Identifying Novel Biomarkers of Broiler Performance and Gut Health

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ABSTRACT

Identifying biomarkers of intestinal health in commercial poultry, particularly broilers, holds immense promise for enhancing poultry welfare and productivity. This study leverages insights from the microbiome, mycobiome, metabolome, and immune system to attempt to identify novel biomarkers for broiler gut health and performance.

Commercial broilers of different ages, sexes, and genotypes were examined, employing genomic DNA extraction from faeces and other gastrointestinal contents, shotgun metagenomic analyses, ITS1 rRNA barcoding, untargeted ^{1}H-NMR of poultry faeces and serum and immunological analyses. Biological efficiency parameters, including body weight, growth, and feed conversion ratio, were recorded. Small intestine morphological development was assessed.

Results highlight optimised methods for identifying microbiome and serum-sourced biomarkers. A screening protocol for phytase activity in chick intestinal microbiome fed a phytase-free diet was developed. Faeces and serum proved to be reproducible sample types, showing remarkable consistency in the faecal microbiome composition of broilers across different genotypes and collection dates. Arabinitol and trimethylamine metabolism emerged as potential metabolome markers in both faeces and serum. Differences in *Lactobacilli* population composition, *Alistipes* presence, and *Akkermansia muciniphila* abundance in faeces were associated with improved performance. The discovery of the yeast *Diutina*, particularly in the context of phosphorus utilisation by the mycobiome, was linked to performance. Positive trends between microbiome and mycobiome diversity in chicks suggest the mycobiome could play a role in gut microbiome maturation and stability. Core members of the broiler faecal mycobiome across life stages were identified including species of *Alternaria*, *Nakazawaea* and *Leucosporidium*. Activation of natural antibodies during the broiler life cycle was observed. The microbiome community structure and metabolome were established. Comparisons were drawn between genotypes, performance, and growth stage.

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LIST OF ABBREVIATIONS

ABBREVIATION	DEFINITION
^{1}H-NMR	Proton Nuclear Magnetic Resonance
AGP	Antimicrobial Growth Promoter
AMR	Antimicrobial Resistance
ASV	Amplicon Sequence Variant
BHI	Brain Heart Infusion
BWG	Body Weight Gain
CV	Coefficient of Variance
DPH	Days Post Hatching
dsDNA	double-stranded DNA
ELISA	enzyme-linked immunosorbent assay
FCR	Feed Conversion Ratio
FDR	Fale Discovery Rate
GALT	Gastrointestinal Associated Lymphoid Tissue
GI	Gastrointestinal
H&E	haematoxylin and eosin
HILIC-MS	Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry
Ig	Immunoglobulin
IQR	Interquartile Range
ITS	Inter Transcribed Region
JIC	John Innes Centre
KLH	Keyhole limpet hemocyanin
MM-	Minimal Media without phytase
MMPa	Minimal media with phytase
MRS	De Man–Rogosa–Sharpe
MSC	microbial safety cabinet
NAB	Natural Antibody
NBF	Neutral Buffered Formalin
OD	Optical Density
OTU	Operational Taxonomic Unit
Pa	Phytic Acid

PCOA	Principle Co-ordination Analysis
PCR	Polymerase Chain Reaction
QC	Quality Control
QIB	Quadram Institute Bioscience
RB+C	Rose-Bengal with Chloramphenicol
rRNA	Ribosomal RNA
SCFA	Short Chain Fatty Acid
SD	Standard Deviation
SI	Small Intestine
sPLSDA	Sparse Partial Least Squared Discriminant Analysis
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
TSP	Trimethylsilylpropanoic
VLP	Virus-like particle
WHO	World Health Organisation
YPD	Yeast Peptone Dextrose

SYMBOLS AND UNITS

SYMBOL/UNIT	DEFINITION
%	Percentage
<	Less than
>	Greater than
±	Error range
µg	Microgram
µl	Microliter
µm	Micron
µm	Micromolar
Bp	Base pairs
FTU	Phytase units
G	Gram
Gb	Gigabase pairs
Kb	Kilobase
m/s	Metre per secnd
mg	Milligram
min	Minute(s)
mL	Millilitre
ng	Nanogram
nm	Nanometers
^{o}C	Celsius
PH	Potential of Hydrogen
ppm	Parts per million
R^2	Coefficient of correlation
s	Seconds
sino	Signal to Noise Ratio
u/µl	Units per microlitre
x g	Relative Centrifugal Force (RCF) or g force
α	Alpha
β	Beta

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My deepest gratitude goes to my supervisory team, who endured my endless chicken puns and provided unwavering support during challenging times. Professor Arjan Narbad, my primary supervisor, recognised my potential, pushed me to excel, and offered invaluable support for both my research and personal growth. Dr Melinda Mayer, my secondary supervisor, provided a wealth of knowledge, insightful questions, and innovative ideas that were instrumental in achieving my research objectives. Her continuous patience, compassion, and emotional support were truly invaluable, shaping me into the scientist I aspire to be.

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maintain a life beyond my research and affirming my worth beyond any experimental outcome.

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Lastly, to my husband, Marcel. Your steadfast support has been the foundation of my journey. This achievement is as much yours as it is mine. Thank you for keeping me grounded, nourished, loved, and encouraged throughout this adventure. I am forever grateful for your presence in my life.

DEDICATION

To my 7-year-old self, whose boundless love for animals, particularly my beloved pet chicken, Princess Zara Fidget (pictured below), inspired a lifelong passion. With the innocence of childhood, the unyielding curiosity, and the support of those closest to me, I have achieved a dream that once seemed unattainable. This PhD is dedicated to the spirit of that young child, to my journey of growth and self-discovery, and to the well-being of chickens around the world.

