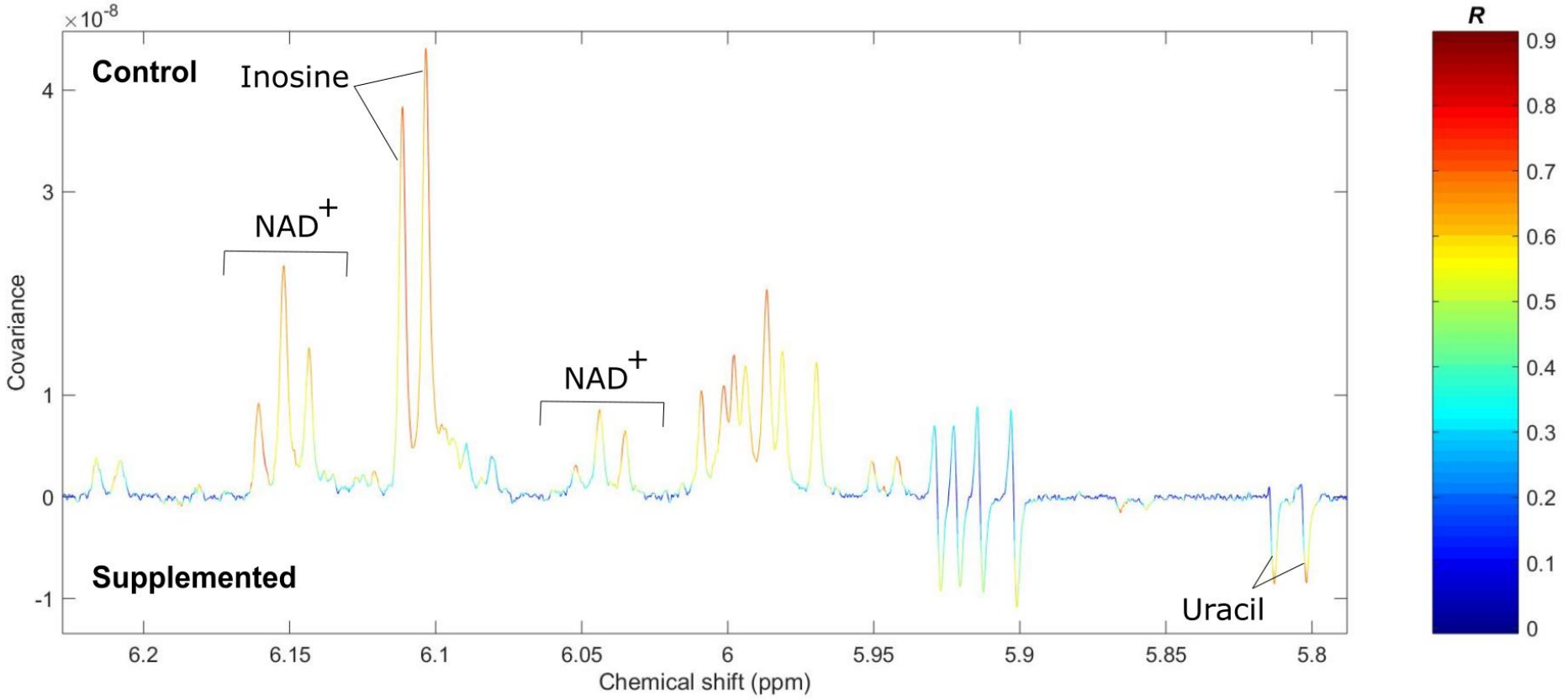

Figure 6.10 *OPLS-DA correlation plot of ^{1}H NMR spectra of liver metabolic extracts from day 22, indicating peaks at differing intensities depending upon bedding type (clean/supplemented bedding; n=6/treatment). Warmer colours such as reds indicate a difference in peak intensity, and therefore suggest a difference in metabolite concentration between treatment groups. Suggested identities of peaks are based upon existing literature and data from the Human Metabolome Database (Wishart* et al., *2018).*

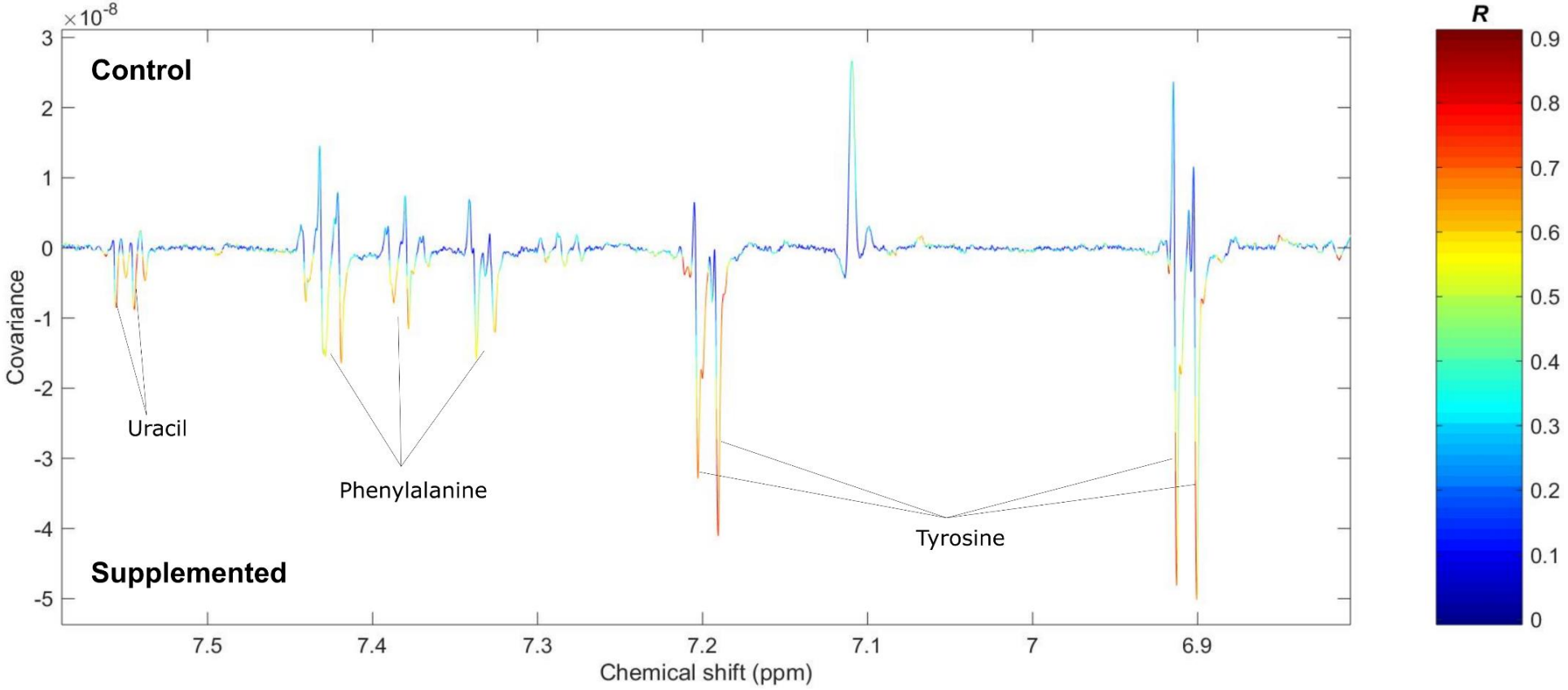

Figure 6.11 *OPLS-DA correlation plot of ^{1}H NMR spectra of liver metabolic extracts from day 22, indicating peaks at differing intensities depending upon bedding type (clean/supplemented bedding; n=6/treatment). Warmer colours such as reds indicate a difference in peak intensity, and therefore suggest a difference in metabolite concentration between treatment groups. Suggested identities of peaks are based upon existing literature and data from the Human Metabolome Database (Wishart* et al., *2018).*

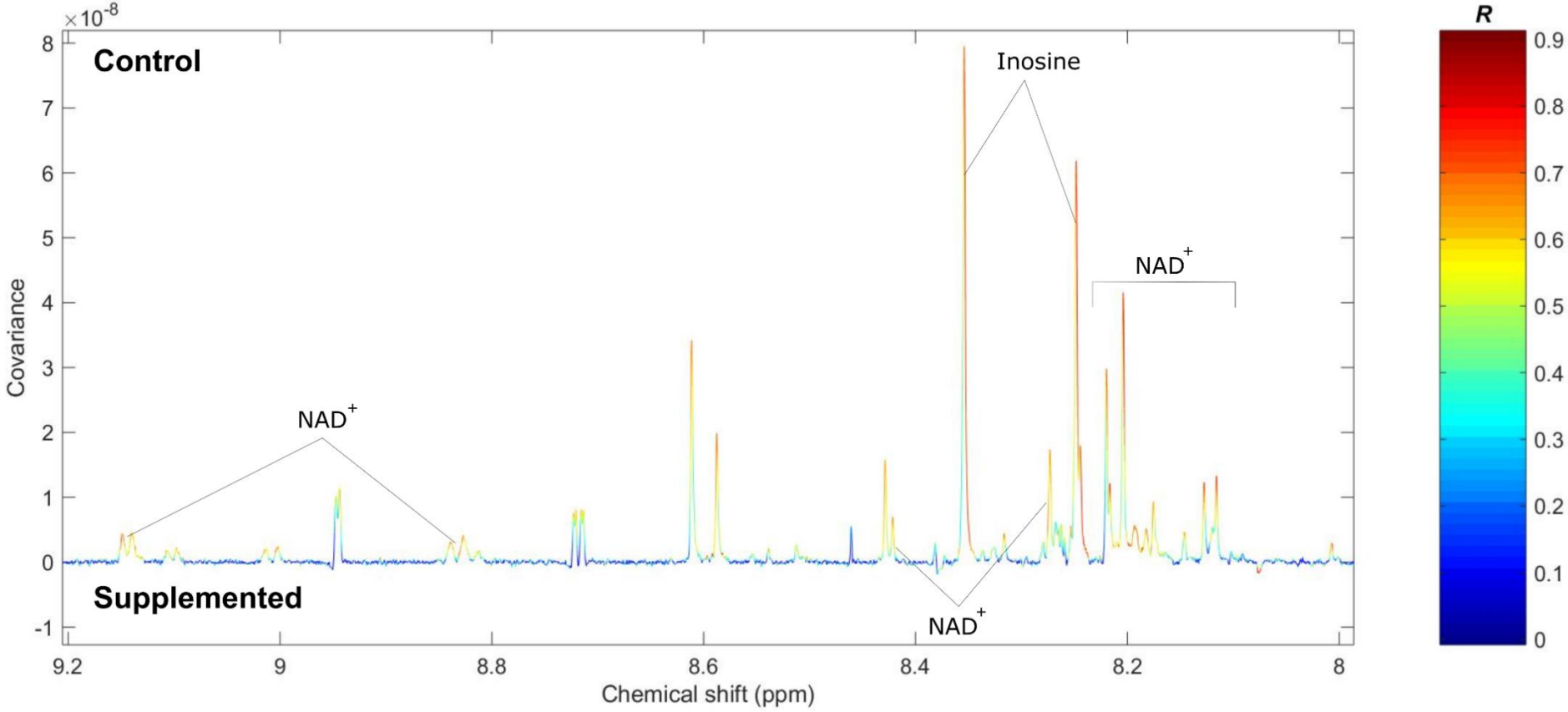

Figure 6.12 *OPLS-DA correlation plot of ^{1}H NMR spectra of liver metabolic extracts from day 22, indicating peaks at differing intensities depending upon bedding type (clean/supplemented bedding; n=6/treatment). Warmer colours such as reds indicate a difference in peak intensity, and therefore suggest a difference in metabolite concentration between treatment groups. Suggested identities of peaks are based upon existing literature and data from the Human Metabolome Database (Wishart* et al., *2018).*

The identities of red/orange peaks were suggested based upon existing literature and metabolic databases, though the identities of a small number of peaks could not be suggested due to a lack of existing data. These include singlet peaks in the region of δ = 8.60 ppm, and multiplets in the region of δ = 5.95 ppm. Target peaks were integrated and concentrations per gram of starting material in individual samples were calculated by a comparison against a known concentration of TSP. Differences in average concentrations between treatment groups were explored through ANOVA (**Table 6.3**).

Concentrations of amino acids (valine, alanine, tyrosine and phenylalanine), an RNA nucleobase (uracil), and energy-associated metabolites (isobutyrate and acetate) were elevated in liver extracts taken from birds on supplemented bedding. Conversely, concentrations of a single amino acid (asparagine), three metabolites involved in energy production (fumarate, succinate and NAD^+), and a single nucleoside (inosine) were elevated in controls.

Correlations between hepatic metabolites and bacterial taxa could not be explored at days 15 and 22 since consistent differences in each parameter were not identified. Differences were only observed in either the hepatic metabolome or the caecal microbiome, but not both simultaneously.

Table 6.3 *Assignments and concentrations of differentially abundant hepatic metabolites affected by bedding (clean shavings/excreta supplementation; n=6/treatment) per gram of starting material at day 22, calculated by peak integration of ^{1}H NMR spectra ± standard deviation. Identifying peak positions, splitting patterns and coupling constants (*J*) are listed. Peaks listed in bold were integrated concentration calculation. (Singlet, s; doublet, d; triplet, t; multiplet, m*). P*-values have been obtained from ANOVA where significance was denoted as* P*<0.05 and means were separated by Tukey's test.*

	Peak assignment			**Concentration (mmol/g)**			
Metabolite	**Position (ppm)**	**Splitting pattern**	***J* (Hz)**	**Control**		**Supplemented**	***P*-value**
Acetate	**1.92**	**s**	**-**	0.754 ± 0.113	<	0.910 ± 0.147	0.065
Alanine	**1.48**	**d**	**7.7**	0.923 ± 0.111	<	1.462 ± 0.128	<0.001
Asparagine	**2.89**	**d**	**7.7**	0.093 ± 0.018	>	0.054 ± 0.008	<0.001
	2.94	d	7.7				
Fumarate	**6.52**	**s**	**-**	0.004 ± 0.003	>	0.0006 ± 0.0004	0.012
Inosine	6.11	d	5.6	0.136 ± 0.028	>	0.074 ± 0.008	<0.001
	8.25	**s**	**-**				
	8.35	s	-				
Isobutyrate	**1.01**	**d**	**7.7**	0.168 ± 0.026	<	0.285 ± 0.037	<0.001

NAD^+	4.28	m	-	0.009 ± 0.002	>	0.004 ± 0.001	0.001
	4.44	m	-				
	4.48	t	5.6				
	4.51	m	-				
	6.15	m	-				
	8.12	d	8.4				
	8.15	**s**	**-**				
	8.22	m	-				
	8.42	s	-				
	8.83	d	8.4				
	9.14	d	6.3				
Phenylalanine	7.33	d	7.0	0.079 ± 0.008	<	0.121 ± 0.014	<0.001
	7.38	d	7.0				
	7.43	**m**	**-**				
Succinate	**2.41**	**s**	**-**	0.233 ± 0.034	>	0.092 ± 0.092	<0.001
Tyrosine	**6.91**	**d**	**9.8**	0.184 ± 0.023	<	0.308 ± 0.043	<0.001
	7.20	d	9.1				
Uracil	**5.81**	**d**	**7.7**	0.033 ± 0.011	<	0.062 ± 0.006	<0.001
	7.55	d	7.0				
Valine	**0.99**	**d**	**7.2**	0.387 ± 0.052	<	0.621 ± 0.079	<0.001
	1.01	d	7.0				
	2.14	m	-				

6.3.5.1. Amino acids

The concentrations of several amino acids appeared to be elevated in the livers of birds on supplemented bedding at day 22. A number of amino acids are considered to be 'essential' in the diet, since they cannot be synthesised by the host. These include phenylalanine, tyrosine and valine, all of which were found at elevated concentrations in supplemented birds. Since feed, and therefore crude protein, intake was not altered by bedding type, differences in the concentrations of these amino acids may be a result of differences in crude protein digestion and amino acid absorption, or as a result of microbial synthesis.

Phenylalanine is an aromatic amino acid that is obtained from the diet and *via* bacterial synthesis by species including *Escherichia coli* (Simmonds *et al.,* 1953). It is enzymatically converted to tyrosine, concentrations of which were also elevated in supplemented birds. Relationships with bacteria were not found, since differences in the caecal microbiome were absent at day 22. This does not, however, mean that bacteria were not responsible for differences in concentrations of metabolites: relationships between bacterial abundance and metabolite production may not be linear and alternative factors including enzyme or substrate availability may affect production (Gottlieb *et al.,* 2014). Alternatively, bacteria higher up the GIT may be more closely linked with amino acid metabolism – further investigations into these relationships are warranted.

Valine, also found at elevated concentrations in supplemented birds, is a branched chain amino acid (BCAA) and is the preferred amino acid substrate of colonic bacteria (Dai *et al.,* 2010). The same authors report that BCAA proportions relative to other amino acids are higher in the colon, suggesting an increase in synthesis, a decrease in degradation, or an increase in utilisation of other BCAA to alter overall proportions. The inhibition of the growth of *E. coli* by valine is a widely accepted phenomenon and is also secreted by gram-negative bacteria in biofilms when carbon sources are not

limited (Valle *et al.,* 2008). It is suggested that it is not necessarily the *numbers* of bacteria, but merely their *presence* and, more importantly, their *ability* to synthesise valine that that affects concentrations. It therefore seems reasonable to suggest that differences in valine concentrations in the present study may not be as a result of bacterial abundance in the caeca. Instead it is hypothesised that differences in valine concentrations are more likely to be caused by carbon availability, differences in which may stem from the microbiome further up the gastrointestinal tract where the majority of digestion occurs.

Though not considered an essential amino acid since it can be synthesised *in vivo*, concentrations of alanine were elevated in birds on supplemented bedding. Research suggests that alanine may have positive effects on liveweight gain and FCR coupled by the upregulation of carnosine synthesis (Qi *et al.,* 2018), though these effects were not observed in the present study. Negative relationships with *Bacteroides* and *Enterococcus* have previously been suggested (Neis *et al.,* 2015). Dodd *et al.* (2017) also suggest that alanine is metabolised by species of *Clostridia,* though a lack of difference in the caecal microbiome prevented the analysis of microbe-metabolite relationships.

The only amino acid to be found at suppressed levels in birds on supplemented bedding was asparagine. This is a non-essential amino acid and can be synthesised *in vivo* from aspartate in an ATP-dependent reaction (Milman *et al.*, 1979). Similarly, there is evidence of microbial synthesis (Min *et al.,* 2002; Baruch *et al.,* 2014) and *de novo* synthesis in the livers of chicken embryos (Arfin, 1967), though the contribution to asparagine concentrations through these pathways in the present study are unclear.

Feed, and therefore protein, intake was not significantly altered by bedding type. Since three out of five amino acids that were affected by bedding were considered to be essential and therefore obtained in the diet, it is reasonable to suggest that either feed

was metabolised differently depending on bedding type, or that differences were as a result of microbial synthesis, perhaps in alternative gut locations such as the small intestine. Irrespective of this, bird growth was not altered which suggests that differences in amino acid concentrations were not crucial in determining productivity. Exploring the microbiomes of differing physiological compartments may elucidate potential relationships with metabolites.

6.3.5.2. Energy metabolism

NAD^+ (nicotinamide adenine dinucleotide), observed at suppressed levels in birds on supplemented bedding at day 22, is a coenzyme comprising an adenine base and nicotinamide linked by two phosphate groups. It can exist as either its oxidised form (NAD^+) or its reduced form (NADH). Their facile redox reactions allow the transfer of energy between molecules in cellular metabolism (Belenky and Bogan, 2007). This perhaps suggests that this pathway of energy production was elevated in controls.

There is evidence that glucose deprivation, caloric restriction and circadian fluctuations can all result in an elevation in NAD^+ concentrations (Fulco *et al.,* 2008; Chen *et al.,* 2008; Ramsey *et al.,* 2009). Increasing NAD^+ concentrations may also prevent age-related ailments in mammals due to an increase in metabolic efficiency (Canto *et al.,* 2015). Pirinen *et al.* (2014) suggest that an increase in mitochondrial function under stress can result in elevated NAD^+ levels, to protect against the aforementioned biological stressors such as glucose deprivation.

The majority of NAD^+ is synthesised *de novo* from a number of molecules including tryptophan and nicotinic acid (Hara *et al.,* 2007), perhaps providing an explanation for differences in concentration between treatment groups in the present study. Differences in hepatic concentrations of tryptophan and nicotinic acid were not observed, suggesting an alternative synthetic route or perhaps dietary NAD^+. Despite this, all birds were fed identical diets and there was no evidence of a difference in feed

intake, suggesting that modulations in NAD^+ concentrations were not as a result of differences in feed consumption. Since caecal bacterial populations were not significantly altered at day 22, relationships with the microbiome could not be explored. Birds frequently consume their bedding and, though not assessed in the present study, intake may have differed between birds on each bedding type. This potentially may have provided birds with an additional source of nutrients including NAD^+. Alternatively, differences in hepatic NAD^+ concentrations may have been a result of bacterial populations in the proximal gut, such as the ileum.

Succinate, also found at depleted concentrations in birds on supplemented bedding, is a dicarboxylic acid and an intermediate in tricarboxylic acid (TCA) cycle of energy production. It therefore plays an important role in oxidative phosphorylation and cellular metabolism. The oxidation of succinate by succinate dehydrogenase leads to the production of fumarate, another dicarboxylic acid that was found at higher concentrations in controls. Other intermediates in the TCA cycle include malate, oxaloacetate and citrate, though these metabolites were not found at different concentrations between treatment groups. Despite this, the observed differences in succinate and fumarate concentrations perhaps indicate differences in the TCA cycle, and thus energy production.

Isobutyrate is one of three branched chain volatile fatty acids (BCVFA) alongside isovalerate and 2-methylbutyrate, that are produced through feed protein degradation by anaerobic bacteria (Allison, 1978; Eugene *et al.,* 2004). It was observed at significantly higher concentrations in birds on supplemented bedding compared with controls, suggesting an increase in microbial degradation of dietary protein. Since all birds were fed the same diet and average feed intake was not altered between treatment groups, it is unlikely that this difference is a result of differences in nutrient consumption. It is more likely that differences arose due to differing capacities of birds to utilise these nutrients, perhaps enzymatically or through microbial digestion.

Alternatively, there may have been differences in bedding consumption that provided birds with an exogenous supply of nutrients.

Relationships between the microbiome and isobutyrate production have previously been identified. *Ruminococcus albus, Ruminococcus flavefaciens* and *Fibrobacter succinogenes* have been positively related to isobutyrate concentrations and are therefore potential candidates for its production (Liu *et al.,* 2014; Wang *et al.,* 2015; Zhang *et al.,* 2015). Bacteria could not be identified to species level in the present study, but relationships between isobutyrate concentrations and bacterial families (*Ruminococcaceae* and *Fibrobacteriaceae*) were not found. This suggests one of three things: that alternative bacteria are responsible for isobutyrate production, that isobutyrate concentrations are independent of bacterial abundance, or that caecal bacteria are not linked to hepatic isobutyrate concentrations.

Acetate, a SCFA, was also elevated in birds on supplemented bedding and was positively correlated with isobutyrate concentrations - it is therefore reasonable to suggest that microbial fermentation was elevated these birds. A lack of relationships between caecal bacterial populations and both isobutyrate and acetate suggest that bacteria in an alternative location, perhaps the proximal GIT, may be responsible for differences in metabolite concentrations.

6.3.5.3. Nucleosides

Concentrations of uracil, a nucleoside that replaces thymine in nucleic acids, were significantly elevated in supplemented birds. Uracil is enzymatically catabolised to β-alanine (Fritzson, 1961). It seems sensible to suggest that if the observed elevated concentration of uracil in supplemented birds was due to a reduction in catabolic rate, it would be coupled with a decrease in β-alanine concentrations. The present results

indicate an elevation in both uracil and β-alanine concentrations, making differences in the rate of catabolism an unlikely explanation for differences in uracil concentrations.

Kurthkoti and Varshney (2010) report that the deamination of cytosine to uracil allows bacteria, specifically *Mycobacterium*, to adapt to microaerophilic conditions. Since the environment in the chicken gut is anaerobic, it is reasonable to suggest this as a plausible explanation for differences in uracil concentrations. This would perhaps suggest a decrease in cytosine concentrations: though not assigned in the spectra in the present study, signals in the regions identifying cytosine (two doublet peaks at δ=5.96 ppm and 7.49 ppm), were potentially affected by bedding type due to the orange-red regions on the OPLS-DA correlation plot. Future work should perhaps involve the fractionation of metabolic extracts to aid resolution and the assignment of peaks in such crowded areas of the spectrum.

Work by Lee *et al.* (2013) found that pathogen-derived uracil is a driving force for cell homeostasis and epithelial cell proliferation following infection by activating reactive oxygen species. This is important in gut immunity, particularly in terms of pathogen clearance, since uracil appears to only be secreted by pathogenic bacteria (Valanne and Rämet, 2013).

A second nucleoside, inosine, was observed at elevated concentrations in controls. Research indicates that inosine can be formed through the deamination of adenosine in the rumen of calves (McAllan and Smith, 1972), suggesting that the same may take place in the broiler gut. Deamination is a spontaneous process that may lead to differences in the composition of nucleoside pools. This may suggest differences in transcription between treatment groups, perhaps in response to differences in immune responses as a result of modulations of the gut microbiome. Though differences in caecal bacteria were absent, the potential for bedding to have differentially altered other gut compartments such as the small intestine cannot be ruled out.

6.3.5.4. SCFA

Though differences were not suggested by OPLS-DA models, peaks assigned to SCFA were integrated and concentrations were analysed through ANOVA. Supplementing bedding with excreta from mature birds significantly increased concentrations of hepatic butyrate and lactate at day 22 (**Table 6.4**).

Table 6.4 *ANOVA exploring the effects of bedding (fresh shavings/excreta supplementation; n=6/treatment) on concentrations of hepatic SCFA, calculated by integrating ^{1}H NMR peaks. Statistical significance is denoted as* P*<0.05 and means were separated by Tukey's test.*

	Bedding			
	Control	**Supplemented**	**SEM**	***P*-value**
Day 15				
Acetate	0.15	0.15	0.005	0.655
Butyrate	2.04	2.50	0.273	0.429
Propionate	0.75	0.63	0.080	0.466
Lactate	1.20	1.14	0.102	0.752
Day 22				
Acetate	0.27	0.24	0.014	0.297
Butyrate	1.13	1.73	0.054	<0.001
Propionate	0.99	0.99	0.030	0.902
Lactate	0.94	1.52	0.039	<0.001
Day 35				
Acetate	0.22	0.22	0.018	0.839
Butyrate	1.85	1.99	0.152	0.639
Propionate	0.98	1.03	0.071	0.765
Lactate	1.43	1.51	0.134	0.776

LEfSe analysis found no differences in bacterial taxa abundance at day 22, suggesting that differences in SCFA concentrations were not of caecal microbial origin. Instead, it is hypothesised that the aforementioned acceleration of the development of a mature caecal microbiome by supplemented bedding primed said birds to process the dietary change at day 15 more effectively than those on fresh bedding. Said birds did not need

to adapt their microbiome to such an extent as controls and may therefore have been able to utilise their feed more efficiently for longer, increasing concentrations of hepatic butyrate and lactate.

Metabolic pathways of energy production appeared to be differentially active within treatment groups at day 22, suggesting that differences in bedding type may have altered the phenotypic metabolome of birds. Since neither liveweight nor feed intake differed between treatment groups, it is thought that rates of total energy production were similar and that metabolic pathways were merely active to different extents, resulting in similar functional metabolomes.

Bacteria play key roles in feed utilisation and energy production. Since differences in the caecal microbiome were evident at the point of dietary change, though differences in metabolites were not found until a week later, it is hypothesised that birds were differentially prepared to utilise their feed from the onset of the dietary change. This may have forced birds to make use of differing metabolic pathways to meet their energy requirements for growth, since a difference in performance was also not observed. It is also hypothesised that differences in the microbiome of the proximal gastrointestinal tract may alter the hepatic metabolome to a greater extent than that of the distal gastrointestinal tract.

The present data give only a ‘snapshot’ of growth and metabolism. Had birds been monitored on a more regular, even daily, basis, we may have observed gradual shifts in metabolism. What was not analysed, however, was the lipid fraction of the liver since methanolic extraction of metabolites and subsequent dissolution in D_2O excludes hydrophobic molecules. It is suggested that future work could explore a biphasic extraction of metabolites, perhaps *via* chloroform:methanol, to explore differences in a wider range of metabolic pathways.

6.4. Conclusion

The present study aimed to investigate the effects of bedding supplementation on the performance, caecal microbiome and hepatic metabolome of growing broiler chicks. No difference in performance was identified, suggesting that exposure to excreta from mature birds is at no detriment to the growing broiler.

Supplementation accelerated the development of a stable adult caecal microbiome, but this did not alter bird performance; suggesting a lack of interaction between the rate of microbiome development and bird growth. Though differences in the hepatic metabolome were identified, there were no relationships with the caecal microbiome. It is anticipated that bedding supplementation will have also altered the microbiome of the proximal gastrointestinal tract. Since the majority of digestion and nutrient absorption occurs in the small intestine, it is anticipated that differences in bacterial composition may have also altered metabolic pathways and explain the observed differences in the liver.

It is suggested that there is a relationship between the phenotypic and functional microbiomes, whereby different bacterial taxa are capable of performing the same role within the gut, allowing phenotypic diversity without altering the functional capacity. These results may suggest a lack of an ‘optimum’ microbiome for bird growth, provided that the functional microbiome is sufficient to meet the needs of the host. It is anticipated that birds are able to cope with differences in their microbiome by utilising different chemical pathways to still meet their metabolic needs. Feed may be utilised differently by individuals but may still result in similar phenotypic responses. In conclusion, the present work suggests that differences in the phenotypic metabolome may not necessarily result in differences in the functional metabolome, and that the rate at which the caecal microbiome develops does not alter performance.

7. Identification of persistent trends - effect of age and management

The studies presented in this thesis were designed to explore the influence of management strategies on the caecal microbiome and hepatic metabolome of growing broiler chickens. Here, effects of bird age and treatment on caecal bacteria, hepatic metabolite concentrations and relationships between the two across all studies are discussed to highlight trends that may help to develop current knowledge. Data from the study exploring protein source were not included in age-related analyses since samples were taken at day 17 alone: data from birds fed fishmeal or synthetic amino acids could therefore not be explored longitudinally.

7.1. Caecal microbiome: bacterial diversity

7.1.1. Alpha diversity

7.1.1.1. Age

The effects of age on alpha diversity were explored through ANOVA where means were separated by Tukey's test. Alpha diversity increased with bird age irrespective of study or treatment (**Figure 7.1**; $P<0.001$): this observation is consistent with existing literature and is widely accepted (Awad *et al.,* 2016; Ballou *et al.,* 2016).

These data indicate a significant increase in diversity between days 4 and 9 (0.338 to 0.672; $P<0.001$), which may have been a result of birds ingesting bacteria from their environment, since the microbiome is relatively naïve in young birds and sensitive to change. Data from all subsequent sampling points were significantly higher than those recorded at day 4. There was not a significant difference between days 9 and 15 ($P=0.218$), but diversity significantly increased between days 15 and 22 (0.705 to 0.777; $P=0.001$), which may have been a result of diet, since a phase change from starter to grower consistently took place at day 15. Providing different levels of nutrients in the grower diets compared with starter diets may have favoured the growth of a wider range of microbes. Similarly, the feed itself may have contained different bacteria that, when ingested, colonised the GIT, but this was not tested. Given that

feed is milled and heated, it is possible that the range and diversity of organisms would be limited although it may be anticipated that, if the 'grower' feed had different source material, novel organisms may have been present.

There were no differences in alpha diversity between day 22 and subsequent sampling points (P>0.05), meaning that the data explored here can be roughly divided into three phases: day 4, days 9 to 15, and days 22 to 35, between which there were significant increases in diversity, but no differences within phases. The persistent increase in diversity with age, also means that birds from days 22 to 35 exhibited significantly higher measures of Simpson's Diversity Index than all birds from days 4 to 15 (P<0.001).

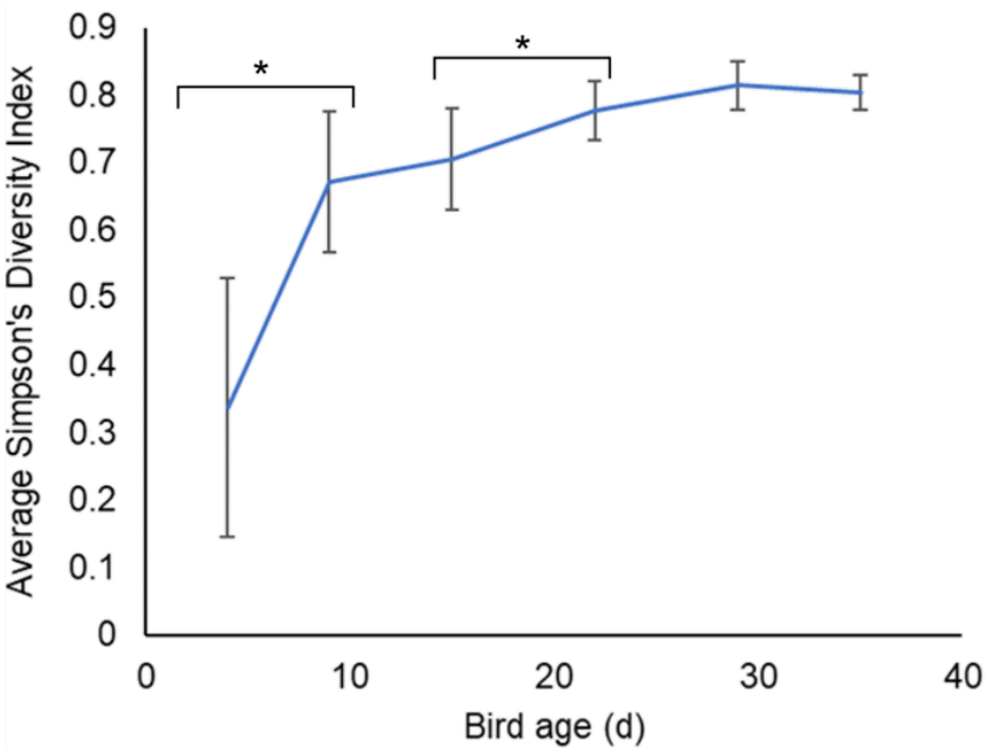

Figure 7.1 *Average Simpson's Diversity Index across all experiments. Error bars represent standard deviation with respect to each bird age. Statistically significant differences (P<0.05) from ANOVA where means were separated by Tukey's test is indicated by *.*

7.1.1.2. Treatment

Alpha diversity was also sensitive to the effects of management with initial heightening by feeding a maize-based diet over a wheat-based diet ($P<0.001$; **Figure 5.1**) although this difference narrowed as the birds aged until indistinguishable. This may be a result of differences in nutrient composition of maize fuelling the growth of a wider range of bacteria, or perhaps due to ingestion and colonisation of bacteria present in the feed itself. Diversity was also initially increased by supplementing bedding with excreta from mature birds (Chapter 6; $P=0.002$) - again, differences reduced over time until diversity could not be distinguished from the control birds on clean shavings. It seems likely that the initial increase was a result of exposure to a more complex environmental microbiome since excreta added to bedding originated from mature birds. Conversely, treatment with Linco-spectin (LS) initially decreased alpha diversity, suggesting that bacteria were sensitive to antibiotic treatment. Again, a plateau was reached with all mature birds exhibiting comparable measures of alpha diversity; the rate at which this plateau was reached was merely altered by management. Subsequent treatment with Aviguard® did not consistently improve diversity, but instead introduced variation between birds and obscured any resultant effect. This particular finding is surprising given Aviguard® is a quality controlled commercial product.

It appears that alpha diversity is sensitive to the effects of management strategy, irrespective of whether that strategy is environment- or diet-based, but that sensitivity decreases as birds age.

7.1.2. Beta diversity

Irrespective of treatment, beta diversity differed with bird age (**Figure 7.2**). This observation is widely accepted in existing literature (Awad *et al.,* 2016; Ballou *et al.,* 2016) and demonstrates how the profile of the caecal microbiome develops over time.

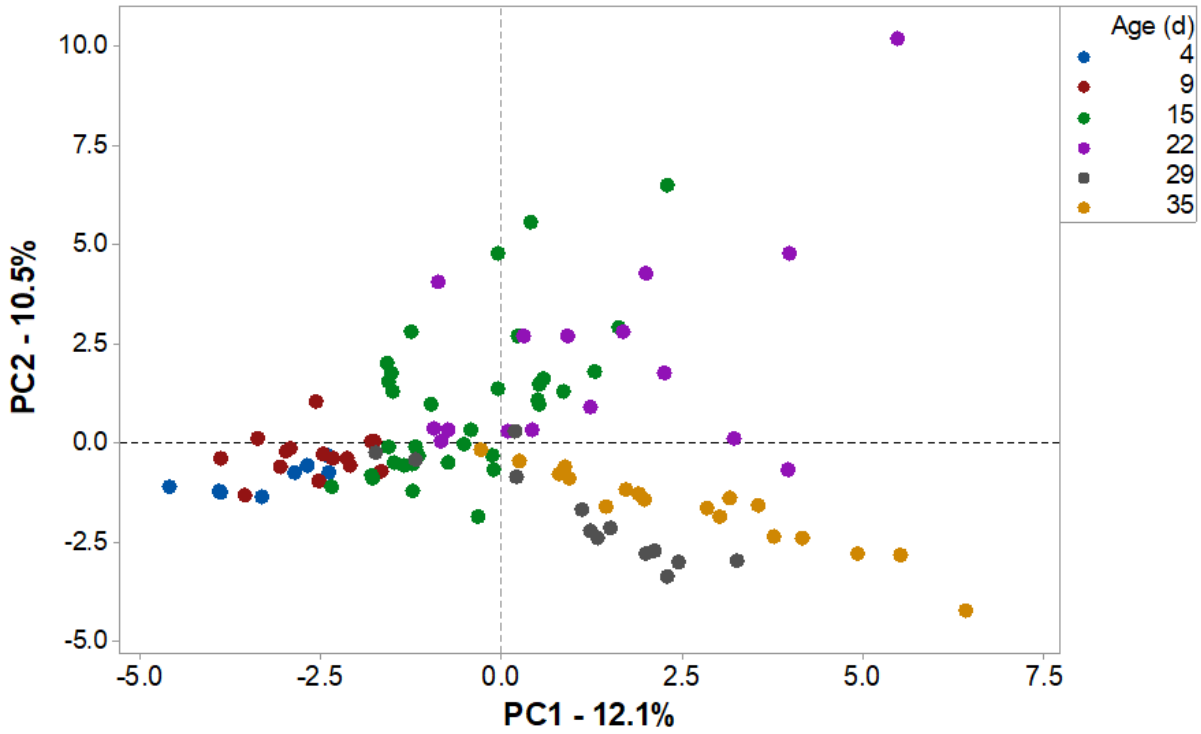

Figure 7.2 *Unsupervised PCA score plot illustrating changes in the profile of the caecal microbiome, analysed through next-generation sequencing. This analysis encompasses data from four studies exploring the effects of antibiotics/probiotics, dietary protein source, dietary cereal base, and bedding supplementation with excreta from mature birds on caecal bacterial populations at a range of bird ages (n=113 birds).*

In spite of the apparent clustering of datapoints between bird ages, there was still a large amount of variation between individual birds. This shows that, whilst the general profile of the microbiome of individual birds develops in a similar manner, it is unlikely that two birds are identical. Despite this, all sampled birds appeared to be healthy and productive, suggesting that individual variation does not necessarily alter the 'success' of the bird. It is therefore hypothesised that a so-called 'model' microbiome does not

exist, and that the most important factor is the ability of said microbiome to develop alongside the bird. Also, there is probably a high degree of metabolic redundancy within any one microbiome meaning that a particular essential biochemical reaction may be achieved by many different bacterial species. If this is the case, then it stands to reason that different microbial populations can still deliver the same biochemical outcome. This raises the issue as to whether population profiling is the most useful measure or whether true metagenomics, which will define all metabolic capabilities of the microbiota genetically, is more appropriate to answer this question.

Aside from bird age, alterations in the general profile of the caecal microbiome were also caused by diet, bedding and antibiotic/probiotic treatments. Differences were mainly observed within the first two weeks of life, after which the microbial profiles appeared to be fairly similar between treatment groups within a study. Only antibiotic and probiotic treatment caused shifts in microbial profiles later in life, with differences evident up to day 29. These results suggest that the microbiome is sensitive to change during the starter and grower phases, but that the bacterial profile reaches a similar profile over time. It appears that any perturbations in the said profile at a young age are generally ‘corrected’ as the bird matures. Antibiotic and probiotic treatments appear to have a longer lasting effect than diet and bedding, perhaps suggesting a more dramatic impact upon the birds, and should perhaps be explored in more depth in future work. It is also suggested that future work explores the effects of sudden perturbances on the microbiome, and whether the health and productivity of the bird are affected whilst the microbiome recovers.

7.2. Caecal microbiome: phyla

7.2.1. Age

Proteobacteria was initially the most abundant phylum identified in caecal digesta, though levels were dramatically depleted by day 9 and were replaced by *Firmicutes* (P<0.001), indicating an age-related shift in the profile of the microbiome (**Figure 7.3**). This observation is concurrent with existing literature (Awad *et al.,* 2016). Subordinate phyla included *Tenericutes*, levels of which increased between days 9 and 15 (P=0.011). *Bacteroidetes* did not appear to increase until after day 9 and were found at significantly higher proportions at day 29 than any other age (P<0.001), though this was likely an effect of study since only birds from Study 1 were sampled at this time.