"Don't try to convey your enthusiasm for chickens to anyone else" – The Hen, and Appreciation by E.B. White, 1944

BEYOND THE BENCH: Broadening Perspectives and Enhancing Impact

This thesis represents the primary outcome of my research journey, yet it is just one facet of my comprehensive PhD experience. I am grateful for the opportunities provided by the communications team, which allowed me to disseminate my passion and gain insight into its broader societal and governmental impact. I actively engaged in public outreach through workshops, talks, radio features, podcasts, articles, videos, and stand-up comedy, aiming to inspire interest in science and advocate for diversity. Additionally, I've contributed to ecological workshops, panel discussions, and mentoring programs, striving to promote inclusivity and decolonisation within the scientific community. My multifaceted involvement reflects a commitment to communication, education, and advocacy across various platforms and initiatives.

Academic engagement took various forms, including conference presentations at the International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health and Society of Feed Technologists conferences, securing first place in a poster competition at QIB, and delivering guest lectures at prestigious institutions such as Johns Hopkins Medicine and Anglia Ruskin University on the interdisciplinary concept of One Health from a zoologist's perspective.

Engaging with industry was not only a mandatory placement but also temporary employment as a laboratory technician at Aviagen, along with receiving the Young Persons Poultry Initiative award to attend the Egg & Poultry Industry Conference.

Government engagement included serving as a scientific advisor for Nuffield Farmers Scholarships and delivering a talk to former science minister George Freeman MP. I also ventured into innovation and entrepreneurship through my biotech start-up "Zoolutions," earning recognition and delivering presentations at Anglia LLP Partnerships enterprise events. Community engagement was integral, with contributions to the Quadram Student Forum as engagement officer and organising the first recreational student retreat.

I have organised public engagement workshops, including "What’s Up with the Chicken’s Gut?" at Norwich Science Festival and "What the Cluck?" at Kid’s County Food and Farming festival. Additionally, I created a “Chickens or Dickens” webcast and quiz for the

Festival of Nature. I engaged with young people through talks at Wellcome Connecting Science's “My STEMM Future” and John Innes Centre’s “Women of the Future,” where I shared my journey and experiences in science to inspire more young women. I am fortunate to have been featured on radio programs such as BBC Radio 4 Farming Today and BBC Radio Norfolk's Stephen Bumfrey’s “Appliance of Science,” as well as hosting my own weekly live show on More Muzic Radio called “Nature+Nonesne.” As a podcast guest on various platforms like “Planted: Unearthed,” “Over the Farmgate,” and “Black Women in Science Podcast” by the Black Women in Science Network, I've shared insights into agriculture and sustainability.

My work has been featured in articles by publications including Eastern Daily Press, Woman-ology, and Gal-Dem. I have engaged creatively through video projects like “Day in the Life of a Zoologist” for the BioDiverse Project and created a parody song and music video titled “Shout Out to My Eggs” for Global Science Show. I performed stand-up comedy at Zoo-LOL-ogy twice and written blogs on topics like “5 things about chicken health” and my capabilities for the British Dyslexia Association. I participated in panel talks on “Women in STEM” at Norwich Science Festival, “Hear my Voice” for University of Glasgow, and UEA Women in Science Education Black History Month, and served as a food champion for “I’m a Scientist.” I'm particularly proud of my involvement with the REED Ecological Network, where I served as vice-chair and committee member. Notably, I've facilitated workshops for the British Ecological Society, including sessions on “Effective Allyship in Ecology” and “Decolonising the Curriculum” at the Ecology Across Borders international conference. I've taught and mentored undergraduates at the British Ecological Society undergrad summer school, covering topics such as “Social Media Skills” and “Decolonising Ecology.” I've also consulted and facilitated events like the Natural History Museum's “MIND BODY SPACE: Exploring Joy and Justice in Nature.”

I was recognised with the UEA Student Communications Award and ARU Alumni Sustainability Champion and featured scientist for British Science Week. My journey has been marked by diverse and impactful communication endeavours that I am immensely proud of.

These activities have broadened my perspective on the diverse audiences impacted by my research, emphasising the importance of making science accessible and relevant to a wide range of stakeholders, including industry, government, and society at large.

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE IMPORTANCE OF POULTRY

Chickens comprise three-quarters of all land-reared animals, and they reign supreme among birds and within the broader poultry collective, encompassing turkeys, geese, and ducks. The domestication of chickens dates back 7,000 to 10,000 years to the red junglefowl of Southeast Asia. Initially revered in rituals, human fascination with chickens dates back to 6,000 BC, as evidenced by archaeological discoveries in China (Hata et al., 2021). They were dietary staples for Ancient Egyptians, sources of philosophical inspiration for Aristotle, and catalysts for advancements in human physiology since the 16th century. Throughout the 20th century, chickens played pivotal roles in Nobel Prize-winning cancer biology research and the validation of Mendelian Genetics.

The chicken marked a milestone as the first bird to have its entire genome sequenced in the early 21st century. Poultry is the primary global source of animal protein, holding significant economic importance. Beyond their scientific and societal contributions, chickens hold considerable economic value, with a ratio of four chickens to every human on Earth (Nations, 2019), underlining their significance industrially and as a species.

As of 2019, chickens represent the leading global source of protein, surpassing pigs despite yielding less meat per animal (Aho, 2019). This shift is partly attributed to the impact of African swine flu. Still, ongoing consumer preferences for poultry meat deemed a more sustainable option amid contemporary climate crises, are expected to sustain this trend.

1.2 MANAGING POULTRY GUT HEALTH TO IMPROVE WELFARE AND SUSTAINABILITY

Managing poultry intestinal health is paramount for bolstering welfare, productivity, and food safety. The microbial community within the chicken's gastrointestinal (GI) tract plays a pivotal role in influencing performance, health, and sustainability, thus guiding breeding strategies, diet formulations, and veterinary interventions. Recent technological breakthroughs have amplified the recognition of how the microbiome and associated analyses can revolutionise the poultry industry.