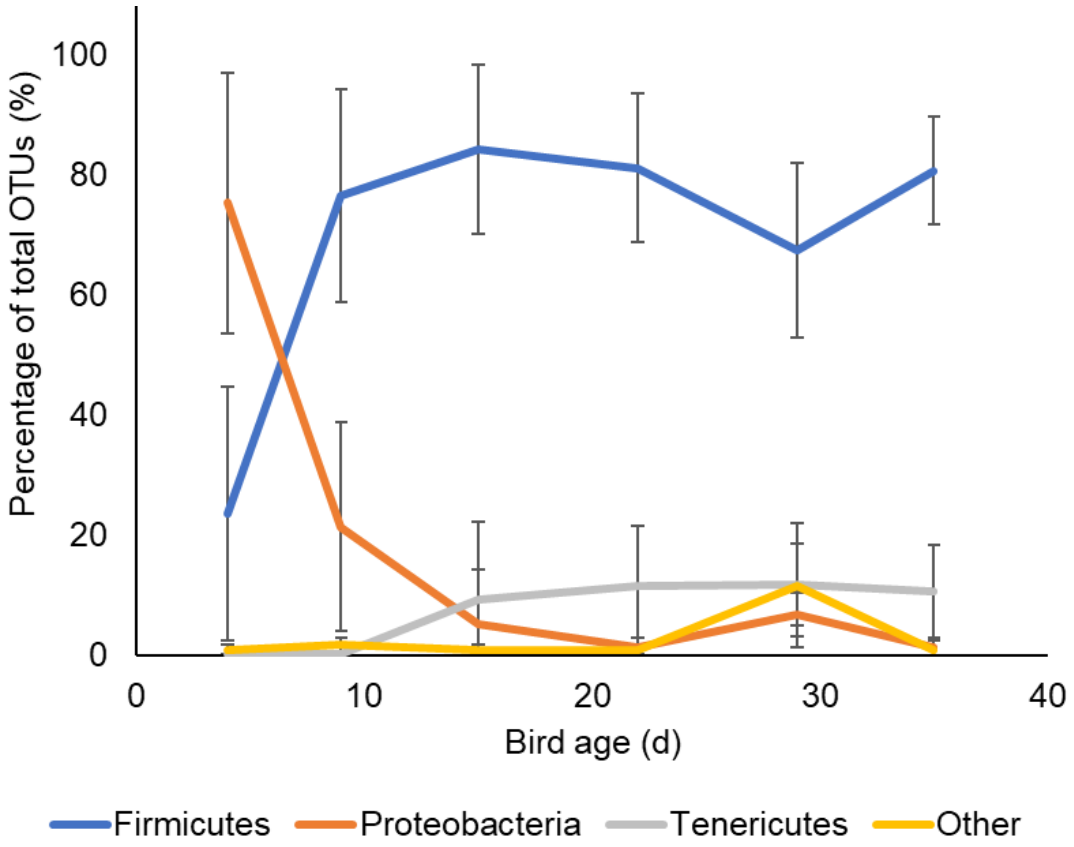

Figure 7.3 *Line plot illustrating changes in the relative abundances of phyla within the caecal microbiome, profiled through next-generation sequencing of caecal digesta, with age. Error bars represent standard deviation within phyla at each bird age.*

The large shift in the abundances of *Proteobacteria* and *Firmicutes* may be attributed to oxygen levels in the gut. Evidence suggests that the gut is first colonised by facultative anaerobes, such as Enterobacteriaceae from phylum *Proteobacteria*, whereby oxygen is consumed *via* aerobic respiration. Depleting oxygen levels thereby allow the colonisation of obligate anaerobes such as *Lachnospiraceae* and *Ruminococcaceae* from phylum *Firmicutes* (Wise and Siragusa, 2006).

Bars in **Figure 7.4** show the relative abundances of phyla, as discussed in Chapter 1, alongside bars representing data from the present studies. There is agreement that *Firmicutes* is generally the dominant phylum, but *Bacteroidetes* and *Tenericutes* appear to be under- and over-represented, respectively, in the present studies. Though this may be a genuine difference between birds used in the present studies and published literature, there may also have been a bias caused by limitations of the NGS approach used. This will be discussed in a later section.

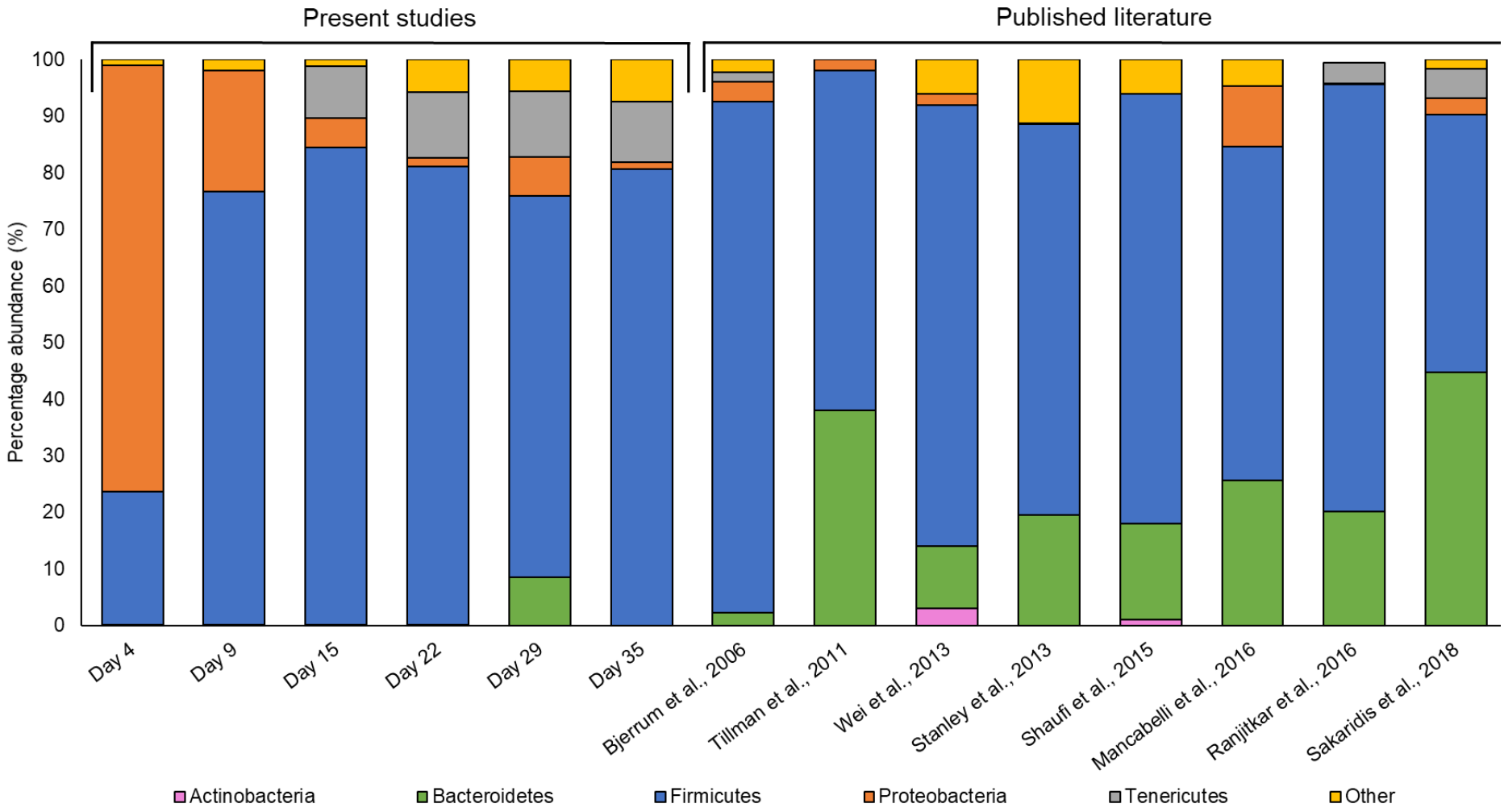

Figure 7.4 *Summary of bacterial phyla, profiled by next-generation sequencing, in the caecal digesta of birds of varying ages in the present studies, alongside adult birds (approximately 35 days of age) in published literature.*

7.2.2. Treatment

As well as an age-related effect, phyla were significantly altered by management strategy. The abundance of *Firmicutes* was sensitive to the effects of treatment during the starter phase, with reductions at day 15 caused by bedding supplementation (*P*=0.018) and the feeding of fishmeal (*P*=0.037) or a maize-based diet (*P*=0.001). Since *Firmicutes* abundance was low in young birds, it is suggested that these treatments perhaps reduced their ability to colonise the caeca. These effects were not long-lasting, suggesting that exposure to environmental bacteria has a larger long-term impact on phylum composition.

Tenericutes were significantly affected by cereal base at day 15 (*P*=0.001) with trends observed at days 22 and 25 (*P*=0.054 and 0.073, respectively). Although extensive research into these bacteria in poultry is lacking, Munyaka *et al.* (2015) found levels to be 3.4 times higher in wheat-fed than maize-fed broilers. Differences were far more pronounced in the present study, where the proportion of OTUs attributed to *Tenericutes* was a factor of 179 times higher in wheat-fed birds. Niu *et al.* (2015) suggested a positive relationship with apparent crude fibre digestibility and this should be considered for future research. Furthermore, relationships between dietary flavonoid concentrations (De Nardi *et al.,* 2016), resistant starches (Kang *et al.,* 2014), fat content (Umu *et al.,* 2015), genetic modification of maize (Buzoianu *et al.,* 2012) and season (Bergmann *et al.,* 2015) exist in other species. This suggests that *Tenericutes* are sensitive to a range of factors that could be further explored in future work, particularly in the context of poultry.

Phylum *Tenericutes* is comprised entirely of bacteria from class *Mollicutes,* hereby denoted as order 'RF39'. These bacteria are primarily scavengers, with a limited metabolic potential (Mitchell and Finch, 1977) – they therefore rely on the host for nutrition and could potentially be a detriment to the bird. The current inability to classify

below order level makes contextualising their potential effects on health and metabolism difficult, though roles in disease have been suggested (Miles, 1992).

7.2.3. Phyla relationships

Correlation analysis highlighted a significant negative relationship between *Firmicutes* and *Tenericutes*, suggesting that these phyla compete for colonisation in the caeca (**Figure 7.5**; r=−0.282, *P*=0.001). The reported Pearson correlation coefficient is perhaps lower than expected given such a low *P*-value, since *Firmicutes* and *Tenericutes* did not heavily colonise until days 9 and 15, respectively, and *Proteobacteria* were dominant at day 4.

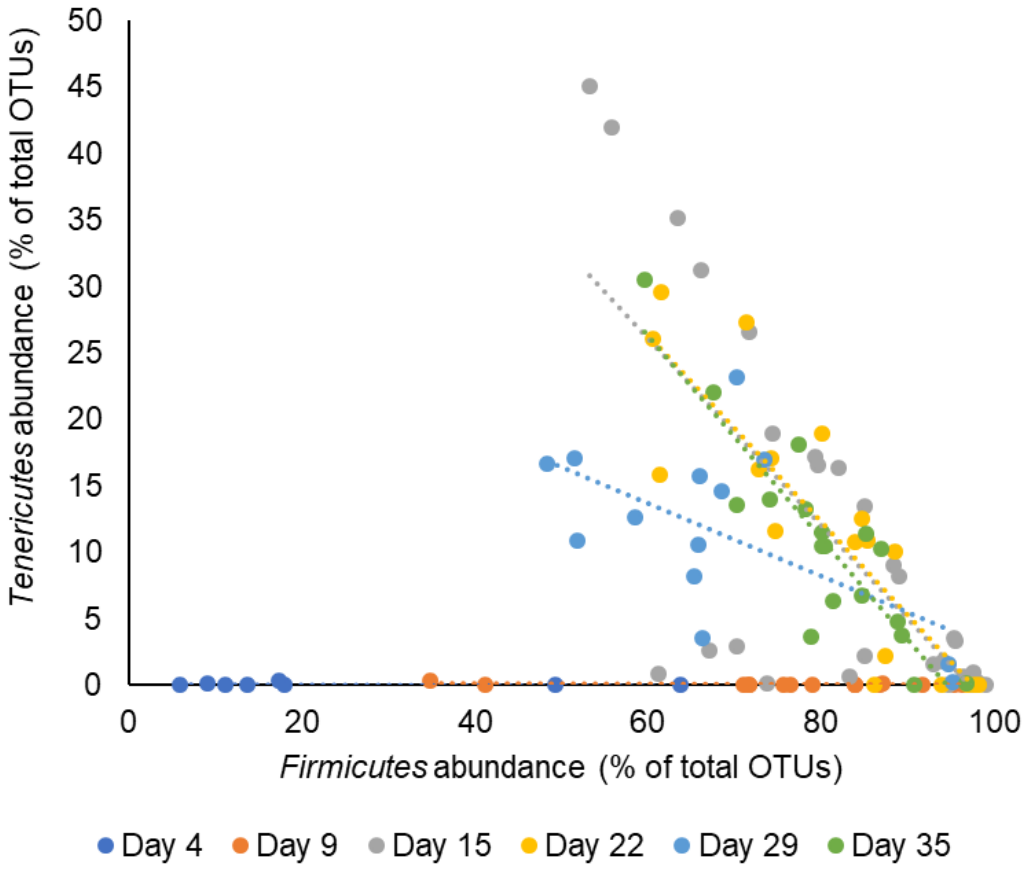

Figure 7.5 *Scatter plot illustrating the relationships between relative abundances of* Firmicutes *and* Tenericutes, *profiled by next-generation sequencing of caecal digesta. Pearson's correlation suggests a significant relationship between phyla (r=-0.202,* P=*0.001).*

7.2.4. Firmicutes:Bacteroidetes *ratio*

The ratio of *Firmicutes* to *Bacteroidetes* (F:B) is often explored since a high F:B ratio has been linked to obesity (Ley *et al.,* 2005). On average, the ratio of *Firmicutes* to *Bacteroidetes* increased with age to a peak at day 22 where levels were significantly higher than at day 4 (P=0.046) followed by a significant reduction between days 22 and 29 (P<0.001) (**Figure 7.6**).

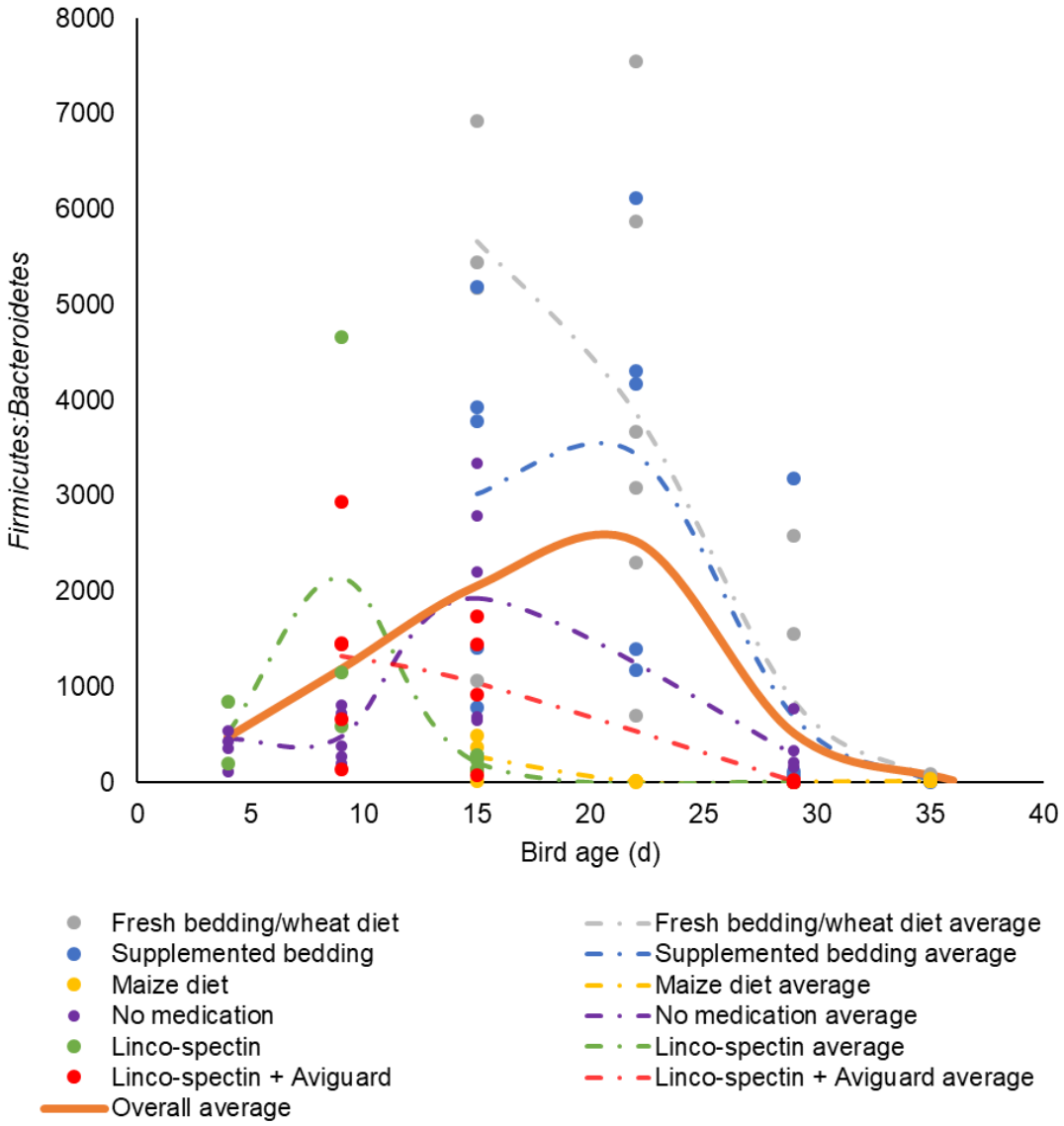

Figure 7.6 *Scatter plot illustrating the effects of bird age on* Firmicutes:Bacteroidetes *ratio, where bacterial populations were profiled by next-generation sequencing of caecal digesta. Dotted lines illustrate averages within treatment groups, solid line illustrates overall average irrespective of treatment.*

Age-related profiles differed between treatments: birds dosed with Linco-spectin and Aviguard® peaked earliest at day 9, whereas those on supplemented bedding peaked latest at day 22. Birds with less exposure to exogenous bacteria, *i.e.* those on clean bedding and not dosed with Aviguard® peaked at day 15, suggesting that management alters the rate at which the ratio of *Firmicutes* to *Bacteroidetes* develops.

Human and murine models suggest that leanness is related to higher levels of *Bacteroidetes*, whereas obesity is related to higher levels of *Firmicutes* (Kotzampassi *et al.,* 2014; Ley *et al.,* 2005). This may be a result of a positive relationship between *Firmicutes* and the ability of the host to harvest energy, particularly from dietary carbohydrates (Turnbaugh *et al.,* 2006). Fewer studies have explored relationships in avian species, though Singh *et al.* (2014) suggest that high levels of *Bacteroidetes* are linked to poor FCR, seemingly consistent with human and murine models. No relationships between FCR and either *Firmicutes* (r=-0.125, *P*=0.261) or *Bacteroidetes* (r=0.057, *P*=0.606) were identified in the present studies. It would perhaps be beneficial to explore F:B ratio in alternative gut compartments such as the ileum in future work. It should be noted that the studies presented here do not directly reflect commercial productivity, so FCR values are of limited value but observations may prompt future work.

7.3. Caecal microbiome: families

Though the caecal microbiome is an unequivocally complex ecosystem, there are a number of key families that have been regularly identified in existing literature. These were introduced in Chapter 1 and their populations will now be discussed in the context of bird age and management practices. Relationships between taxa are also explored through correlation analysis: based upon the number of degrees of freedom (n=113), the critical Pearson correlation coefficient value where $P<0.05$ was calculated as ± 0.195. This highlighted a huge number of apparent relationships, where a large

proportion were an incidental result of bird age. The threshold was therefore increased to ± 0.254 (P<0.01) to focus upon the most significant relationships.

7.3.1. Lachnospiraceae

7.3.1.1. Age

Existing work highlights a high abundance of *Lachnospiraceae* (phylum *Firmicutes*), in a range of animals including broilers (Stanley *et al.,* 2016). There was a significant increase in abundance from day 4 to a peak at day 9 (P<0.001), followed by a gradual decrease to day 29 where levels were significantly lower than at day 9 (**Figure 7.7**; P<0.001).

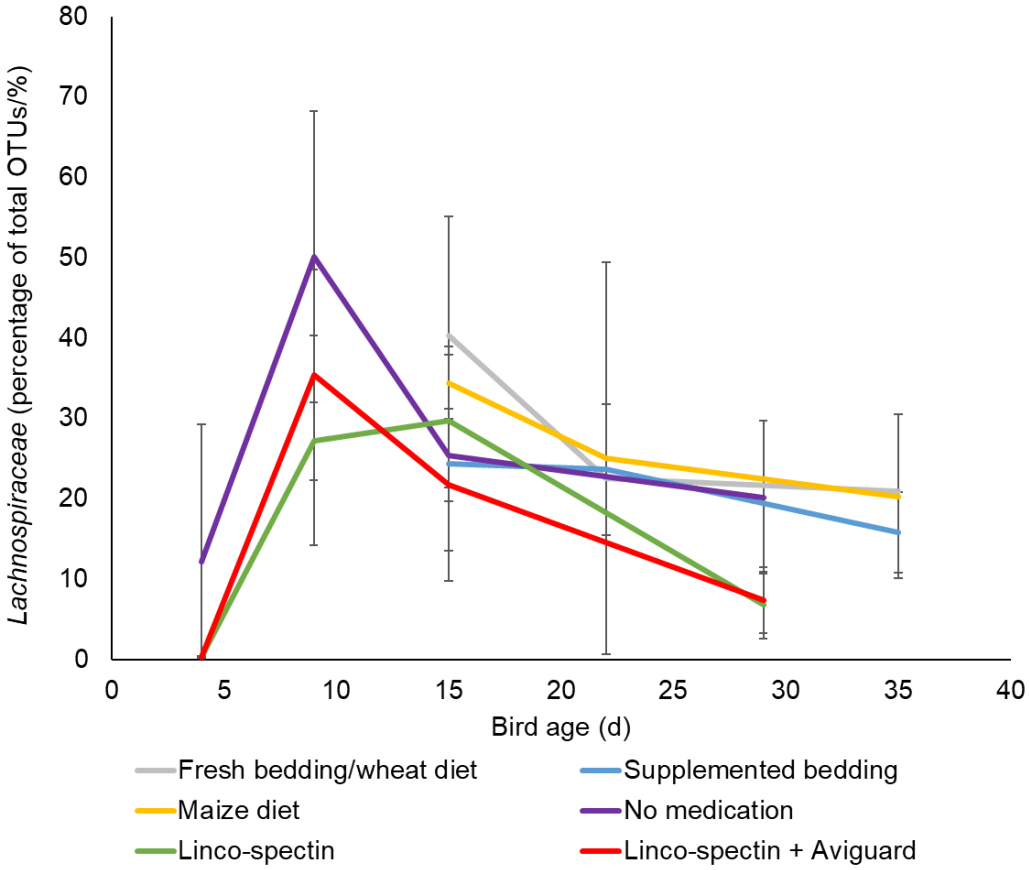

Figure 7.7 *Line plot illustrating the effects of bird age on the abundance of* Lachnospiraceae, *profiled through next-generation sequencing of caecal digesta, within treatment groups. Error bars represent standard deviation within treatment groups at each age.*

The sharp increase between days 4 and 9 was likely a result of reducing levels of *Proteobacteria*, leaving niches available for colonisation by other bacteria. The gradual decrease after day 15 may be a result of diet, since all birds were transferred to a grower diet at this point. The differences in nutrient composition may therefore have fuelled the growth of alternative species.

7.3.1.2. Treatment

Bacterial abundance was extensively affected by treatment, including the feeding of a maize-based diet or fishmeal as a protein source (P=0.009): since *Lachnospiraceae* digest complex carbohydrates, differing diets may have provided alternative compositions and concentrations of carbohydrates that fuelled differential bacterial growth. Birds within a study were fed the same diet and feed intake between treatments within a study did not differ: this suggests that differences in *Lachnospiraceae* abundance were more likely to be a result of nutrient composition rather than a direct result of feed intake. It is also thought that *Lachnospiraceae* are sensitive to interactions with other bacteria, since levels were reduced by supplementing bedding with excreta from mature birds at day 15 (P=0.010). It is hypothesised that this is a result of increased competition from other taxa for colonisation of niches within the gut: relationships with other bacteria were therefore explored through correlation analysis.

7.3.1.3. Relationships between taxa

Significant relationships between levels of *Lachnospiraceae* and other bacterial families are listed in **Table 7.1**.

Table 7.1 *Significant relationships between relative abundances of* Lachnospiraceae *and other bacterial families, profiled by next-generation sequencing of caecal digesta. Data were analysed through Pearson's correlation where threshold for statistical significance (*P*<0.001) was set as ± 0.254.*

Family	**r**	***P*-value**
Leuconostocaceae	0.40	<0.001
Coriobacteriaceae	0.38	<0.001
Erysipelotrichaceae	0.36	<0.001
Staphylococcaceae	0.27	0.004
Unassigned	-0.27	0.004
Rikenellaceae	-0.30	0.001
Rikenellaceae	-0.30	0.001
Peptococcaceae	-0.31	0.001
Christensenellaceae	-0.32	0.001
Mollicutes RF39	-0.41	<0.001
Unknown *Clostridiales*	-0.41	<0.001

Lachnospiraceae appeared to exhibit more negative than positive relationships, suggesting that relative abundance is fairly sensitive to the presence of other families, or different species expressed differing affinities for resources that determined bacterial growth. The negative relationship between *Lachnospiraceae* and unknown *Clostridiales* (r=-0.41, *P*<0.001) is likely to be a result of age, since each family respectively decreased and increased over time. It is unclear whether these relationships are a result of direct competition between bacteria for resources, or whether changes in factors such as diet and metabolism fuelled the growth of different taxa.

Due to the observed positive correlations, it is suggested that *Lachnospiraceae* do not directly compete with *Staphylococcaceae*, *Erysipelotrichaceae, Coriobacteriaceae* or *Leuconostocaceae*, but there is the possibility that the abundance of one relies upon the other. Existing literature hints at a potential negative relationship between both *Lachnospiraceae* and *Erysipelotrichaceae* and FCR, suggesting positive effects on performance, though this observation has been inconsistent between studies (Stanley *et al.,* 2016). The relative abundances of neither *Lachnospiraceae* nor *Erysipelotrichaceae* were significantly associated with FCR in the present studies (r=-0.173, *P*=0.124; r=0.008, *P*=0.944, respectively). Despite this, the strong positive relationship between *Lachnospiraceae* and *Erysipelotrichaceae* in the present data (r=0.36, *P*<0.001) suggests a lack of competition for colonisation: it is hypothesised that either both utilise the same resource, but that said resource is in excess and does not limit bacterial growth, or that different resources fuel the growth of each family. Similarly, the growth of one family may rely upon coexistence with the other if nutrients that are essential for the growth of one species are metabolically produced by the other. This is worthy of further examination.

There is the possibility that the positive relationship between *Lachnospiraceae* and *Coriobacteriaceae* (phylum *Actinobacteria*) was an effect of the study, since the latter was found at particularly low levels in birds from the medication study, irrespective of treatment. Despite this, Pitta *et al.* (2018) suggest that *Lachnospiraceae* and *Coriobacteriaceae* (phylum *Actinobacteria*) may be linked to alterations in the composition of fatty acid isomers caused by dietary starch. Both species were found to be positively correlated with isomers of conjugated linoleic acid (*i.e.*, fatty acids which are 18 carbons in length and contain two double bonds of either *cis*- or *trans*-geometry) in the rumen. Though the profile of the rumen ecosystem is different to that of the chicken caecum, it is thought that bacteria may interact in similar ways between species. Evidence also suggests interactions between *Lachnospiraceae* and *Coriobacteriaceae* and the regulation of glucose and cholesterol metabolism (Martinez

et al., 2013; Gomez-Arango *et al.,* 2016). Though extensive work into *Leuconostocaceae* in poultry is lacking, they have been identified as heterolactic fermentation bacteria, meaning that they ferment glucose to lactic acid, acetic acid and CO_2 (Sikora *et al.,* 2013). It is therefore reasonable to suggest that each of these bacteria interact with similar pathways, potentially explaining the positive relationship between their abundances.

7.3.2. Ruminococcaceae

7.3.2.1. Age

The abundance of *Ruminococcaceae* significantly increased between days 4 and 9 in birds from the medication study (**Figure 7.8**; P=0.001). Relative abundance was then, on average, unaltered by age, though high levels of variation between treatment groups and studies at days 15 and 22 suggest sensitivity to management strategies. It should be noted that the abundances of *Ruminococcaceae* in controls and LS-treated birds from the medication study were significantly higher than any other group at day 15 (P<0.001). Reasons for this difference are unclear, particularly since birds treated with both LS and Aviguard®, from the same study, were comparable to all other treatment groups in the remaining studies.

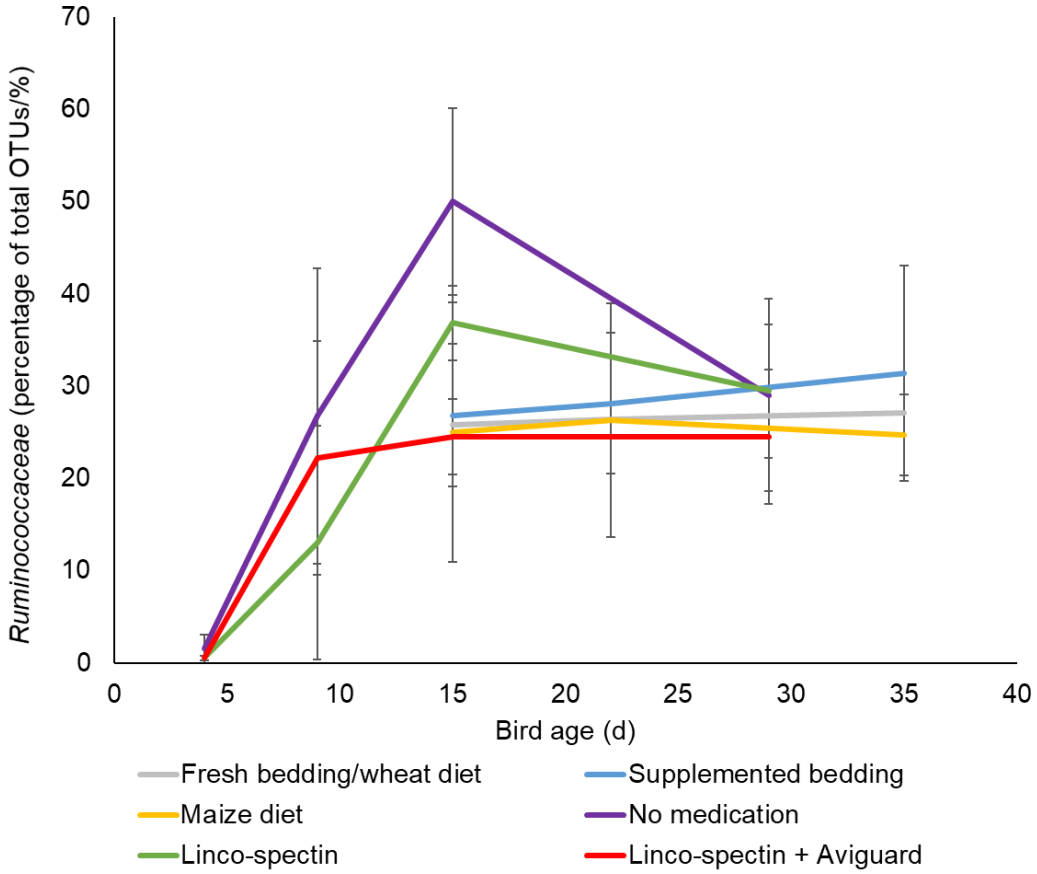

Figure 7.8 *Line plot illustrating the effects of bird age on the abundance of* Ruminococcaceae, *profiled through next-generation sequencing of caecal digesta, within treatment groups. Error bars represent standard deviation within treatment groups at each age.*

7.3.2.2. Treatment

Levels of *Ruminococcaceae* were highly sensitive to treatment in young birds in the medication study (*P*=0.046). The administration of Aviguard® following LS treatment suppressed numbers, suggesting that they are poor competitors in highly diverse microbiomes in young animals; this is supported by the concurrent lack of effect of bedding supplementation. Relative abundances were also significantly affected by dietary cereal base at days 15 (*P*=0.003) and 22 (*P*=0.016), and by protein source at day 17 (*P*=0.047), which suggests sensitivity to nutrient composition and availability. Differences within a study were only observed up to day 22; this may suggest that, once stable colonies are established, populations are resistant to diet-related changes.

7.3.2.3. Relationships between taxa

Significant relationships between levels of *Ruminococcaceae* and other bacterial families are listed in **Table 7.2**.

Table 7.2 *Relationships between relative abundance of* Ruminococcaceae *and other bacterial families, profiled by next-generation sequencing of caecal digesta. Data were analysed through Pearson's correlation where threshold for statistical significance (*P*<0.001) was set as ± 0.254.*

Family	r	*P*-value
Mogibacteriaceae	0.26	0.006
Clostridiaceae	-0.35	<0.001
Unknown *Lactobacillales*	-0.42	<0.001
Enterobacteriaceae	-0.57	<0.001

As with *Lachnospiraceae*, *Ruminococcaceae* (phylum *Firmicutes*) appeared to exhibit more negative than positive relationships with other taxa, suggesting competition for niches. The negative relationship with Enterobacteriaceae (phylum *Proteobacteria*) is likely to be a result of bird age and shifts in oxygen availability, due to the aforementioned shift in phyla between days 4 and 9. Conversely, the positive relationship with *Mogibacteriaceae* suggests absence of competition for colonisation.

7.3.3. Erysipelotrichaceae

7.3.3.1. Age

Levels of *Eryspielotrichaceae* (phylum *Firmicutes*) were significantly altered by age and were lowest at day 4 followed by a general increase to day 9 (*P*=0.067) and a general decrease for the remainder of life (**Figure 7.9**; *P*=0.004).

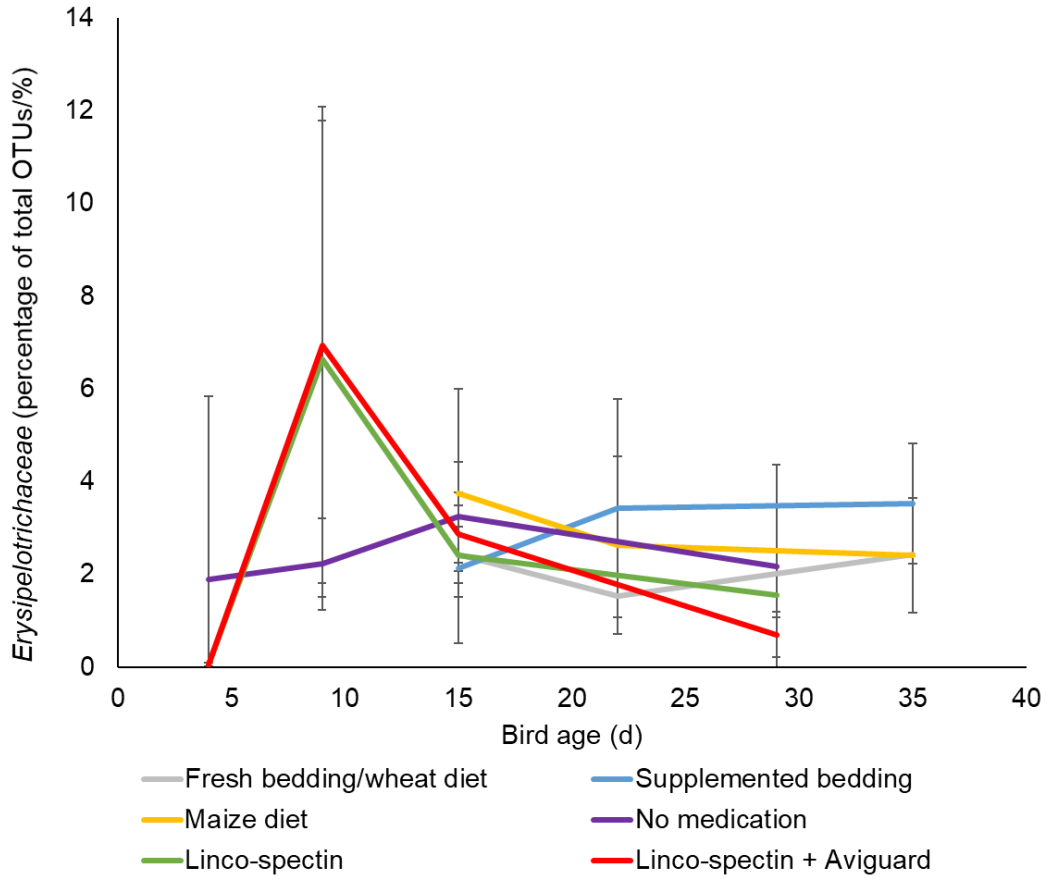

Figure 7.9 *Line plot illustrating the effects of bird age on the abundance of* Erysipelotrichaceae, *profiled through next-generation sequencing of caecal digesta, within treatment groups. Error bars represent standard deviation within treatment groups at each age.*

7.3.3.2. Treatment

Relative abundances within a study were affected by diet alone, with an increase in birds fed a wheat-based rather than a maize-based diet at day 15 (P=0.003), and soybean meal supplemented with synthetic amino acids over fishmeal as a protein source at day 17 (P=0.009). A lack of effect of bedding supplementation and antibiotic/probiotic treatment suggests that *Erysipelotrichaceae* are resistant to the effects of environment and are more sensitive to nutrient composition and availability.

Existing research suggests relationships between dietary fat content and levels of *Erysipelotrichaceae* (Turnbaugh *et al.,* 2009). Such relationships could not be explored

in the present study since birds were not fed individually and therefore an accurate prediction of individual feed, and therefore fat, intake could not be made. Further research highlights a potential immunogenic role of fat content with evidence of alterations of the inflammatory status in humans (Palm *et al.*, 2014). Kaakoush *et al.* (2015) suggests a relationship between diet, levels of *Erysipelotrichaceae* and inflammation – the immunological statuses of birds used in the present studies were not explored but could be considered for future work.

Also of note is the peak in bacterial abundance in antibiotic and probiotic-treated birds at day 4. This peak was not observed in controls, so it is reasonable to suggest that bacteria were sensitive to LS, despite the lack of data from other studies at this sampling point. The fact that both LS and LS+Aviguard® appeared to follow the same profile suggests a lack of sensitivity to Aviguard® and that, once levels are perturbed, they struggle to return to 'normal' levels. This theory should be explored by profiling the microbiome in young birds in a range of studies to ensure that levels observed in control birds are representative of normal populations.

7.3.3.3. Relationships between taxa

Significant relationships between levels of *Erysipelotrichaceae* and other bacterial families are listed in **Table 7.3**.

Table 7.3 *Relationships between relative abundance of* Erysipelotrichaceae *and other bacterial families, profiled by next-generation sequencing of caecal digesta. Data were analysed through Pearson's correlation where threshold for statistical significance (*P*<0.001) was set as ± 0.254.*

Family	**r**	***P*-value**
Bifidobacteriaceae	0.45	<0.001
Lachnospiraceae	0.36	<0.001

No negative relationships between taxa were observed, suggesting a lack of competition for resources. The positive relationship between *Erysipelotrichaceae* and *Lachnospiraceae* were previously discussed in section 7.1.3.3. Levels of *Erysipelotrichaceae* were most strongly associated with *Bifidobacteriaceae,* a family that has been linked to the degradation of carbohydrates to produce lactic and acetic acids (Lee and O'Sullivan, 2010) and is often included in probiotics to promote gut health. Relationships between taxa abundance and SCFA concentrations are discussed in section 7.6.

7.3.4. Lactobacillaceae

7.3.4.1. Age

Lactobacillaceae is a common lactic acid-producing family from phylum *Firmicutes*. Levels fluctuated with age but were not significantly affected (**Figure 7.10**; *P*=0.164). Relative abundance appeared to continuously increase in control birds from the medication study, but this trend was not observed in older birds from other studies. Whether this increase would have continued beyond day 29 is therefore unclear.

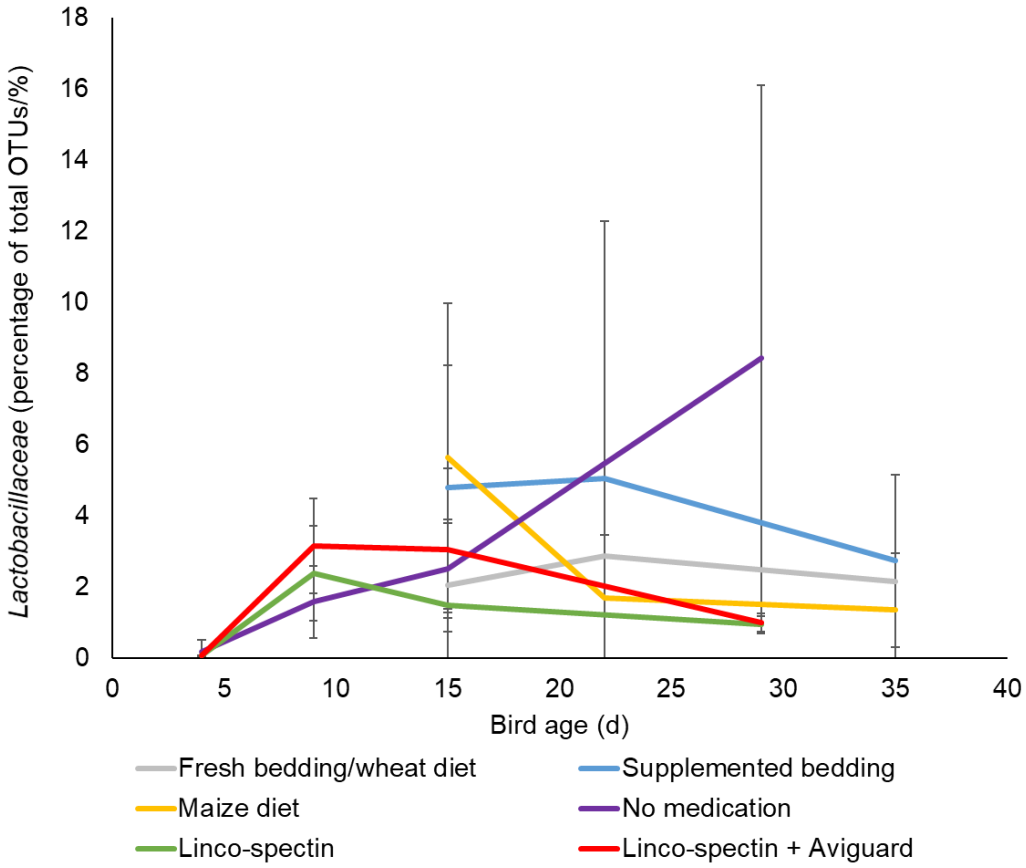

Figure 7.10 *Line plot illustrating the effects of bird age on the abundance of* Lactobacillaceae, *profiled through next-generation sequencing of caecal digesta, within treatment groups. Error bars represent standard deviation within treatment groups at each age.*

7.3.4.2. Treatment

Relative abundance was mainly affected by diet, with a reduction in maize-fed birds at days 15 (P=0.003) and 22 (P=0.047) and those fed fishmeal as a protein source at day 17 (P=0.047). Wu *et al.* (2014) found genera from family *Lactobacillaceae* to be differently affected by feeding fishmeal, with an increase in *L. reuteri* and *L. animalis* and a decrease in *L. johnsonii* and *L. acidophilus.* Unfortunately bacteria could not be taxonomically identified to species level in the present study, and therefore effects on individual taxa could not be explored. Reduced levels of *Lactobacillaceae* have also been linked to dietary supplementation of phytase (Akyurek *et al.,* 2011; Witzig *et al.,* 2015), an enzyme that hydrolyses phytic acid to release useable phosphorus. Mikulski and Klosowski (2014) report concentrations of phytic acid to be almost nine times

higher in maize grain than wheat grain. *Lactobacillaceae* were reduced in maize-fed birds at days 15 (*P*=0.003) and 22 (0.047) in the present study, suggesting a potential negative relationship with dietary phytic acid and scope for future work.