Leveraging these advancements will fortify research endeavours to identify new biomarkers and interventions to enhance poultry welfare and production efficiency. Poultry intestinal health encompasses the functionality of digestive and immunological systems,

profoundly impacting various physiological responses. Coupled with a growing emphasis on animal welfare and escalating consumer demands for sustainability, chickens have emerged as the primary global protein source.

Consumer apprehensions have catalysed industry transformations geared towards enhancing poultry welfare, especially amidst the escalating global challenge of AMR. The intestinal microbiome contributes to nutrient digestion, vitamin and metabolite production, and immune system fortification. As antimicrobial usage in poultry farming undergoes reductions, identifying robust intestinal health biomarkers can significantly reduce mortality rates and veterinary interventions, benefiting the industry.

Advanced technologies are rapidly infiltrating commercial agriculture (Ricke et al., 2022a). Bioinformatic analyses play a pivotal role in microbiome research by facilitating the interpretation of vast sequence data generated from sequencing of microbial communities. These analyses enable researchers to characterise microbial communities' taxonomic composition, functional potential, and dynamics across diverse environments. By employing different computational tools and utilising various reference databases, bioinformatics allows for identifying microbial species, predicting their metabolic pathways, and inference of interactions within microbial communities. Moreover, bioinformatic approaches enable the comparison of microbiome data across different samples or experimental conditions, aiding in discovering microbial biomarkers associated with broiler performance or gut health. There is a growing appreciation for utilising bioinformatics to monitor and manage poultry intestinal microbiome and nutrition (Olson et al., 2022). Microbiome analysis is becoming an increasingly accessible and accepted method for managing flock health and production (Ricke et al., 2022b).

Concurrently, our understanding of the relationship between a chicken's intestinal microbiome and welfare is expanding. A recent study sought to establish connections between welfare and broiler microbiota, employing Welfare Quality™ protocols to gauge animal welfare status in conjunction with caecal microbiome composition and *Campylobacter* colonisation using 16S ribosomal RNA (rRNA) gene metabarcoding (Di Marcantonio et al., 2022). Broilers with high welfare exhibited higher microbial richness, with Bacteroidetes as the dominant phylum, while low-welfare animals showed an increased abundance of *Proteobacteria* (Di Marcantonio et al., 2022). Whilst this data showed an association but does not demonstrate a causal link between welfare and gut microbiome composition.

The perceived conflict between good welfare and efficient farming is evident in agricultural practices. For instance, in response to public pressure, the industry transitioned to open housing following the discontinuation of battery cages. However, open housing can exacerbate disease spread among flocks due to chickens' practice of coprophagy (Richards-Rios et al., 2020b). While economic arguments centred on services cannot replace moral or aesthetic considerations, they can complement and reinforce these concepts. Improvements in meat quality and reductions in mortality rates have economic implications for individual farmers and mitigate the risk of antibiotic usage and zoonotic outbreaks (Lillehoj and Lee, 2012). Such interdisciplinary considerations align with the World Health Organisation's (WHO) One Health initiative, which promotes the holistic integration of human, animal, and environmental health for mutual benefit.

The poultry industry is in constant flux, adapting swiftly to cater to consumer preferences and adhere to evolving local legislation and regulations. Globally, there's mounting pressure on livestock farming to enhance efficiency and sustainability to meet the needs of a growing population while mitigating the impacts of global warming (Walters et al., 2019). The rising public concern over animal welfare is added to the complexity of addressing modern society's poultry needs. Many believe that intensive commercial poultry farming contributes to welfare issues like lameness and obesity (Sommerfeld et al., 2020).

1.2.1 Current Markers of Poultry Gut Health

The discovery of a biomarker holds promise for managing the broiler microbiome and monitoring bird health, welfare, and performance, which is crucial for enhancing productivity and sustainability amidst population growth and climate challenges (Ducatelle et al., 2018b). Age serves as a recognised host factor shaping the microbiome composition in chickens, while different biogeographical locations within the broiler GI tract harbour distinct microbiome compositions (Jurburg et al., 2019). The microbiome and immune system present promising avenues for identifying novel biomarkers for poultry intestinal health. Integrating these biomarkers into broiler intestinal health management could significantly impact breeding strategies, diet formulation, and veterinary interventions. Ensuring the detectability and robustness of biomarkers is paramount, given the substantial influences of host and environmental factors (Robinson et al., 2022a, Walters et al., 2019).

No universal signature of dysbacteriosis in any species has yet been specified. The aetiology and definition of dysbacteriosis itself are undefined, although some microbial signatures have been identified in humans and laboratory models using proteobacteria abundance (Brüssow, 2020, Olesen and Alm, 2016). In human studies, expression of MDR1 in the intestinal epithelium was associated with inflammation; unfortunately, it is still unclear whether this is relevant to poultry (Haritova et al., 2010). Similarly, myeloid protein 1 (MIM1) has been identified as an inflammation marker in humans, detected within faeces using liquid chromatography and mass spectometry (De Meyer et al., 2019). Known for their expression on myeloid cells, all secretory granules of heterophiles contain considerable amounts of MM1, which may represent a helpful intestinal biomarker of inflammation (De Meyer et al., 2019). Ovotransferrin was identified using proteomics, single-dimensional gel electrophoresis and LC-MS as a marker in chicken faeces with induced necrotic enteritis or coccidiosis (Goossens et al., 2018). A biomarker capable of detecting abnormalities of intestinal health, not specific intestinal disease, would be of greater benefit.

The primary bile acids prevalent in poultry, sodium taurochenodeoxycholate and sodium taurocholate, are conjugated with taurine. Elevated bile acid deconjugation may signify an increase in bacterial species with this capability, impacting microbial nutrient availability and thus influencing microbiome composition (Bailey, 2010). Dietary supplementation with taurine has been linked to detrimental effects on broilers' intestinal growth and mucosal structure, leading to higher levels of toxic bile acids (Huang et al., 2014). Additionally, chickens fed a taurine-enriched diet exhibited reduced intestinal mucus layer thickness, impaired villus development in the jejunum and ileum, decreased jejunum weight, and elevated serum total bile acid levels (Yuan and Wang, 2010).

1.3 BIOGEOGRAPHY OF THE CHICKEN GASTROINTESTINAL TRACT

The GI tract of chickens exhibits compartmentalisation, with distinct ecological and functional differences across its sections. Locations of the broiler GI tract are known to have different microbiome compositions. The spatial variation of the microbiome is pertinent to the functional and physiological differences within the intestine.

The crop (Fig. 1.1A) functions as an outgrowth of the oesophagus, serving as a reservoir for ingested food and water before proceeding through the rest of the tract. This

anatomical feature allows avian species to consume a large quantity of food before seeking a safe location for energy-intensive digestion (Yeoman et al., 2012). Despite the presence of oral amylase, minimal digestion occurs within the crop, leading to the transient or environmental nature of the associated microbiota (Shang et al., 2018). With approximately 10^8-10^9 bacteria per gram, lactic acid-producing bacteria dominate, aiding lactate fermentation, including *Lactobacillaceae*, *Bifidobacteria* and *Enterobacteria* (Gong et al., 2007).

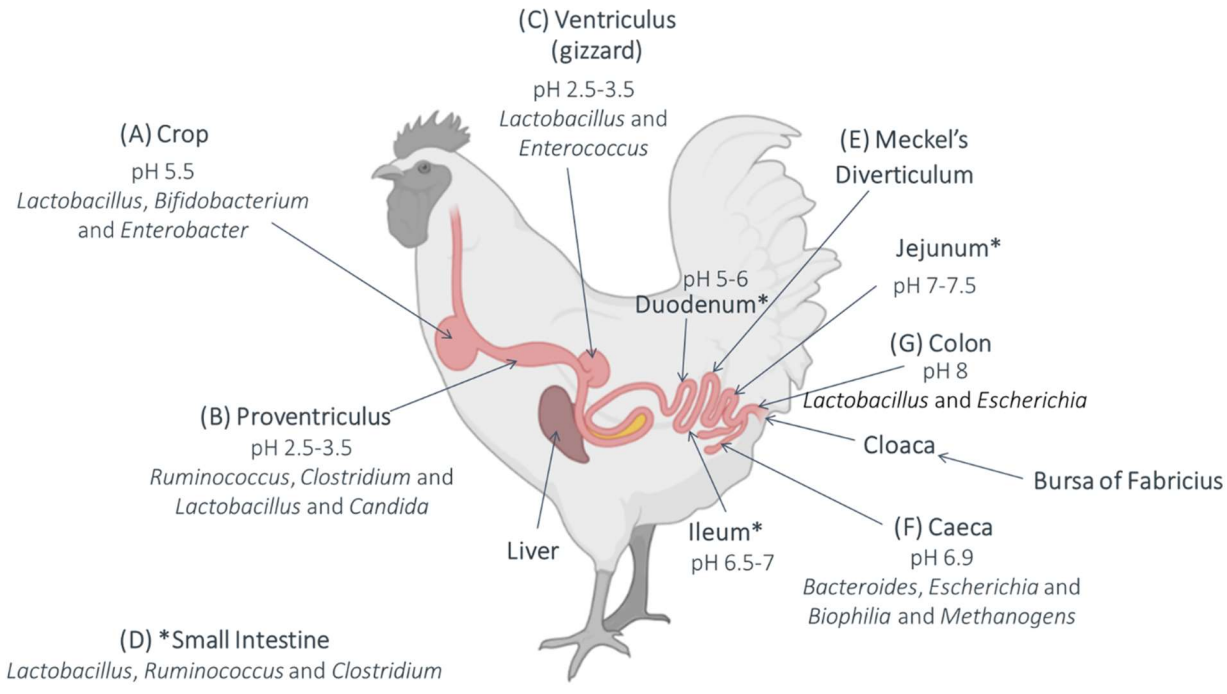

Figure 1.1 Diagram of the chicken GI tract demonstrating the compartments and varying pH, (Glendinning et al., 2019, Gong et al., 2007, Yeoman et al., 2012, Shang et al., 2018).

To initiate digestion and facilitate mineral solubilisation (e.g., calcium), the proventriculus (Fig. 1.1B) and ventriculus (Fig. 1.1C) represent the most acidic segments of the intestinal tract, with pH levels ranging from 2.5 to 3.5, subject to host physiological factors. The proventriculus initiates genuine digestion, hosting approximately 10^7-10^8 bacteria per gram, with prevalent species including those belonging to the genera *Ruminococcus*, *Clostridium*, and *Lactobacillus* (Mohd Shaufi et al., 2015), alongside detectable fungi like *Candida* and *Aspergillus* (Shang et al., 2018). Unique to avian species, the ventriculus, or gizzard, engages in the mechanical breakdown of ingested feed, incorporating previously secreted chemicals. It boasts a robust lining to shield muscles from acidity. While foraging, chickens naturally ingest small stones, partially softened by the

acidic environment of the proventriculus and ventriculus, aiding in their further fragmentation into smaller pieces. These stones persist, facilitating mechanical digestion akin to mammalian teeth. Grit supplementation is often necessary however feeding chickens whole grain diets reduce the need for grit as the whole grains act like grit in the grinding process (Abdollahi et al., 2013). *Lactobacillus* and *Enterococcus* are among the most frequently identified microorganisms in the ventriculus (Gong et al., 2007, Yeoman et al., 2012, Shang et al., 2018). Another crucial function of the gizzard is to regulate the rate at which feed passes into the small intestine (SI) (Svihus, 2014); large feed particles or stones stimulate gizzard activity, slowing the passage of feed into the SI and thereby enhancing digestion. Additionally, the gizzard aids in the precipitation and pre-digestion of proteins through the action of acid and pepsin, which is essential for efficient digestion.