The relative abundance of *Lactobacillaceae* was elevated when birds were dosed with LS, irrespective of whether they also received subsequent probiotic treatment or not (*P*<0.05). Previous studies have found conflicting effects of antibiotics on levels of *Lactobacillaceae*, with evidence of an increase with bacitracin methylene disalicylate (Fasina *et al.,* 2015) and a decrease with amoxicillin (Schokker *et al.,* 2017) during the first two weeks of life. Discrepancies between studies are potentially attributed to the antibiotic of choice, since each antibiotic listed here plus lincomycin and spectinomycin, that comprise LS, are each from a different class and therefore function mechanistically in different ways.

Generally, *Lactobacillaceae* are considered beneficial bacteria (Fuller, 1989) which are often utilised as probiotics to improve gastrointestinal health. It can therefore be hypothesised that alterations in levels of *Lactobacillaeae* may have altered gut health – this could be explored through histological approaches in future work.

7.3.4.3. Relationships between taxa

Significant relationships between levels of *Lactobacillaceae* and other bacterial families are listed in **Table 7.4**.

Table 7.4 *Relationships between relative abundance of* Lactobacillaceae *and other bacterial families, profiled by next-generation sequencing of caecal digesta. Data were analysed through Pearson's correlation where threshold for statistical significance (*P*<0.001) was set as ± 0.254.*

Family	**r**	***P*-value**
Peptostreptococcaceae	0.63	<0.001
Unknown *Firmicutes*	0.32	0.001

These data suggest that levels of *Lactobacillaceae* were not suppressed by other taxa, since positive correlations alone were made. The strongest association was made with *Peptostreptococcaceae* (phylum *Firmicutes*) which has been shown to express the gene for lactate dehydrogenase, an enzyme that catalyses the conversion of lactate to pyruvate in mechanisms of energy production (Uniprot, 2012). It is therefore hypothesised that *Lactobacillaceae* produce the lactic acid that is subsequently metabolised by, and therefore fuels the growth of, *Peptostreptococcaceae.*

7.3.5. Alcaligenaceae

7.3.5.1. Age

Alcaligenaceae were found in the medication study alone: the apparent difference in abundance with age (**Figure 7.11**) was not significant and is most likely a study effect.

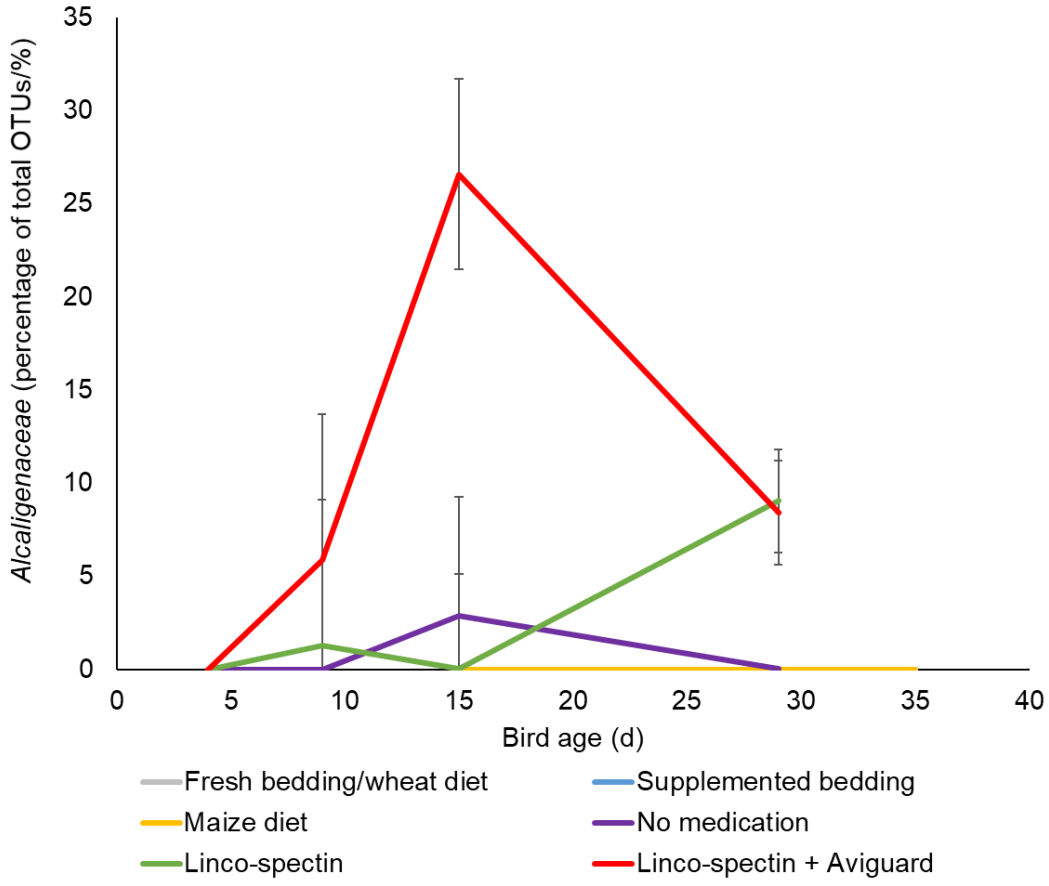

Figure 7.11 *Line plot illustrating the effects of bird age on the abundance of* Alcaligenaceae, *profiled through next-generation sequencing of caecal digesta, within treatment groups. Error bars represent standard deviation within treatment groups at each age.*

7.3.5.2. Treatment

Since *Alcaligenaceae* were found in the only study which utilised EnviroBed^{TM}, it is hypothesised that the bedding acted as a reservoir from which bacteria were ingested. EnviroBed^{TM} was used with the intention of reducing the extent to which birds consumed their bedding (personal communication), though birds appeared to consume just as much bedding as would be expected from those on wood shavings. Shavings were subsequently reinstated for the remaining studies since this is a more common practice in industry. Levels of *Alcaligenaceae* were highest in birds dosed with Aviguard® at day 15, suggesting that the majority of these bacteria may also have been of probiotic origin, though there is also evidence of bacterial colonisation at lower levels in other treatments, particularly since levels were almost identical in AB and AB+PRO birds at day 29 (P=0.772).

Further taxonomic analysis revealed that every read assigned to *Alcaligenaceae* was further assigned to genus *Sutterella. Sutterella* spp. have been found in humans, with a potential relationship with inflammatory bowel disease, though bacteria have also been identified in healthy patients so a consistent aetiopathological effect is unclear (Hiippala *et al.,* 2016). Bae *et al.* (2017) report that levels of *Sutterella* spp. decrease along the GIT, meaning that exploration of abundance higher up the digestive tract is warranted. Reponses of *Sutterella* spp. to environment have not been explored in great depth. Carrasco *et al*. (2018) report a reduction when broilers are fed tannins at an inclusion level of 1 g/kg of feed but found no response to bacitracin at the same level of inclusion. It was noted in the present studies that *Sutterella* spp. was significantly increased in birds dosed with Aviguard® and was also found in the probiotic product itself, suggesting its origin and supporting Ravindran (2014). It is likely that Aviguard® exposed birds to higher levels of *Sutterella* spp., resulting in an increase in colonisation. Biological effects cannot be hypothesised at this point since existing literature is lacking.

7.3.5.3. Relationships between taxa

Significant relationships between levels of *Alcaligenaceae* and other bacterial families are listed in **Table 7.5**.

Table 7.5 *Relationships between relative abundance of* Alcaligenaceae *and other bacterial families, profiled by next-generation sequencing of caecal digesta. Data were analysed through Pearson's correlation where threshold for statistical significance (*P*<0.001) was set as ± 0.254.*

Family	**r**	***P*-value**
Unassigned	0.36	<0.001
Bifidobacteriaceae	0.27	0.004

Zhao *et al.* (2017) found both *Alcaligenaceae* and *Bifidobacteriaceae* to be negatively correlated with carbohydrate and cholesterol intake. If both of these families are indeed affected by the intake of these nutrients, it is reasonable to suggest that this is the cause of the relationship between their abundances observed in the present studies: correlations with nutrient intake could not be made since feed data for individual birds were not collected.

7.3.6. Enterobacteriaceae

7.3.6.1. Age

The aforementioned shift in dominant phylum from *Proteobacteria* to *Firmicutes* between days 4 and 9 was mirrored in Enterobacteriaceae. ANOVA indicated a significant decrease between days 4 and 9 ($P<0.001$) and 9 and 15 ($P<0.001$) followed by a plateau at around 1.5% (**Figure 7.12**). Further work should consider exploring a wider range of sampling points during early life to more accurately map the transition of Enterobacteriaceae from high to low levels and explore any related biological effects.

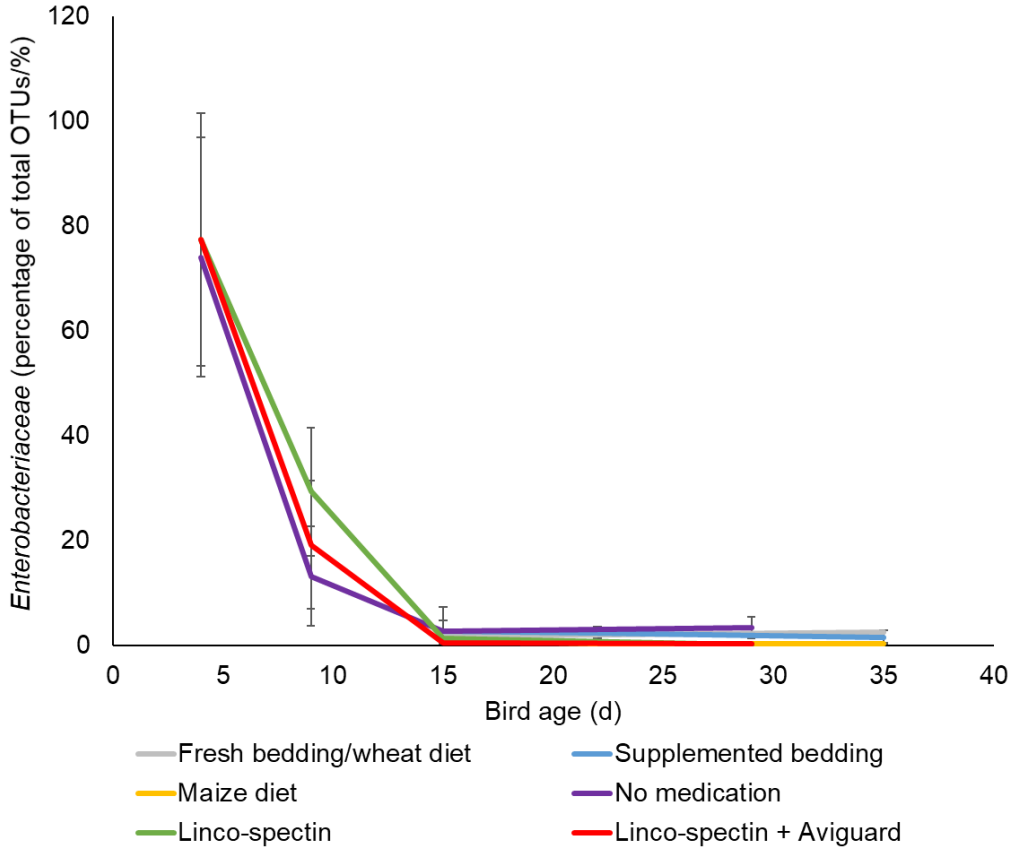

Figure 7.12 *Line plot illustrating the effects of bird age on the abundance of Enterobacteriaceae, profiled through next-generation sequencing of caecal digesta, within treatment groups. Error bars represent standard deviation within treatment groups at each age.*

7.3.6.2. Treatment

Enterobacteriaceae were mainly affected by cereal base, with evidence of significantly lower levels in birds fed a maize-based diet over those fed a wheat-based diet, irrespective of age (day 15, P=0.003; day 22, P=0.009; day 35, P=0.004). This may be a result of bacteria within the diet itself, leading to different levels of exposure in the gut, or differences in ingredient composition. There was also a significant reduction in birds dosed with LS or LS+Aviguard® but this effect was less pronounced with significant differences observed at day 29 alone (P=0.014).

Enterobacteriaceae are commonly found in the gut of animals, where they generally reside as commensals but are also opportunistic pathogens. None of the animals in

the present studies appeared to be of ill-health, suggesting that the gut was consistently healthy enough to prevent proliferation of pathogenic species. Of particular note is *E. coli*, which is also a human pathogen and therefore potentially a concern of food safety. Taxa in the present studies could not be consistently identified to genus level, so the effects of treatment on levels of *E. coli* in particular could not be explored. Future work may consider the use of microbial plating to enumerate *E. coli*, perhaps with the addition of antibiotics to explore the effect of management strategy on the development and persistence of antibiotic resistance.

7.3.6.3. Relationships between taxa

Significant relationships between levels of Enterobacteriaceae and other bacterial families are listed in **Table 7.6**.

Table 7.6 *Relationships between relative abundance of Enterobacteriaceae and other bacterial families, profiled by next-generation sequencing of caecal digesta. Data were analysed through Pearson's correlation where threshold for statistical significance (*P*<0.001) was set as ± 0.254.*

Family	**r**	***P*-value**
Unknown *Lactobacillales*	0.61	<0.001
Enterococcaceae	0.48	<0.001
Clostridiaceae	0.46	<0.001
Mogibacteriaceae	-0.32	0.001
Mollicutes RF39	-0.33	<0.001
Unknown *Clostridiales*	-0.48	<0.001
Ruminococcaceae	-0.57	<0.001

The significant negative relationships are likely a result of the large shift in the microbiome profile in young birds, particularly where *Ruminococcaceae*, unknown *Clostridiales* and *Mogibacteriaceae* are concerned since these families are all from the phylum *Firmicutes.* Both Enterobacteriaceae and *Lactobacillales* are lactic acid

producing taxa (Wust *et al.,* 2011): their positive relationship may therefore be a result of high levels of substrates available for fermentation.

7.3.7. Rikenellaceae

7.3.7.1. Age

Relative abundances of *Rikenellaceae* were very low at all sampling points except day 29 at an average of 7.6%. Though ANOVA suggests that abundance was significantly higher at day 29 than all other points (**Figure 7.13**; $P<0.001$), this is a result of samples from the medication experiment alone and suggests an effect of study. It is therefore hypothesised that this apparent spike at day 29 is not truly representative of 'normal' levels of *Rikenellaceae* in terms of bird age and may be a result of bedding material.

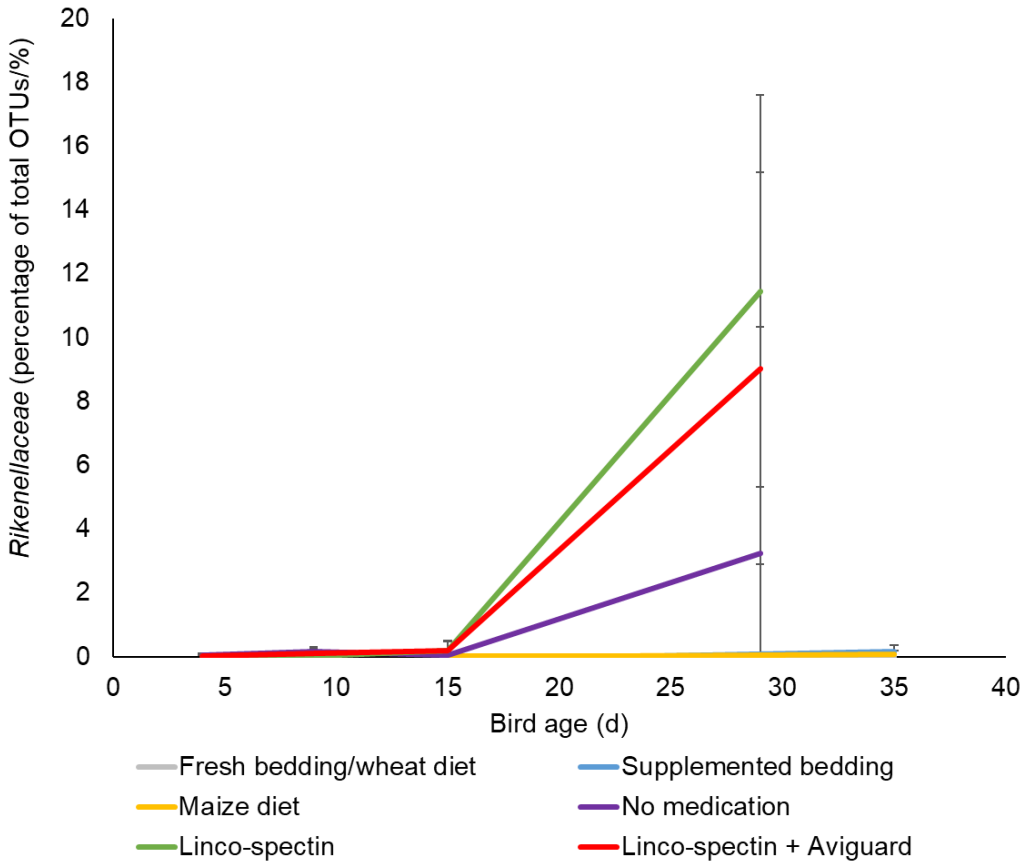

Figure 7.13 *Line plot illustrating the effects of bird age on the abundance of* Rikenellaceae, *profiled through next-generation sequencing of caecal digesta, within treatment groups. Error bars represent standard deviation within treatment groups at each age.*

NGS reads were unable to classify bacteria below family level, though *Rikenellaceae* comprises only three genera (*Acetobacteroides, Alistipes* and *Anaerocella*). Ranjitkar *et al.* (2016) found only *Alistipes* in the caeca of growing broilers, where levels significantly increased between days 15, 22 and 29, where they were identified as the most abundant family. This observation was certainly not made in any of the present studies, where bacteria from phylum *Firmicutes* dominated.

7.3.7.2. Treatment

Aside from the effects of study in terms of medication, populations of *Rikenellaceae* were significantly reduced by a wheat-based diet (P=0.009) and soybean meal supplemented with synthetic amino acids at day 15 (P=0.008), suggesting sensitivity to nutrient composition and availability during the grower phase. Relative abundance was significantly depleted in AB birds at day 15 (P=0.028), but this effect was not evident at any other sampling point within the same study.

7.3.7.3. Relationships between taxa

Significant relationships between levels of *Rikenellaceae* and other bacterial families are listed in **Table 7.7**.

Table 7.7 *Relationships between relative abundance of* Rikenellaceae *and other bacterial families, profiled by next-generation sequencing of caecal digesta. Data were analysed through Pearson's correlation where threshold for statistical significance (*P*<0.001) was set as ± 0.254.*

Family	**r**	***P*-value**
Odoribacteraceae	0.59	<0.001
Unassigned	0.51	<0.001
Bacteroidaceae	0.42	<0.001
Lachnospiraceae	-0.30	0.001
Peptococcaceae	-0.40	<0.001

Clarke *et al.* (2013) noted that supplementation with a bacteriocin-producing strain of *Lactobacillus salivarius* significantly increased proportions of *Peptococcaceae* whilst reducing proportions of *Rikenellaceae*, which is characteristic of a negative relationship. This suggests that their relative abundances may not interact with each other, but that they may be differentially affected by exogenous factors.

The strongest positive relationship was observed between *Odoribacteraceae* and *Rikenellaceae* (r=0.59, *P*<0.001). It is hypothesised that this was actually an artefact of the effect of study, since the samples responsible for said apparent positive correlation were all attributed to samples taken at day 29 in the medication study. Furthermore, to the author's knowledge, existing literature has not previously reported any relationship between these families.

7.3.8. Odoribacteraceae

7.3.8.1. Age

Odoribacteraceae, from the phylum *Bacteroidetes*, were only identified from day 15 onwards. This suggests an effect of age on colonisation, characterised by a significant increase between days 15 and 22 (*P*=0.002) and 22 and 35 (*P*=0.014) (**Figure 7.14**). Levels were much lower in the medication experiment, suggesting an effect of study and that these data are outliers.

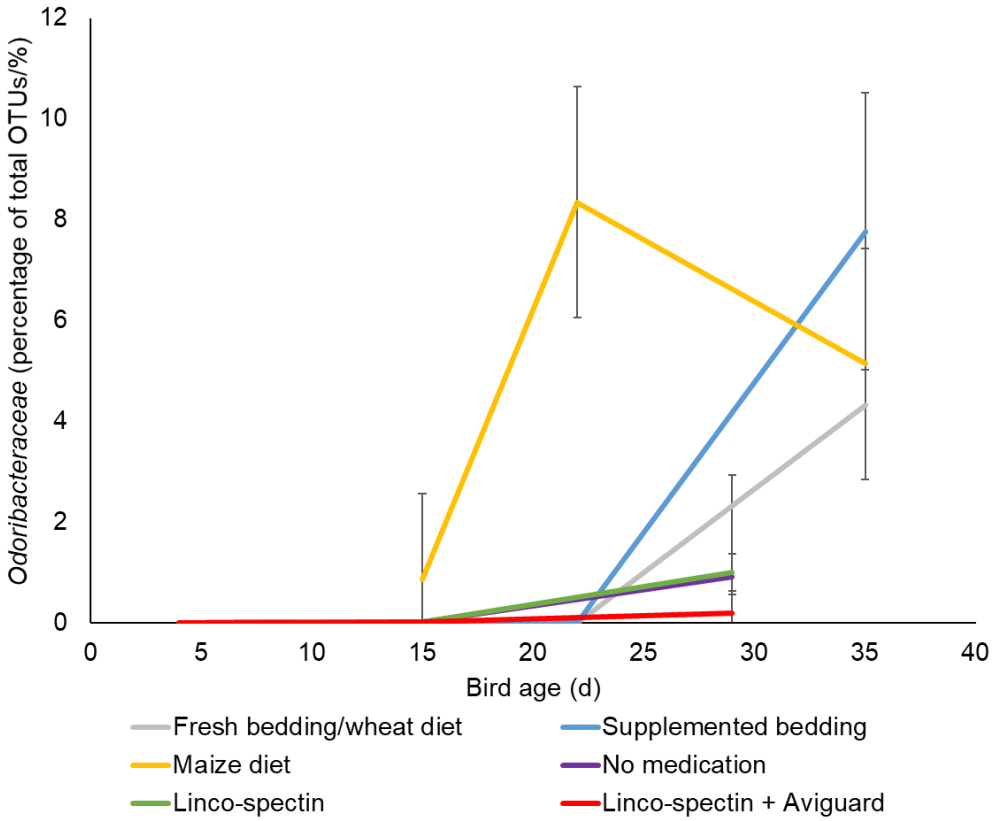

Figure 7.14 *Line plot illustrating the effects of bird age on the abundance of* Odoribacteraceae, *profiled through next-generation sequencing of caecal digesta, within treatment groups. Error bars represent standard deviation within treatment groups at each age.*

7.3.8.2. Treatment

Relative abundance was reduced by feeding a wheat-based diet over a maize-based diet and supplemented over fresh bedding at day 15 (P=0.004). It is therefore hypothesised that these treatments may have delayed said age-related increases. The lack of differences between Linco-spectin and Aviguard® treatments (P>0.05) may also suggest an effect of study. The bedding and diet base studies were run concurrently at a different time to the medication study, suggesting that differences in levels of *Odoribacteraceae* may be more of an effect of housing or environment rather than management strategy. It would be beneficial to explore dynamics of *Odoribacteriaceae* in a more controlled experiment to reduce the impact of study.

7.3.8.3. Relationships between taxa

Significant relationships between levels of *Odoribacteriaceae* and other bacterial families are listed in **Table 7.8**.

Table 7.8 *Relationships between relative abundance of* Odoribacteriaceae *and other bacterial families, profiled by next-generation sequencing of caecal digesta. Data were analysed through Pearson's correlation where threshold for statistical significance (*P*<0.001) was set as ± 0.254.*

Family	**r**	***P*-value**
Rikenellaceae	0.59	<0.001
Peptococcaceae	0.30	0.001
Unassigned	0.28	0.003

The relationship between *Odoribacteriaceae* and *Rikenellaceae* was previously discussed in section 8.3.7.3. Again, the apparent correlation with *Peptococcaceae* appears to be an artefact of the effect of study, with the majority of the positive relationship accounted for by samples taken from the medication study at day 29. Despite this, removing said samples from the analysis actually strengthened the statistical significance (r=0.404; *P*<0.001), though reasons for this relationship are unclear.

7.4. Relationships between performance and the caecal microbiome

The difference between caecal bacterial populations was numerically characterised as the sum of all LDA scores for each treatment group at each sampling point, where a higher score highlighted a larger difference in microbiomes. Differences in performance were determined by calculating the difference in average FCR for the same treatment/age comparisons. Relationships between these data explored through Pearon's correlation coefficient.

FCR did not consistently differ between treatment groups within a study, though there were consistently differences in the composition of the caecal microbiome. No relationship between numerical differences in FCR and accumulative LDA scores was observed (*r*=0.386; *P*=0.270; **Figure 7.15**).

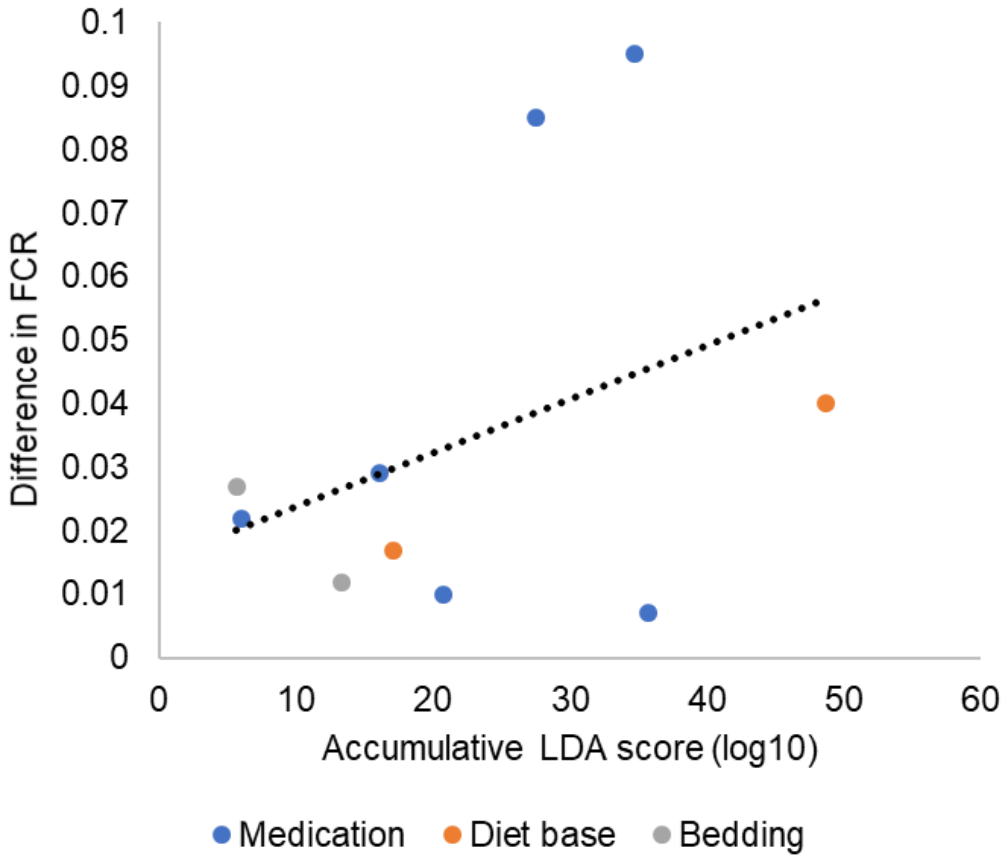

Figure 7.15 *Scatter plot illustrating the relationship between numerical differences in performance assessed through FCR, and differences in caecal bacterial populations, profiled through next-generation sequencing and calculated as accumulative LDA scores obtained from LEfSe analysis of bacterial abundances. Data points are coloured by study origin for reference.*

The two datapoints at the top of the plot that lead to the suggestion of a positive correlation are derived from comparisons of birds dosed with LS and Aviguard® with controls and those dosed with LS alone between days 9 and 15. The difference in FCR was only significant between controls and AB+PRO birds (*P*=0.042), suggesting that the use of Aviguard® following antibiotic treatment may have a positive influence on

productivity – this warrants further exploration in future work. The fact that this relationship was not observed in any other treatment groups suggests that, in general, the profile of the caecal microbiome is not reflected in differences in performance.

7.5. Hepatic metabolome

Consistent changes in concentrations of hepatic metabolites were not observed throughout the presented studies although, according to OPLS-DA analysis, bedding and diet base had the most effect on metabolic profiles.

7.5.1. Energy metabolism

Though differences in the same metabolites were not observed between studies, a common theme appeared to be energy metabolism. Differences in concentrations of glucose, NAD^+, succinate and isobutyrate were observed, though these were not consistently related to a single management factor, with evidence that concentrations were altered by bedding and diet. It is thought that these differences may be a result of feed intake, though relationships cannot be fully explored since birds were fed on a pen-basis and individual feed intake is unknown. If, however, these differences are not a result of feed intake, and that metabolic pathways are truly affected by management, the apparent lack of differences in health and growth suggest that birds alter their metabolism in response to their environment. This area warrants more in-depth work to fully understand how energy metabolism may be altered by management to meet demands for maintenance and growth.

7.5.2. SCFA

Despite the lack of consistent differences in individual hepatic metabolites, exploratory integration of SCFA peaks revealed differences in metabolite concentrations that were not highlighted in OPLS-DA models – the limitations of this technique will be discussed

later. Peaks attributed to SCFA are easily identifiable, well-resolved, and suffer little overlap with neighbouring NMR peaks, and can therefore be investigated fairly reliable using the present techniques.

7.5.2.1. Age

ANOVA of all NMR data revealed that concentrations of SCFA significantly increased with age (**Figure 7.16**). Concentrations of acetate and butyrate increased between all sampling points until day 22, followed by a dip to day 29 (P<0.001), but recovered by day 35 (P<0.001). This dip is likely an effect of study, since only birds from Study 1 (medication) were sampled at day 29. Concentrations of propionate significantly increased between days 4 and 15 (P<0.001) and 15 and 22 (P=0.011) but plateaued at subsequent sampling points. Concentrations of lactate significantly increased between days 4 and 15 (P<0.001) and numerically decreased at days 22 and 29, but these was not deemed to be statistically significant. Concentrations returned to match those at day 15 by day 35 – it is hypothesised that this dip is a result of a sudden dietary change, and that the gradual return to day 15 concentrations reflects the ability of the birds to adapt to and utilise their new diets.

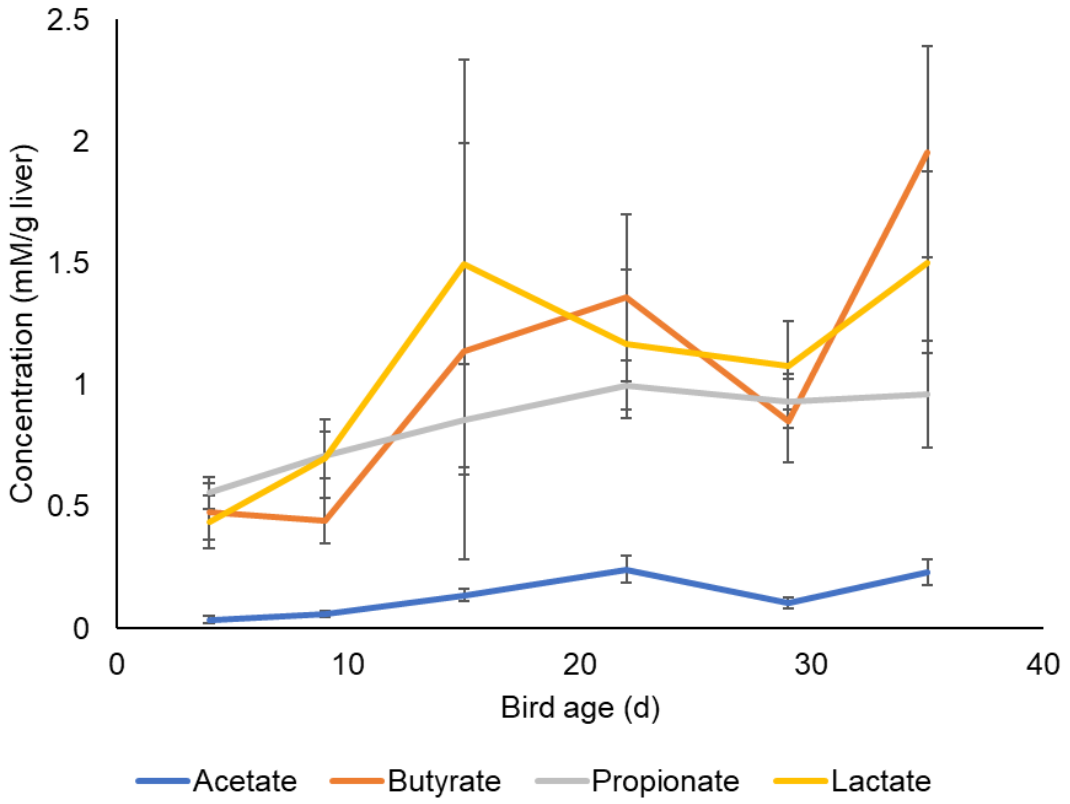

Figure 7.16 *Line plot illustrating the effects of bird age on concentrations of hepatic SCFA per gram of liver tissue, concentrations of which were calculated through the integration of peaks in ^{1}H NMR spectra. Error bars represent standard deviation within treatment groups at each age.*

The overall increase in SCFA concentrations with age suggests an increase in carbohydrate metabolism – this may be a result of an increase in feed consumption with age, since SCFA are products of carbohydrate fermentation. Increasing feed intake, and therefore substrate availability, will naturally increase concentrations of metabolic products.

Data for day 29 suggest a reduction in SCFA concentrations, particularly of butyrate, though this was most likely a result of study. Only data from the antibiotic/probiotic study were recorded at day 29 – analysis of earlier datasets, where samples were taken from multiple studies, suggest that SCFA concentrations were lower in all birds from the antibiotic/probiotic study, irrespective of treatment. The apparent reduction in SCFA concentrations at day 29 are therefore not representative of the true effects of

age, a significant drawback of this analysis. Future work should aim to explore the same sampling points within all experiments to attempt to reduce the effects of 'study' as a variable. Despite this, the overall effects of age on SCFA concentrations are still likely to be valid.

Concentrations of SCFA have been linked with gut health in terms of cellular architecture, development of the immune system and prevention of disease by reducing pH and preventing bacterial colonisation. Though these parameters were not explored in the present studies, it is hypothesised that each would have increased with bird age. Future work should consider exploring relationships between SCFA concentrations in a range of biological samples effects on enteric cellular architecture and immune responses to further develop management strategies that positively influence bird health and productivity.

7.5.2.2. Treatment

The only treatment to affect concentrations of hepatic SCFA was cereal base (Chapter 5) during the starter phase. Concentrations of butyrate were higher in wheat-fed birds (P=0.006), whereas concentrations of propionate and lactate were higher in maize-fed birds (P=0.012 and P<0.001, respectively). The lack of effect of protein source, bedding, LS or Aviguard® (P>0.05) suggests that these treatments do not affect metabolism, or that effects may not be reflected in the liver. It is thought that the former is more likely, with suggestions that the composition of complex carbohydrates is the most influential factor and should be explored in future work.

7.5.2.3. Performance

Correlation analysis revealed significant positive relationships between FCR and concentrations of butyrate and acetate (P=0.001), whilst a trend was observed between FCR and propionate (**Figure 7.17**; P=0.098).

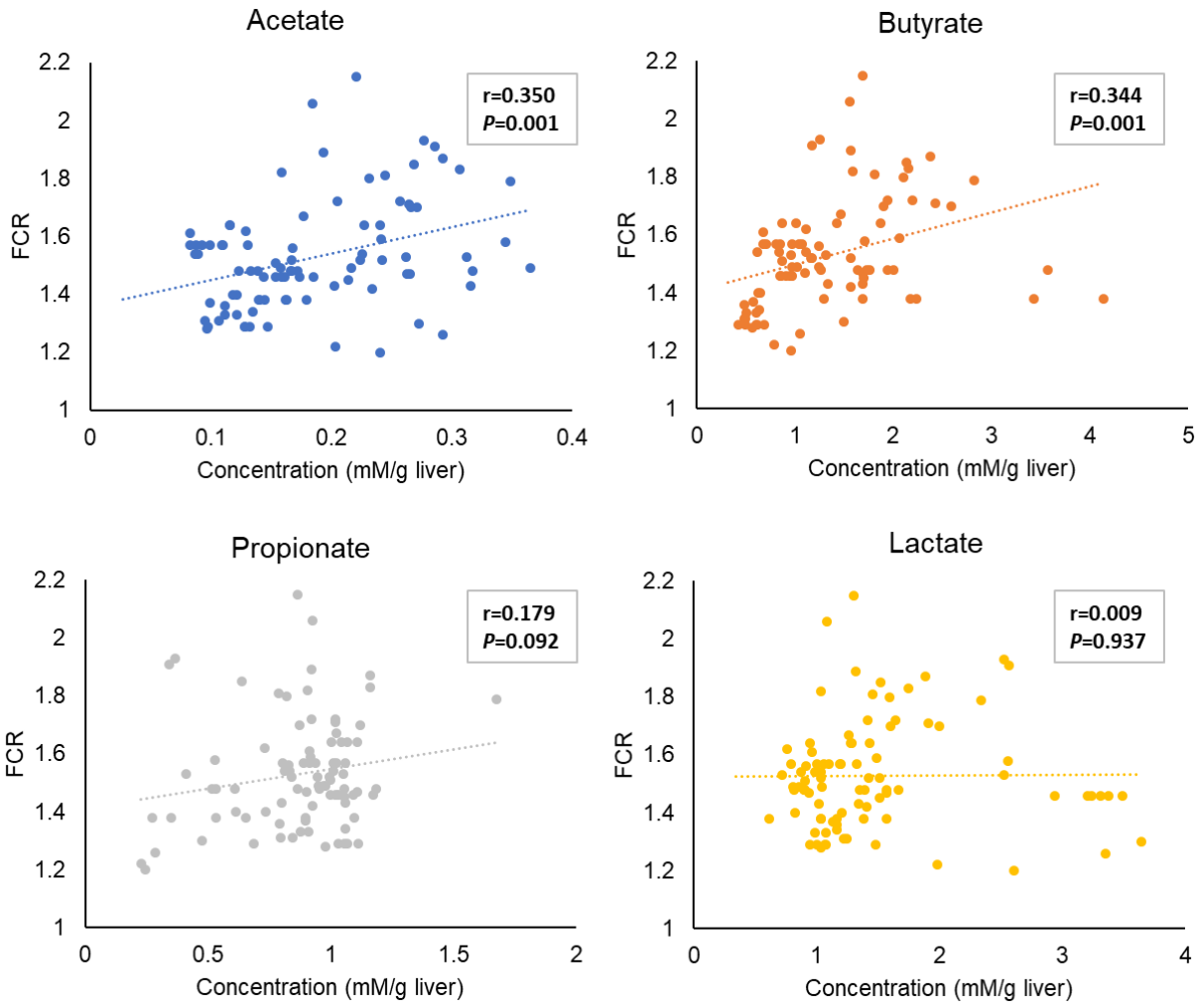

Figure 7.17 *Scatter plots illustrating the relationships between hepatic SCFA and lactate concentrations per gram of liver tissue of individual birds, and the FCR of the pen from which each bird was taken. SCFA and lactate concentrations were calculated through the integration of peaks in ^{1}H NMR spectra. Relationships were explored through Pearson's correlation coefficient where statistical significance was denoted as* P*<0.05.*

These relationships are likely to be artefacts of a concurrent increase in both parameters with age. Data were therefore reanalysed at each timepoint within studies to explore whether the frequent differences in SCFA concentrations were reflected in performance: no relationships were identified (*P*>0.05). A range of studies have also failed to identify such relationships, despite the plethora of evidence that SCFA exert beneficial effects on broiler physiology, growth and health. It is thought that this is because birds in said studies were unchallenged, and that positive effects of SCFA on

growth are exerted when birds are kept under challenging conditions (Pinchasov and Jensen, 1989; Hernandez *et al.,* 2006; Isabel and Santos, 2009; Świątkiewicz and Arczewska-Wlosek 2012). If this theory is correct, it is reasonable to suggest that differences in the caecal microbiome did not challenge birds, since there were no relationships between performance and hepatic SCFA concentrations.

7.6. Multi-omics: metagenomic and metabolomic correlations

One of the aims of these studies was to explore the relationships between the caecal microbiome and hepatic metabolome. Since OPLS-DA was unable to highlight metabolites that were consistently affected by treatment but exploratory analysis of SCFA found consistent differences, concentrations of SCFA alone were correlated against bacterial abundances (**Figure 7.18**).