The small intestine (Fig. 1.1D) is the site of the majority of chemical breakdown of the feed and absorption of nutrients and is comprised of the duodenum loop followed by the jejunum and ileum. The pancreas secretes bicarbonate and digestive enzymes to neutralise the hydrochloric acid from the proventriculus, elevating the pH to 5-6 (Glendinning et al., 2019). Bile acid, produced by the liver and released from the gall bladder at the duodenum site, is crucial for digesting fat-soluble vitamins and lipids while also aiding in the production and dispersion of short-chain fatty-acids (SCFA) in the lower GI tract (Yeoman et al., 2012). The SI is primarily populated by *Firmicutes*, with dominant species including *Lactobacillus*, *Ruminococcus*, and *Clostridium*, alongside some *Enterococcus* and *Escherichia* (Glendinning et al., 2019). Most studies on the SI focus on the ileum, which harbours approximately 10^8-10^9 bacteria per gram (Shang et al., 2018). *Candidatus arthromitus* is specific to the jejunum and ileum, while the composition of *Lactobacillus* in the SI serves as an indicator of microbiome development (Richards-Rios et al., 2020a, Pokorná et al., 2019). For instance, *Ligilactobacillus (*formerly *Lactobacillus*) *salivarius* in the ileum suggests a mature, stable microbiome (Pokorná et al., 2019).

At hatching, the yolk sac is located inside the chick. Prior to hatching, the yolk is enveloped via the navel (Kaspers and Schat, 2012, Feye et al., 2020). Absorption of the yolk occurs through the yolk stalk, which subsequently develops into Meckel's diverticulum. Meckel's diverticulum (Fig. 1.1E) marks the transition from the end of the jejunum to the beginning of the ileum, signalling the commencement of the distal gut.

The caeca (Fig. 1.1F) plays a pivotal role in regulating the GI microbiome through nutrient recycling and fermentation (Ocejo et al., 2019b). The caeca harbour the most

dense and diverse microbiome among all intestinal sections, with an impressive population ranging from 10^{10} to 10^{11} bacteria per gram (Shang et al., 2018). This microbial community includes *Bacteroides*, *Escherichia*, and *Bilophilia*, alongside various *Methanogens* (Ocejo et al., 2019a). The presence of *Alistipes* is particularly indicative of a stable, mature caecal microbiome (Richards et al., 2019).

The large intestine (colon) (Fig. 1.1G) serves as the site for the final reabsorption of water. Inadequate water reabsorption can manifest as liquid retention in faeces, leading to diarrhoea and dehydration, indicative of intestinal stress (Grond et al., 2018). This may stem from dysbacteriosis or physiological limitations that require management. Inflammation disrupts normal intestinal mucosal function, while alterations in the microbiome affect fermentation and absorption processes (Yadav and Jha, 2019). Infectious diseases can damage the intestinal lining, reducing water absorption capacity (Roberts et al., 2015). Dietary factors like high-fibre diets or nutrient deficiencies may also impede water absorption (Desbruslais et al., 2021). Environmental stressors such as heat stress or overcrowding can compromise gut health and function (Nanto-Hara et al., 2019). Implementing strategies to promote optimal gut health, balanced nutrition, suitable environmental conditions, and effective disease prevention can mitigate these challenges and enhance water absorption in the large intestine of chickens. Although high water content in faeces can indicate intestinal stress, it is important to consider that this condition may also be caused by excessive water intake.

Dominant genera in the large intestine include *Lactobacillus* and *Escherichia* (Shang et al., 2018). The faecal microbiome has been described as variable; however, the use of faecal samples is least invasive and most efficient sample method that avoids sacrificing birds. The predominant factors influencing the faecal microbiome composition are age, feed and the environment (Schokker et al., 2021). Within the faeces, resident microbiota of the ileum and cecum are combined, giving a better overview of the entire GI microbiome (Schokker et al., 2021). A recent study suggested that caecal communities in adult broilers (19-31 dph) are more variable than the cloaca. It is estimated that cloacal swabs capture ~60% of the taxa identified within the caeca of broilers (Andreani et al., 2020).

Avian species possess a single opening for eliminating digestive and reproductive waste, known as the cloaca (**Fig. 1.1H**). Faeces comprise a mixture of intestinal and urinary waste, referred to as urates, identifiable by their white crystalline appearance on the exterior of faecal droppings. Excess protein, absorbed from the SI, is converted to uric acid,

excreted into the cloaca, and then transported to the caeca via retroperistalsis through the colon. Bacterial species with proteolytic and ureolytic activity tend to proliferate in birds fed a protein-rich diet (Bailey, 2010). Urates in poultry faecal samples can pose challenges for downstream microbiota analysis. Additionally, avian immune systems lack lymph nodes but include an additional lymphoid organ such as GI associated lymphoid tissues (GALTs) along the gut and the Bursa of Fabricius, located within the cloaca. However, the Bursa Fabricius is only present until the juvenile stage of development (Kaspers and Schat, 2012). Rare taxa in the caeca were less well represented in the cloacal samples, although no functional evaluation of these taxa was assessed. This shows that cloacal swabs are a promising method for high-throughput studies but less valuable for precise estimates. A recent study suggested that caecal communities in adult broilers (19-31 dph) are more variable than the cloaca. It is estimated that cloacal swabs capture ~60% of the taxa identified within the caeca of broilers (Andreani et al., 2020). Rare taxa in the caeca were less well represented in the cloacal samples, although no functional evaluation of these taxa was assessed. This shows that cloacal swabs are a promising method for high-throughput studies but less valuable for precise estimates.

The high density and diversity of bacteria in the caecum has been valuable for research causing a bias in current literature to this intestinal location compared to other sections of the broiler gut. Content from the crop, stomach and SI will contain undigested food. Analysis of this content gives insights into environmental microbiota and digestive function (Bortoluzzi et al., 2019). The poultry gut microbiome can be costly and beneficial to the host with environmental competition between the bacteria in the proximal gut (e.g. ventriculus and SI) and the distal gut (caeca and colon). Both produce toxic metabolites, such as amino acid catabolites, and catabolise bile acids, depressing growth and decreasing host fat digestibility (Shang et al., 2018), further emphasising the importance of intestinal health in the management of poultry.

Understanding the intricacies of the chicken gut microbiome is vital for identifying markers of performance and health. This research requires consideration of GI physiology and its influence on data interpretation. The GI tract is compartmentalised, with distinct sections harbouring diverse microbial communities influenced by factors like pH. When studying the chicken gut microbiome, it is imperative to account for these physiological variations and selective environments. By understanding these dynamics, researchers can better identify biomarkers for performance and health.

1.3.1 Intestinal Structure

Histopathology enables the measurement of the morphological structures of the chicken GI tract, offering insights into intestinal development and damage. The length of the intestinal villi, depth of the crypts, and their relative sizes can give an indication of ongoing intestinal health status (Kaspers and Schat, 2012). Rapid villus elongation occurs between 4-10 dph, a process stimulated by *Lactobacilli*, while decreased villus length often indicates severe GI issues. The intestinal lumen is lined with a single layer of epithelial cells containing crypt-based stem cells for continual renewal (Zhang et al., 2015). Cells located towards the villus tip play a crucial role in nutrient absorption, undergoing differentiation and programmed cell death before exfoliating from the villus (Zhang et al., 2015).

Table 1.1 Reference values of broiler villus length and crypt depth in different locations of the SI at 23 dph, adapted from (De Verdal et al., 2010).

	VILLUS HEIGHT (MM)	CRYPT DEPTH (MM)	CRYPT DEPTH RATIO
DUODENUM	1400	190	8
JEJUNUM	900	170	6
ILEUM	700	160	5

1.3.2 Lumen and Mucosa

Researching the chicken microbiome for gut health is further complicated by significant differences in the composition of the caeca-luminal and caeca-mucosal microbiome (Richards et al., 2019), (Gong et al., 2007), (Volf et al., 2016). These studies observed notable variations in the abundance of amplicon sequence variants for

Lachnospiraceae, *Ruminococcaceae*, *Christensenellaceae*, and *Bacillaceae* between the mucosal caeca and the lumen (Richards et al., 2019).

The GI microbiome encompasses luminal and mucosal components, each with distinct metabolic and immunological functions. The composition of the luminal microbiome is shaped by factors such as nutrient availability, the presence of antimicrobial substances, and the rate of feed passage. Conversely, the composition of the mucosal microbiome is influenced by the expression of specific adhesion sites on enterocyte membranes, the secretion of secretory immunoglobulins (Ig), and the rate of mucus production (Gong et al., 2007). Additionally, the microaerobic environment of the mucosal layer, resulting from oxygen diffusion (Adhikari and Kwon, 2017), imposes additional selection pressures on intestinal microbiota. Despite these differences, there is a mutual influence between mucosal and luminal-associated bacterial populations. However, it is important to note that mucosal bacterial populations may be underrepresented in faecal sampling, as intestinal tissue is required for their accurate identification (Volf et al., 2016).

The GI mucosal lubrication and development process is energetically demanding and can influence performance (Volf et al., 2016). As the gut matures, the mucus layer thickens, promoting mucin secretion and turnover of epithelial cells. This lubricates the GI tract and acts as a barrier against microorganisms attempting to invade intestinal epithelial cells. Moreover, these bacteria play a role in producing essential vitamins (such as K and B groups), SCFA, organic acids (like lactic acid), antimicrobial compounds (such as bacteriocins), and reducing triglyceride levels. They also stimulate non-pathogenic immune responses, contributing to the nutrition and protection of the host (Shang et al., 2018).

Bile acids are regulators of the microbiome, playing roles in the digestion and absorption of lipid and fat-soluble dietary nutrients while also exerting significant antimicrobial effects (Liao et al., 2020). Synthesised by the liver and released into the intestinal lumen, bile acids are typically reabsorbed in the distal SI and recycled by the liver (Chen et al., 2015). Secondary metabolites, such as SCFA and nutrients, bind to the host's intestinal epithelial cells, influencing nutrient availability and performance parameters such as growth. The intestinal microbiome composition associated with feed conversion ratio (FCR) differs distinctly from those linked to the promotion or inhibition of growth. However, investigations into the correlations between performance metrics and the gut microbiome have not been conducted at the pedigree level (Díaz-Sánchez et al., 2019).

1.3.3 Epithelial Barrier Integrity

A key indicator of GI health is the integrity of the epithelial barrier, which allows molecules to pass through via non-mediated passive diffusion (Zhang et al., 2015, Garcia-Hernandez et al., 2017). Microorganisms adhere to the enterocyte epithelial lining of the GI tract, forming a protective barrier that inhibits pathogenic colonisation. To counteract potentially harmful molecules, a family of plasma-membrane-bound efflux pumps, including ATP-binding cassette transporters or multi-drug-resistant pumps, is deployed (Ducatelle et al., 2018a). These pumps are expressed throughout the intestinal tract of all animals, including poultry (Haritova et al., 2010). Broilers with compromised gut barriers exhibit noticeable increases in endotoxins in the serum, which diminish feed intake by indirectly enhancing satiety peptide secretion (Chen et al., 2015), consequently negatively impacting performance.