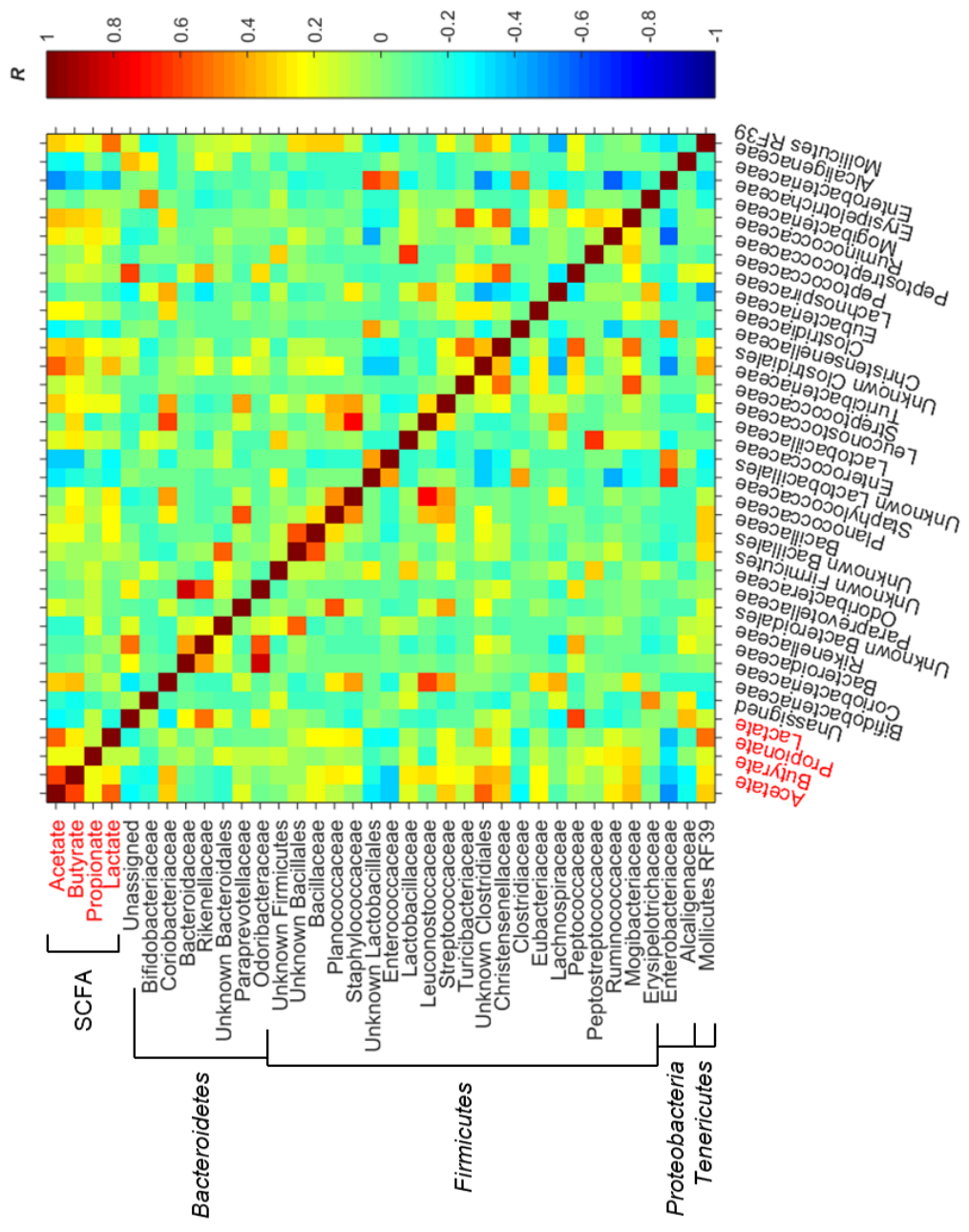

Figure 7.18 *Heat map illustrating relationships between hepatic SCFA and lactate concentrations, calculated through the integration of peaks in ^{1}H NMR spectra and labelled in red, and the relative abundances of caecal bacteria, profiled through next-generation sequencing and labelled in black. Relationships were characterised through Pearson's correlation coefficient, where a significant relationship* ($P<0.05$) *was denoted at a correlation coefficient threshold of ± 0.164.*

Table 7.9 lists significant correlations between hepatic SCFA concentrations and the relative abundances of bacterial families identified in caecal content. Based upon a sample size of 113 birds, the correlation coefficient threshold where $P<0.05$ was set as ± 0.195. Since a vast number of potential relationships were highlighted, the *P*-value threshold for statistical significance was subsequently altered to $P<0.001$ with a new coefficient threshold of ± 0.254 of to focus on the most significant correlations.

These data suggest relationships between concentrations of hepatic SCFA and the relative abundances of bacterial families in caecal digesta. Despite this, these associations may not represent absolute relationships, since data were taken from birds of different ages and may actually represent coincidental associations between developments in metabolism and the microbiome with age. An example of this is Enterobacteriaceae, which were negatively correlated with concentrations of all SCFA. As previously discussed, levels of Enterobacteriaceae significantly decrease whilst concentrations of SCFA significantly increase with age. The apparent negative correlation may suggest that Enterobacteriaceae reduced the production of SCFA, when, in fact, it is more likely that SCFA were simply not produced at high concentrations in young birds. The suggested relationships between Enterobacteriaceae and SCFA concentrations are therefore more likely to be a result of the natural shifts in the microbiome with age, rather than direct relationships between bacteria and metabolism.

Table 7.9 *Significant SCFA- bacteria relationships, analysed through the integration of ^{1}H NMR peaks and next-generation sequencing, respectively. Relationships were explored through correlation analysis where a correlation coefficient threshold for statistical significance was set as ± 0.254 (*P*<0.001).*

SCFA	**Bacteria**	***r***	***P*-value**
Acetate	Unknown *Clostridiales*	0.55	<0.001
	Mogibacteriaceae	0.35	<0.001
	Mollicutes RF39	0.34	<0.001
	Coriobacteriaceae	0.32	0.001
	Christensenellaceae	0.32	0.001
	Streptococcaceae	0.32	0.001
	Ruminococcaceae	0.27	0.003
	Clostridiaceae	-0.26	0.005
	Unassigned	-0.28	0.002
	Unknown *Lactobacillales*	-0.29	0.002
	Enterococcaceae	-0.36	<0.001
	Enterobacteriaceae	-0.50	<0.001
Butyrate	Unknown *Clostridiales*	0.38	<0.001
	Christensenellaceae	0.36	<0.001
	Coriobacteriaceae	0.36	<0.001
	Mogibacteriaceae	0.33	<0.001
	Leuconostoaceae	0.30	0.001
	Lactobacillaceae	0.28	0.003
	Alcaligenaceae	-0.26	0.006
	Enterococcaceae	-0.35	<0.001
	Enterobacteriaceae	-0.35	<0.001
Propionate	*Mogibacteriaceae*	0.30	0.001
	Ruminococcaceae	0.26	0.005
	Enterobacteriaceae	-0.31	0.001
Lactate	*Mollicutes* RF39	0.50	<0.001
	Unknown *Clostridiales*	0.36	<0.001
	Enterococcaceae	-0.26	0.006
	Enterobacteriaceae	-0.39	<0.001

It should also be noted that, since bacterial abundance is not an absolute measure of the number of target bacteria in a sample but merely indicates the proportion that bacteria contribute to the whole microbiome, relationships between bacterial counts and metabolite concentrations as a predictor of SCFA production cannot be analysed from the current data. The associations listed here, however, can be drawn upon for future work to further explore relationships between the caecal microbiome and hepatic SCFA concentrations. Quantification of bacteria and metabolites could perhaps be achieved through quantitative PCR (qPCR) of unique genes and calibrated gas chromatography (GC) to more conclusively define microbiome-metabolome relationships.

8. Discussion

The results presented in these studies suggest that alpha diversity of caecal bacterial populations of growing broiler chickens is most sensitive at a young age, but effects generally dissipate over time. Differences in management strategies may alter bacterial diversity, but it is thought that these effects are neither of detriment or benefit to the bird in terms of performance due to a lack of significant relationships in the present data. It is thought that differences in alpha diversity are a result of exposure to environmental bacteria – future work should therefore explore relationships between the environmental and gastrointestinal microbiomes to highlight which species are most commonly transferred between ecosystems and how this may affect biological processes in the host.

It is also suggested that beta diversity is sensitive to change during the first two weeks of life, with reduced sensitivity with age. It is thought that diversity naturally reaches a fairly common profile over time, irrespective of perturbations induced by treatment during early life. It is also suggested that differences in beta diversity are not necessarily reflected in differences in alpha diversity, indicating that one may not be an accurate predictor of the other. The microbiome can be equally as diverse in terms of the number and range of taxa present, but the identities of said species may differ therefore causing a difference in beta diversity without a difference in alpha diversity. Given that age is the most significant factor, it would seem important to investigate the contribution of the host genotype upon the microbiota as the host is very likely to be providing the most stringent of selective pressures. Ross 308 birds were used in these studies, and it may be informative to compare the various genetic lines and perhaps even contrast with progenitor wild type species of fowl.

The evidence presented in this thesis illustrates that individual bacterial families are differentially affected by both age and management strategy. It appears that, within a study, bacteria are more sensitive to the effects of diet than environment, potentially

as a result of differences in the provision, levels and composition of nutrients that fuel bacterial growth. It is thought that bacteria are most sensitive to levels of complex carbohydrates, since these are solely metabolised by the gut microbiome and subsequently alters the production of metabolites such as SCFA which may further modulate bacterial populations.

The effect of each study itself must also be considered. The studies exploring bedding supplementation and diet base were run concurrently, whereas the medication and protein studies were carried out at different times. Differences in the environment in terms of human interaction, diet batch, bedding material and cleaning regimens between studies may have affected the types and levels of bacteria that birds were exposed to, despite the fact that every study was conducted in the same environmentally-controlled room. The largest design features that defined the medication study were the use of EnviroBed™ over wood shavings, and the use of a commercial diet during the starter phase. It is hypothesised that these may have been sources of alternative bacteria in comparison with bedding and diets used for the other studies. It is recommended that future work explores the bacterial populations of diet, bedding and transport media to suggest the origins of taxa also identified in the gut microbiome.

Despite the large differences between studies and treatments within a study, it was noted that no single group of birds appeared to perform worse than another, and neither was there any evidence of gross pathologies. There was some evidence that the use of Aviguard® following antibiotic treatment may influence caecal bacterial populations and FCR concurrently – this is something that should be explored further in future work, particularly as there were no concurrent differences in hepatic metabolites. In general, however, the effects of bedding and diet on bacterial populations were independent of any differences in FCR. For this reason, it is hypothesised that the ability of a bird to thrive and grow is independent of the profile

of its microbiome, so long as key microbial metabolic activities are performed. This does, however, lead to an assumption that 'key' bacteria are required, and that these 'key' bacteria will be consistently found, which is perhaps naïve. As mentioned earlier, assuming there are many different bacteria able to perform a key metabolic function, it stands to reason that any one or more of these and in differing numbers and compositions could fulfil the desired metabolic outcome. Hence, different but isofunctional bacterial populations are present. Birds appear to develop alongside their microbiome and develop intricate and unique relationships that, if established from a young age, ultimately result in the same product: a healthy, efficiently-growing bird. To explore this further, future work should consider the implementation of a sudden stressor to older birds to characterise if and how the microbiome can recover, and whether this affects the health and growth of the chicken.

8.1. Technique limitations and future improvements

8.1.1. Next-generation sequencing

8.1.1.1. Identity assignment

There are a number of reasons why identities could not be assigned to some bacteria, including the formation of chimeric sequences during the PCR stage of DNA sequencing. A chimera is a PCR artefact that arises from the premature termination of DNA extension, reattachment to an alternative DNA template and recommencement of synthesis until completion. In short, a chimeric sequence is a mixture of two pieces of DNA that would suggest a new taxon based upon the fact that it would not match existing data stored in genomic databases. It is thought that an imbalance in dNTPs, primers and PCR inhibitors are the main cause of chimera formation, leading to the suggestion that the minimum number of PCR cycles required to obtain sufficient data are used. Smyth *et al.* (2010) suggest that identifying the end point of the log-linear phase of a PCR through qPCR can help one suppress the formation of artificial chimeras, since the system becomes saturated beyond this point. An attempt was

made to remove chimeric sequences from raw NGS data in QIIME, but the efficacy of said data processing could be questioned. It is suggested that this process is reviewed for future work, along with the optimisation of PCR conditions to reduce the potential for chimeras to form.

The complexity of the gut microbiome must also be considered. Quast *et al.* (2013) predict that around 2 million near full-length rRNA gene sequences are available in genetic databases but note that this represents only a fraction of all microbes. Similarly, Aird *et al.* (2011) suggest that reference databases are skewed towards well-studied ecosystems and may therefore present an incomplete view of bacterial populations. Full genome sequencing would allow a more definite assignment of taxa, along with identification of genes of interest that encode metabolic enzymes, virulence factors, antibiotic resistance and the like. Full-genome sequencing is much slower and more expensive process than sequencing of the 16S gene alone and was therefore inappropriate for the present studies. What could be considered for future work, however, is the sequencing of the whole 16S gene rather than selected variable regions (v4 and v5 in the case of the present studies). Goodwin *et al.* (2016) identify issues concerning the speed and cost of sequencing full-length genes, but also comment on the need to carefully design full-length primers to prevent PCR bias. Karst *et al.* (2018) have expanded on this with the development of experimental parameters to generate high-throughput, full-length 16S rRNA sequences without primer bias and an error rate of just 0.17% compared with 0.50% observed in non-full-length sequencing. The use of whole genome sequencing, whereby every gene is characterised, thereby allowing predictions of functionality, may also be considered.

8.1.1.2. Taxa representation

As undeniably useful for bacterial community profiling the 16S gene may be, it must be noted that bacteria often carry a varied number of copies of said gene. This means

that the number of reads associated with taxa may not be directly representative of bacterial abundance if said taxa carry multiple copies of the 16S gene (**Figure 8.1**). If, for example, six copies of a gene were identified, it could be assumed that all of these belong to each of six bacteria from the same taxon (**Panel A**). Alternatively, said bacteria could each carry three copies of the gene, meaning that six reads actually belong to two bacteria (**Panel B**). This difference in the number of gene copies can therefore skew the apparent relative abundances of bacteria.

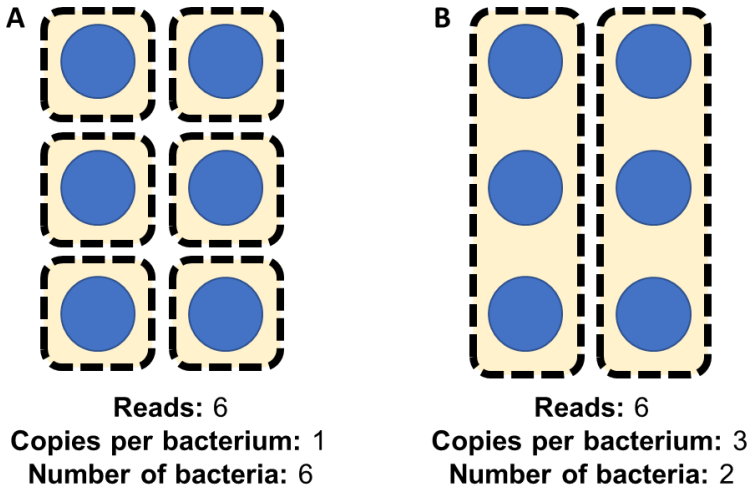

Figure 8.1 *Schematic diagram illustrating how gene copies (blue) can over-estimate the relative abundances of taxa (individual 'bacteria' are contained in dashed line).*

Kembel *et al.* (2012) have identified this as a problem and have subsequently devised a mathematical method of incorporating the number of gene copies into equations for the more accurate calculation of bacterial abundance in a complex ecosystem. They found their method to significantly reduce species richness when considering gene copies rather than solely relying on read numbers and suggest that their method reduces the likelihood that relative levels of highly abundant taxa are over-estimated. This is a consideration that should be made in future work to ensure that levels of bacterial taxa are accurately estimated and not over-represented by exploring raw OTU counts alone.

8.1.2. ^{1}H NMR

8.1.2.1. Metabolite restrictions

The protocol used to extract hepatic metabolites was suitable only for polar metabolites, as a result of the solvents used in extraction steps, so differences in non-polar metabolites may have been missed. Future work should consider adapting the protocol to include a non-polar solvent extraction (*e.g.* chloroform) to extract the lipid phase of tissue extracts. This would allow the exploration of some of the hypotheses suggested here, for example exploration of the relationships between *Lachnospiraceae*, *Coriobacteriaceae* and cholesterol metabolism, particularly given the hypothesised relationship between fat and the immune system.

8.1.2.2. Statistical analysis

Differences in concentrations of SCFA were only found coincidentally - the question therefore remains as to whether differences in other metabolites remain undetected. Due to the chemical complexity of the polar extracts examined here by NMR spectroscopy, it is not possible to assign identities for every peak: the benefit of OPLS-DA is that, in theory, the complexity of spectra is reduced to focus only on differentially represented peaks. It must be questioned, however, whether this is the most appropriate tool for analysing the metabolome if effects of treatment are being missed as a result of insufficient sensitivity within the model construction. In this case, manual deconvolution of the spectra to resolve individual peaks, and assignment of those peaks to metabolites, was far more precise and reliable than the OPLS-DA method. However, the sheer number of peaks makes it unfeasible for the manual approach to be used extensively. OPLS-DA, as a technique, can be exploited in finding molecules to investigate but is no match for a more rigorous manual inspection of the data. Future work should perhaps select common metabolites or metabolites of interest to focus on, which could be explored through alternative techniques including a calibrated form of gas chromatography (GC) or HPLC where the metabolic extract is compared with known reference compounds.

9. Conclusion

The aims of this project set out in Chapter 1 were

1. Explore the effects of management strategies and bird age on the caecal microbiome
2. Explore the effects of management strategies and bird age on the hepatic metabolome
3. Explore the relationships between bacterial taxa and hepatic metabolites

9.1. Aim 1: Explore the effects of management strategies on the caecal microbiome

The composition of the caecal microbiome of growing broilers was characterised through next-generation sequencing (NGS) and explored through analysis of variance (ANOVA) of alpha diversity, principal component analysis (PCA) of beta diversity and linear discriminant analysis effect size (LEfSe) of bacterial abundances.

Data consistently highlighted that, irrespective of study or treatment, measures of alpha diversity increased with bird age and that the general profile of the caecal microbiome, characterised by beta diversity, developed sequentially over time. It appears that measures of alpha and beta diversities were most sensitive to management strategy in young birds and that initial perturbations tended to dissipate as birds aged.

The relative abundances of bacterial taxa were consistently affected by management strategy, with evidence of sensitivity to medication in the forms of an antibiotic and probiotic, diet in terms of protein source and cereal base, and the supplementation of bedding with excreta from adult birds. Though not intentionally explored as an experimental variable, the use of bedding type (EnviroBed *vs* wood shavings) between

studies also appeared to have had a profound impact on the composition of the caecal microbiome. The effects of management are most likely an effect of nutrient availability and exposure to and ingestion of bacteria from the environment.

Whilst differences between treatments and studies existed, there was also notable variation between individual birds both within and between studies. This was not associated with bird performance; it is therefore suggested that birds raised under differing management strategies and environments exhibit different microbiomes, but that each enable the hosts to grow as effectively as one another. It is suggested that the microbiome develops both symbiotically and uniquely with each bird and that a definitive 'ideal' gut microbiome does not strictly exist.

It appears that the microbiome develops alongside individuals in a unique manner, but it is hypothesised that sudden perturbations can be induced by factors such as antibiotics, dietary changes or stress. This is potentially demonstrated in Chapter 5, where it is thought that differences in performance were a result of stress. Future work should therefore explore these relationships through frequent sampling around the point at which birds are exposed to such stressors.

9.2. Aim 2: Explore the effects of management strategies on the metabolic profile of the liver

The effects of management strategy on the hepatic metabolome were explored through ^{1}H NMR profiling and multivariate statistical analyses. Issues with the chosen statistical tests became evident in the late stages of data analysis, and not all differences in metabolite concentrations may have been highlighted. Though a comprehensive analysis was not possible, shifts in energy metabolism were suggested as a result of differences in glucose, glucose, NAD^{+}, succinate and isobutyrate. None of these metabolites were reflected in differences in performance,

suggesting that management strategy caused the metabolic profile of the birds to shift to meet energy requirements.

Short chain fatty acids (SCFA) were easily identifiable in NMR spectra and have been frequently linked to gut health: concentrations of acetate, butyrate, propionate and lactate were therefore explored, irrespective of results of statistical modelling. Concentrations of all SCFA significantly increased as each study progressed, suggesting heightened metabolic activity or merely an increase in feed intake with bird age. Neither antibiotic and probiotic treatment, nor the supplementation of bedding with excreta from mature birds altered the concentrations of SCFA within a timepoint. Of the two studies that explored the effects of diet, only cereal base had any significant effect. Wheat and maize diets comprise different concentrations and types of complex carbohydrates, suggesting that this is the reason behind differing concentrations of SCFA. Merely offering a different diet alone is insufficient to induce changes in SCFA production, as highlighted in Chapter 5 where the effects of protein sources were explored. Despite there being differences in FCR between treatments in this study, no relationships with SCFA concentrations were observed. This means that, despite the evidence that SCFA concentrations positively influence gut health, concentrations in the liver are not indicative of performance.