Consistent observation of a direct connection between intestinal disruption and the loss of intercellular junction integrity suggests a close association between dysbacteriosis and intestinal wall integrity (Garcia-Hernandez et al., 2017). In vitro studies have shown that pro-inflammatory cytokines can elevate epithelial permeability, while differential expression of epithelial claudin proteins regulates mucosal and epithelial homeostasis (Garcia-Hernandez et al., 2017).

The phenotype of intestinal epithelial cells in broilers is altered inducing increased intestinal permeability and altering cell proliferation by inflammation-associated oxidative stress, which could be used as a potential biomarker (Garcia-Hernandez et al., 2017). Validated by serum mRNA levels, an up-regulation of IL-1β, IL-8, TGF-β4 and FABP-6 alongside a down-regulation of FABP-2 and mucin-2 were observed in association with broiler gut barrier failure (Chen et al., 2015). Leakage due to a dysfunctional intestinal barrier leads to blood-dependent activation of systemic inflammation. Acute phase α1-acid glycoprotein has been applied as a systemic inflammation biomarker in poultry (Kogut et al., 2018). To successfully implement a novel biomarker of intestinal health, the collection and quantification need to have a neutral, but preferably positive, effect on poultry productivity and welfare.

Compromised intestinal permeability also impacts bacterial colonization in the liver, with the enumeration of liver bacteria serving as a biomarker of intestinal function and integrity (Tellez et al., 2014). Leakage of epithelial junction complexes allows the passage of lipopolysaccharides, which can be detected and quantified in serum (Ducatelle et al., 2018a). Similarly, FITC-d, a fluorochrome, can be orally administered via gavage, and fluorescence can be measured in serum and the GI tract to assess barrier integrity (Vicuna et al., 2015). However, biomarkers that require sacrificing animals to assess intestinal health are less beneficial to commercial farmers, who seek to minimise bird loss. Leakage from the intestinal epithelial barrier may also lead to the migration of intestinal metabolites such as D-lactate, which can be identified in poultry serum (Ducatelle et al., 2018a, Chen et al., 2015).

1.3.3.1 *Serum Metabolites*

Metabolomics, encompassing both targeted and untargeted approaches, holds significant importance in microbiome research due to its ability to provide insights into the functional activities and metabolic interactions within microbial communities. Targeted metabolomics allows quantifying specific metabolites that play crucial roles in microbial metabolism and host-microbiome interactions, such as bile acid conjugates, short-chain fatty acids and amino acids (Beauclercq et al., 2019a). This approach enables the investigation of the dynamics of critical metabolic pathways, identifies biomarkers, and elucidates the metabolic responses of microbial communities to environmental changes or perturbations. On the other hand, untargeted metabolomics offers a broader view by profiling a wide range of metabolites within a sample (Le Roy et al., 2016), thereby uncovering novel metabolic signatures and pathways relevant to microbiome function (Huang et al., 2022). Integrating metabolomic data with other omics datasets, such as genomics, can provide a comprehensive understanding of microbial communities' metabolic potential and activity (Liu et al., 2022).

Serum metabolites offer a promising avenue for discovering novel biomarkers of intestinal health and welfare. Given the routine blood collection for flock management, obtaining serum samples is readily feasible in commercial practices. The primary techniques for assessing serum metabolites include liquid chromatography-mass spectrometry and

proton nuclear magnetic resonance (^{1}H-NMR) (Le Roy et al., 2016, Jaurila et al., 2020). While acquiring and analysing the data necessitates technical expertise, ^{1}H-NMR is becoming more accessible, with bench-top machines already utilised for diagnostics in humans (Jaurila et al., 2020).

D-lactate is a serum metabolite recently recognised in poultry as a potential biomarker of intestinal health. This metabolite is produced solely by intestinal microbes but can be detected in the serum if the intestinal barrier is compromised (Lei et al., 2013). However, the levels of D-lactate may fluctuate based on the initial concentration in the host's intestine, which is influenced by the host's microbial composition and environmental factors.

Serum metabolites that have translocated from the intestine to the peripheral circulating blood because of compromised intestinal permeability may offer more substantial evidence for confirming poultry intestinal health as a diagnostic tool rather than as biomarkers for health and welfare (Chen et al., 2015). Once the integrity of the epithelial barrier is compromised, intestinal health may already be compromised, which provides less informative value compared to biomarkers that indicate disruption or predict host susceptibility.

1.4 THE BROILER INTESTINAL MICROBIOME AND PERFORMANCE

The broiler GI microbiome is crucial in food digestion, metabolic processes, immune system development and performance (Diaz Carrasco et al., 2019). Imbalances in microbial composition, known as dysbacteriosis, can detrimentally affect poultry productivity, health, and welfare. However, it is important to note that dysbacteriosis lacks a universally agreed-upon definition and aetiology (Ducatelle et al., 2018a). The vague usage of dysbacteriosis is increasingly criticised (Brüssow, 2020), with suggestions to discard it as a critical concept in microbiome science (Olesen and Alm, 2016). Understanding the intricate relationship between hosts and their microbiomes is pivotal for effective poultry production and management (Zduńczyk, 2019). Various intrinsic factors and environmental stressors have been observed to influence the intestinal health and performance of chickens. Developing biomarkers capable of monitoring or predicting intestinal health or performance would benefit the industry significantly. The microbiome of chickens plays a pivotal role in

influencing pathogen colonisation, nutritional effects, and the efficacy of veterinary interventions (Dittoe et al., 2022). Beyond fostering GI health and immune function, the microbial makeup of broilers is increasingly scrutinised concerning performance indicators like growth rate and feed efficiency. Through characterising microbial communities, metabolic profiles, and pathways, efforts are underway to develop a more quantitative approach to predictive tools to enhance poultry health and performance (Dittoe et al., 2022).