9.3. Aim 3: Explore the relationships between bacterial taxa and hepatic metabolites

Correlation analysis of caecal bacteria and hepatic SCFA concentrations suggested a plethora of relationships. Whilst these relationships cannot be fully explored under the current analytical techniques, associations between taxa and metabolites create many hypotheses for future work. Despite the observed differences in SCFA and bacterial abundance, neither were significantly associated with FCR. This suggests that

perturbations in the caecal microbiome that may alter the production of SCFA are not reflected in bird performance.

9.4. Future work

The work presented in this thesis provides a comprehensive overview of the caecal microbiome of the growing broiler chickens under a range of management strategies. There are still yet more management strategies employed by producers that have not been explored in these studies, such as prebiotics, the use of a probiotic without antibiotics, and the use of exogenous enzyme supplements, that could be investigated in future work. The use of enzymes would be of particular interest in terms of metabolic profiling, since the extent to which broilers can utilise their feed may be influenced by the use of said feed additives (Alagawany *et al.,* 2018).

Whilst no individual group of chickens appeared to perform better or worse than another, the lack of relationship between growth, the microbiome and the metabolome could be consolidated by developing a method of recording the performance of individual birds. FCR is calculated on a flock-basis and it is merely assumed that the birds selected for sampling were representative of their flock. There is evidence of differences in bacterial populations between individuals, meaning that true relationships with growth efficiency may have been missed. The main constraint is that of feed intake, since it is difficult to assess the mass of feed that an individual bird has consumed in a group housing system – this could be improved through individual housing of birds. It would also be beneficial to further explore the effects of Aviguard® on gut health, individual performance and bacterial populations along the gut to consolidate the work carried out in Chapter 3.

Whilst the analytical techniques employed during these studies were highly useful, they were not without their flaws. It is suggested that non-polar tissue extracts are

analysed alongside polar extracts, particularly since there have been suggestions of a gut-liver axis in terms of lipid metabolism (Konturek *et al.,* 2018). Similarly, the profiling of bacterial populations through next-generation sequencing does not give an absolute quantification of taxa, but merely a relative abundance. By utilising molecular techniques such as RT-PCR or qPCR, the associations between bacterial taxa and SCFA that are highlighted as potential targets for specific analysis (Chapter 7) could be explored. Target liver molecules such SCFA and bacterial metabolites including TMA and TMAO could also be explored more effectively with techniques such as calibrated GC. By targeting specific molecules, effects such as histological and immunological responses to varying concentrations could be explored. By then linking said effects to molecules and then to bacteria, said bacteria could be manipulated through management strategy to improve the health and productivity of broilers.

9.5. Conclusions

The data presented in this thesis give an overview of how management strategies may affect the composition and development of the microbiome and metabolome of growing broiler chickens. It is apparent that, whilst a plethora of treatment-associated differences exist, each bird is ultimately a unique system that utilises its environment in different ways. The lack of relationship between performance measured through FCR, the relative abundances of bacterial families and the concentrations of hepatic SCFA indicates that, whilst individual birds can appear different from one another, each can still be phenotypically similar. Whether this stands true when a stressor is administered to healthy birds, or whether the chosen analyses failed to identify every effect of treatment on the microbiome and metabolome, the work presented here provides a strong base which can be utilised to compose new hypotheses and explore specific relationships in more depth in future work with improved methods and a greater understanding of metagenomic-metabolomic relationships.

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Appendix 1: Study 1 (Antibiotic/Probiotic) LEfSe Results

Summary of all taxa that were significantly affected by the use of antibiotics and probiotics (AB, Linco-spectin days 1-3; AB+PRO, Lincos-spectin days 1-3 plus Aviguard® day 5), as a result of LEfSe analysis of bacterial abundances analysed through next-generation sequencing.

	Average abundance (%)			
Taxa	**Control**	**AB**	**LDA score**	***P*-value**
Day 4				
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae	12.1	0.3	5.082	0.025
Day 9				
Firmicutes.Clostridia.Clostridiales.Clostridiaceae	1.6	0.3	5.046	0.046
Firmicutes.Clostridia.Clostridiales	21.5	18.6	5.411	0.046
Firmicutes.Clostridia	21.5	18.6	5.403	0.046
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae	26.8	13.0	5.218	0.046
Day 15				
Bacteroidetes.Bacteroidia.Bacteroidales.Bacteroidaceae			3.938	0.011
Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae	0.05	0.21	4.050	0.028
Proteobacteria	1.29	5.52	4.307	0.018
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae	50.01	36.82	4.791	0.045
Bacteroidetes	0.08	0.39	3.673	0.018
Bacteroidetes.Bacteroidia	0.08	0.39	3.673	0.018
Bacteroidetes.Bacteroidia.Bacteroidales	0.08	0.39	3.673	0.018
Day 29				
Firmicutes.Clostridia.Clostridiales.Eubacteriaceae	0.00	0.01	3.411	0.029
Proteobacteria.Betaproteobacteria.Burkholderiales.Alcaligenaceae	0.02	9.04	4.661	0.011
Proteobacteria.Betaproteobacteria.Burkholderiales	0.02	9.04	4.671	0.013
Proteobacteria	3.33	9.20	4.457	0.014
Proteobacteria.Gammaproteobacteria	3.31	0.17	4.188	0.014
Proteobacteria.Betaproteobacteria	0.02	9.04	4.673	0.013
Firmicutes.Clostridia.Clostridiales.Christensenellaceae	0.02	0.18	3.168	0.021
Proteobacteria.Gammaproteobacteria.Enterobacteriales	3.30	0.17	4.187	0.014
Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae	3.30	0.17	4.189	0.014

	Average abundance (%)			
Taxa	**AB**	**AB+PRO**	**LDA score**	***P*-value**
Day 15				
Proteobacteria.Gammaproteobacteria.Enterobacteriales	19.16	29.27	3.695	0.019
Firmicutes.Clostridia.Clostridiales.Eubacteriaceae	0	0.01	4.297	0.006
Proteobacteria.Betaproteobacteria.Burkholderiales.Alcaligenaceae	1.28	5.86	5.124	0.010
Actinobacteria.Actinobacteria.Bifidobacteriales.Bifidobacteriaceae	0.04	0.07	3.901	0.018

Proteobacteria.Betaproteobacteria.Burkholderiales	0.01	26.57	5.086	0.010
Proteobacteria	5.52	27.07	5.113	0.011
Firmicutes.Clostridia.Clostridiales	83.17	61.43	5.043	0.011
Firmicutes	88.39	69.46	4.968	0.019
Proteobacteria.Gammaproteobacteria	1.27	0.49	3.697	0.019
Firmicutes.Clostridia	83.17	61.43	4.990	0.011
Actinobacteria.Actinobacteria	0.00	0.05	3.975	0.018
Proteobacteria.Betaproteobacteria	0.01	26.57	5.112	0.010
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae	36.82	24.51	4.780	0.019
Actinobacteria.Actinobacteria.Bifidobacteriales	0.00	0.05	3.923	0.018
Firmicutes.Clostridia.Clostridiales.Veillonellaceae	0.00	0.26	3.961	0.004
Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae	1.27	0.49	3.676	0.019
Unassigned	0.72	1.77	3.960	0.019
Day 29				
Firmicutes.Clostridia.SHA_98	<0.01	0.04	3.028	0.018
Firmicutes.Clostridia.Clostridiales.Veillonellaceae	0.01	0.19	3.272	0.020

	Average abundance (%)			
Taxa	**Control**	**AB+PRO**	**LDA score**	***P*-value**
Day 9			4.261	0.011
Firmicutes.Clostridia.Clostridiales.Peptococcaceae	0.01	0.08	3.561	0.021
Proteobacteria.Betaproteobacteria.Burkholderiales.Alcaligenaceae	<0.01	5.86	4.572	0.011
Actinobacteria.Coriobacteriia.Coriobacteriales	<0.01	<0.01	4.079	0.021
Firmicutes.Clostridia.SHA_98	<0.01	0.03	3.795	0.041
Proteobacteria.Betaproteobacteria.Burkholderiales	<0.01	5.87	4.592	0.013
Proteobacteria	13.17	25.03	4.348	0.027
Proteobacteria.Gammaproteobacteria	3.30	0.23	4.252	0.014
Proteobacteria.Betaproteobacteria	<0.01	5.87	4.596	0.013
Firmicutes.Clostridia.Clostridiales.Christensenellaceae	0.00	0.01	3.432	0.021
Actinobacteria.Coriobacteriia	<0.01	0.02	4.031	0.021
Firmicutes.Clostridia.Clostridiales.Veillonellaceae	0.00	0.73	3.555	0.007
Cyanobacteria	<0.01	<0.01	4.281	0.011
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae	<0.01	0.02	4.067	0.021
Proteobacteria.Gammaproteobacteria.Enterobacteriales	13.16	29.27	4.252	0.014
Cyanobacteria.4C0d_2	0.21	3.29	4.255	0.011
Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae	13.16	19.16	4.253	0.014
Unassigned	0.96	2.14	4.258	0.027
Day 15				
Firmicutes.Clostridia.Clostridiales.Eubacteriaceae	0.00	0.04	4.686	0.007
Proteobacteria.Betaproteobacteria.Burkholderiales.Alcaligenaceae	2.87	26.57	5.063	0.014
Actinobacteria.Actinobacteria.Bifidobacteriales.Bifidobacteriaceae	0.00	0.05	4.686	0.029
Proteobacteria.Betaproteobacteria.Burkholderiales	2.87	26.57	5.081	0.014

Proteobacteria	5.52	27.07	5.017	0.014
Firmicutes.Clostridia.Clostridiales	85.69	61.43	5.041	0.014
Firmicutes	93.29	68.46	5.097	0.014
Firmicutes.Clostridia	85.69	61.43	5.123	0.014
Actinobacteria.Actinobacteria	0.00	0.05	4.612	0.029
Proteobacteria.Betaproteobacteria	2.87	26.57	5.073	0.014
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae	50.01	24.51	5.122	0.014
Actinobacteria.Actinobacteria.Bifidobacteriales	0.00	0.05	4.667	0.029
Firmicutes.Clostridia.Clostridiales.Veillonellaceae	0.05	0.26	3.869	0.041
Unassigned	0.47	1.76	4.116	0.014
Day 29				
Cyanobacteria.4C0d_2.YS2	0.21	3.29	4.261	0.011
Firmicutes.Clostridia.Clostridiales.Peptococcaceae	0.01	0.08	3.561	0.021
Proteobacteria.Betaproteobacteria.Burkholderiales.Alcaligenaceae	0.03	8.42	4.572	0.011
Actinobacteria.Coriobacteriia.Coriobacteriales	<0.01	0.02	4.079	0.021
Firmicutes.Clostridia.SHA_98	0.01	0.04	3.795	0.041
Proteobacteria.Betaproteobacteria.Burkholderiales	0.03	8.42	4.592	0.013
Proteobacteria	3.33	8.65	4.348	0.027
Proteobacteria.Gammaproteobacteria	3.30	0.23	4.252	0.014
Proteobacteria.Betaproteobacteria	0.03	8.42	4.596	0.013
Firmicutes.Clostridia.Clostridiales.Christensenellaceae	0.02	0.15	3.432	0.021
Actinobacteria.Coriobacteriia	<0.01	0.02	4.031	0.021
Firmicutes.Clostridia.Clostridiales.Veillonellaceae	<0.01	0.19	3.555	0.007
Cyanobacteria	0.21	3.29	4.281	0.011
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae	<0.01	0.02	4.067	0.021
Proteobacteria.Gammaproteobacteria.Enterobacteriales	3.30	0.23	4.252	0.014
Cyanobacteria.4C0d_2	0.21	3.29	4.255	0.011
Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae	3.30	0.23	4.253	0.014
Unassigned	1.73	5.02	4.258	0.027

Appendix 2: Study 2 (Protein Source) LEfSe Results

Summary of all taxa that were significantly affected by protein source (FM, fishmeal; SAA, soya supplemented with synthetic lysine/methionine), as a result of LEfSe analysis of bacterial abundances analysed through next-generation sequencing.

	Average abundance (%)			
Taxa	**FM**	**SAA**	**LDA score**	***P*-value**
Firmicutes.Erysipelotrichi.Erysipelotrichales	1.66	3.43	5.247	0.009
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae	11.32	16.91	5.791	0.009
Firmicutes.Clostridia.Clostridiales	62.18	79.16	6.037	0.016
Firmicutes.Bacilli.Lactobacillales.Enterococcaceae	0.04	0.18	5.704	0.028
Firmicutes.Clostridia	62.18	79.16	6.426	0.016
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae	27.29	32.46	5.996	0.047
Firmicutes.Erysipelotrichi.Erysipelotrichales.Erysipelotrichaceae	1.66	3.43	5.247	0.009
Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae	1.62	4.02	5.283	0.047
Firmicutes.Erysipelotrichi	1.66	3.43	5.247	0.009
Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae	4.98	0.02	5.071	0.008
Firmicutes.Bacilli.Bacillales	0.20	0.51	5.402	0.047

Appendix 3: Study 3 (Cereal Base) LEfSe Results

Summary of all taxa that were significantly affected by the dietary cereal base (wheat/maize), as a result of LEfSe analysis of bacterial abundances analysed through next-generation sequencing.

	Average abundance (%)			
Taxa	**Wheat**	**Maize**	**LDA score**	***P*-value**
Day 15				
Actinobacteria.Coriobacteriia	0.07	0.01	4.226	0.003
Actinobacteria.Coriobacteriia.Coriobacteriales	0.07	0.01	4.226	0.003
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae	0.07	0.01	4.226	0.003
Bacteroidetes.Bacteroidia.Bacteroidales	0.03	0.92	4.664	0.003
Bacteroidetes.Bacteroidia.Bacteroidales.Odoribacteraceae	<0.01	0.86	4.366	0.004
Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae	<0.01	0.04	4.487	0.001
Firmicutes	97.80	70.16	4.855	0.027
Firmicutes.Bacilli.Bacillales	0.08	0.39	4.121	0.028
Firmicutes.Bacilli.Bacillales.Bacillaceae	0.01	0.07	3.813	0.024
Firmicutes.Bacilli.Bacillales.Staphylococcaceae	0.02	0.01	4.045	0.003
Firmicutes.Bacilli.Gemellales	<0.01	<0.01	6.037	0.015
Firmicutes.Bacilli.Lactobacillales.Aerococcaceae	<0.01	<0.01	5.885	0.042
Firmicutes.Bacilli.Lactobacillales.Enterococcaceae	0.02	0.02	3.877	0.010
Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae	5.02	2.15	6.103	0.003
Firmicutes.Bacilli.Lactobacillales.Leuconostocaceae	0.02	<0.01	4.814	0.002
Firmicutes.Clostridia	88.95	62.53	7.312	0.003
Firmicutes.Clostridia.Clostridiales	88.95	62.53	6.171	0.015
Firmicutes.Clostridia.Clostridiales.Christensenellaceae	<0.01	0.09	4.094	0.009
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae	53.51	18.99	7.117	0.003
Firmicutes.Clostridia.Clostridiales.Mogibacteriaceae	0.03	0.01	4.411	0.003
Firmicutes.Clostridia.Clostridiales.Peptococcaceae	<0.01	0.01	6.052	0.004
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae	28.45	21.94	6.771	0.003
Firmicutes.Erysipelotrichi	2.66	3.49	5.826	0.003
Firmicutes.Erysipelotrichi.Erysipelotrichales	2.66	3.49	5.826	0.003
Firmicutes.Erysipelotrichi.Erysipelotrichales.Erysipelotrichaceae	2.66	3.49	5.826	0.003
Proteobacteria	1.70	0.94	5.570	0.003
Proteobacteria.Gammaproteobacteria	1.70	0.94	5.570	0.003
Proteobacteria.Gammaproteobacteria.Enterobacteriales	1.70	0.94	5.570	0.003
Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae	1.70	0.94	5.570	0.003
Tenericutes	0.16	26.98	5.603	0.003
Tenericutes.Mollicutes	0.16	26.98	5.603	0.003
Tenericutes.Mollicutes.RF39	0.16	26.98	5.603	0.003
Day 22				
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae	36.03	11.49	6.718	0.016
Bacteroidetes.Bacteroidia.Bacteroidales.Odoribacteraceae	<0.01	8.34	5.248	0.008
Firmicutes.Clostridia.Clostridiales.Dehalobacteriaceae	<0.01	0.04	6.357	0.005

Firmicutes.Clostridia.Clostridiales	83.17	61.61	6.282	0.028
Actinobacteria.Coriobacteriia.Coriobacteriales	0.04	0.01	4.199	0.047
Proteobacteria	1.96	0.22	5.401	0.009
Firmicutes.Clostridia.Clostridiales.Clostridiaceae	0.54	0.19	4.850	0.016
Firmicutes.Bacilli.Lactobacillales	4.45	2.49	5.627	0.047
Firmicutes	90.02	66.54	5.716	0.047
Proteobacteria.Gammaproteobacteria	1.96	0.22	5.401	0.009
Firmicutes.Clostridia	83.17	61.61	7.015	0.028
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae	27.17	25.56	6.496	0.016
Bacteroidetes.Bacteroidia	0.04	8.46	5.242	0.009
Actinobacteria.Coriobacteriia	0.04	0.01	4.199	0.047
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae	0.04	0.01	4.199	0.047
Proteobacteria.Gammaproteobacteria.Enterobacteriales	1.96	0.22	5.401	0.009
Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae	<0.01	0.03	6.798	0.005
Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae	1.96	0.22	5.401	0.009
Bacteroidetes	0.04	8.46	5.242	0.009
Day 35				
Firmicutes.Clostridia.Clostridiales.Gracilibacteraceae.Gracilibacter	<0.01	<0.01	3.423	0.022
Gammaproteobacteria.Enterobacteriales	2.48	0.22	4.109	0.004
Cyanobacteria.4C0d_2	0.04	1.21	3.699	0.025
Firmicutes.Clostridia.Clostridiales.Dehalobacteriaceae	0.03	0.08	2.844	0.025
Cyanobacteria.4C0d_2.YS2	0.04	1.21	3.699	0.025
Cyanobacteria	0.04	1.21	3.700	0.025
Firmicutes.Clostridia.Clostridiales.Gracilibacteraceae	<0.01	<0.01	3.418	0.022
Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae	2.48	0.22	4.109	0.004
Gammaproteobacteria	2.48	0.22	4.109	0.004
Firmicutes.Clostridia.Clostridiales.Christensenellaceae	0.12	0.32	3.079	0.025

Appendix 4: Study 4 (Bedding Supplementation) LEfSe Results

Summary of all taxa that were significantly affected by bedding (clean shavings/supplemented with excreta from mature birds), as a result of LEfSe analysis of bacterial abundances analysed through next-generation sequencing.

	Average abundance (%)			
Taxa	**Control**	**Supplemented**	**LDA score**	***P*-value**
Day 15				
Firmicutes.Bacilli.Bacillales	0.11	0.37	3.187	0.025
*Firmicutes.Bacilli.Bacillales.*Unknown *Bacillales*	0.08	0.29	3.115	0.034
Actinobacteria.Coriobacteriia	0.08	0.01	3.156	0.006
Firmicutes.Clostridia	90.04	75.99	4.820	0.037
UnknownBacteria	0.11	0.74	3.573	0.004
Firmicutes.Bacilli.Bacillales.Bacillaceae	0.01	0.08	3.249	0.020
Firmicutes.Bacilli.Lactobacillales.Enterococcaceae	0.02	<0.01	3.816	0.037
Tenericutes.Mollicutes.RF39	0.22	12.40	4.767	0.004
Tenericutes	0.22	12.40	4.767	0.004
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae	53.17	24.35	4.497	0.010
Unknown *Firmicutes*	<0.01	0.01		0.037
Firmicutes.Clostridia.Clostridiales	90.04	75.99	4.820	0.037
Firmicutes.Bacilli.Lactobacillales.Leuconostocaceae	0.02	<0.01	4.132	0.016
*Firmicutes.Clostridia.Clostridiales.*Unknown *Clostridiales*	5.84	24.54	4.954	0.016
Actinobacteria	0.08	0.01	3.177	0.016
Tenericutes.Mollicutes	0.22	12.40	4.767	0.004
Firmicutes.Bacilli.Bacillales.Staphylococcaceae	0.02	0.01	4.026	0.025
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae	0.08	0.01	3.156	0.006
Actinobacteria.Coriobacteriia.Coriobacteriales	0.08	0.01	3.156	0.006
Day 35				
Cyanobacteria.4C0d_2.YS2	0.01	0.32	3.422	0.010
Firmicutes.Clostridia.Clostridiales.Dehalobacteriaceae	0.02	0.14	3.844	0.010
Bacteroidetes.Bacteroidia.Bacteroidales.Odoribacteraceae	3.31	7.77	4.367	0.016
Bacteroidetes	3.32	7.92	4.378	0.016
Firmicutes.Clostridia.Clostridiales.Peptostreptococcaceae	2.56	0.54	3.997	0.010
Firmicutes.Clostridia.Clostridiales.Eubacteriaceae	0.01	0.10	3.885	0.016
Firmicutes.Clostridia.Clostridiales.Christensenellaceae	0.08	0.30	3.705	0.004
Bacteroidetes.Bacteroidia	3.32	7.92	4.378	0.016
Cyanobacteria	0.01	0.32	3.464	0.010
Bacteroidetes.Bacteroidia.Bacteroidales	3.32	7.92	4.378	0.016