Efficient performance is a key focus in commercial poultry production for sustainability and provision of affordable animal protein; it is considerably linked to the gut microbiome. The gut microbiome can influence poultry production, including feed efficiency and growth (Clavijo and Flórez, 2018, Stanley et al., 2014). Broilers, raised explicitly for meat production, undergo slaughter at an average rate of 90 million per month in the UK (Affairs, 2020). Selective breeding for broilers adopts a balanced breeding approach to encompass production traits, health and welfare traits and breeder traits. A key production trait is FCR which is calculated as the ratio of feed given to animal weight gain, is a crucial parameter in the poultry industry for evaluating productivity and efficiency (Willems 2013). A lower FCR value is preferred as it indicates that less feed is required for the animals to gain weight and is therefore an important economic driver and improves the sustainability of poultry production. However, it is essential to consider weight alongside FCR, as a low FCR score is most beneficial when accompanied by high weight gain or meat yield. While FCR is widely utilised, other measures of feed efficiency include average daily feed intake, calculated as cumulative feed intake divided by the number of birds and multiplied by the number of days, and average daily gain, which is the difference between final weight gain and initial weight divided by the number of days. While body weight alone is a valid measure of performance and productivity, feed efficiency strengthens the evidence.

1.4.1 Successional Changes

The composition of a chick's GI microbiome is dynamic, often undergoing frequent temporal shifts. Avian species lack a lactation period, which hinders the initial transfer and colonisation of microbiota observed in mammalian livestock. Recent propositions suggest

that approximately 63% of the embryo cecum microbiota originates from the eggshell, while 28% and 17% come from the egg white, oviduct, and cloaca, respectively (Lee et al., 2019). However, other research groups have faced challenges in replicating these findings. Commercial chickens originating from hatcheries would not have experienced hatching from a nest, another source of early colonisers of the microbiome (Waite and Taylor, 2014). The first microbial exposure to many chicks is from the hatcheries. The management of poultry breeding permits hatcheries to contribute to the development of the microbiome meaningfully; for example, the implementation of high biosecurity at farms successfully prevents *Salmonella* (Bailey et al., 2018). Manipulation of this environment may provide an alternative option to manage microbiome development.

Early life exposure to microbes is known to have long-lasting effects on a chicken's microbiome. Despite early bacterial communities not necessarily becoming resident gut microbiota, they influence the microbiome development (Rodrigues et al., 2020). The successional changes in the broiler are influenced by the initial community composition, creating differences in their mature microbiome. Pioneer colonisers delay microbiome composition establishment in the ileum, whereas lactic acid bacteria can support greater colonisation of symbiotic populations in a young broilers' ileum (Rodrigues et al., 2020). Microbiome transplants of caecal material to 1-day-old chicks significantly alter the structure of the mature microbiome (Ramírez et al., 2020). Transplanting microbiomes alters the developmental trajectory, even among broiler lines, potentially affecting desirable traits like enhanced feed efficiency (Zduńczyk, 2019). Caecal content transplants performed on chicks at 7 dph led to initially higher richness and diversity, which continued to increase over time. However, even after a 12-week study period, it did not reach the same levels as those observed in the donors (Zduńczyk, 2019).

Chickens exhibit three stages of rapid successional changes in their GI microbiome development during the post-hatch period. Vertically transmitted rapid colonisers such as *Escherichia/Shigella* or *Streptococcus* are the dominating species initially. Still, they are displaced by fast-growing taxa such as *Ruminococcus*-like species variants within the first week of hatching (Mohd Shaufi et al., 2015, Oakley and Kogut, 2016, Jurburg et al., 2019). The final successional stage, at around 10 dph, sees replacement by specialist slow-growing taxa before reaching a partially stable microbiome at 14 dph (Glendinning et al., 2019, Shang et al., 2018, Mohd Shaufi et al., 2015, Richards et al., 2019).

At 7 dph, the ileum has a high abundance of *Enterobacteriaceae* and *Clostridiaceae* with fewer *Lactobacillaceae*; however, toward the end of a broiler life at 35 dph, the ileum is dominated by *Lactobacillaceae*, fewer *Clostridiaceae* and minimal *Enterobacteriaceae* (Mohd Shaufi et al., 2015). Early colonisation of *Escherichia coli* and *Enterococcus spp*. is the most common cause of mortality in chicks, and competitive exclusion has been proven successful (Richards-Rios et al., 2020b).

Successional changes to the relative abundance of chicken GI microbiome can be observed during development, with some consistency at the family level between studies. Large shifts are observed in faecal and caecal microbiomes from week to week, with increased community complexity observable in the caeca (Jurburg et al., 2019). Microbial communities may even exhibit shifts in composition on a daily scale in early colonisation (Richards et al., 2019). Despite the abundant variations in publications, the recorded relative successional changes suggest community modulation via structured competitive exclusion, resource competition and early colonisation of a transient microbiome (Schneitz, 2005).

While the successional dynamics of bacterial populations within the gut microbiome have been extensively studied, other components, such as fungi and virus-like particles, notably bacteriophages, have received less attention (Robinson et al., 2022a).

1.4.2 Beyond Bacteria

Maintaining a comprehensive understanding of poultry health involves exploring all relevant parameters concerning their intestinal well-being. Recent technological advancements have revolutionised our insight into the composition of the intestinal microbiome through culture-independent techniques, previously unavailable (Glendinning et al., 2019). Early culture-based identification methods did not capture many microbiota members, estimated to represent only 10-20% of the microbiome (Zduńczyk, 2019). Implementing new high-throughput techniques such as transcriptomics, metagenomics, proteomics, and metabolomics allows a deeper understanding of poultry intestinal health. Understanding the ecology of intestinal microorganisms beyond bacteria and their interactions with the host and environment is crucial for enhancing stability and quality in poultry production.

As shotgun metagenomic sequencing becomes more affordable, researchers increasingly acknowledge the importance of non-bacterial components in poultry studies (Mulholland et al., 2021, Gilroy et al., 2021). This technology enables the analysis of functional genes and pathways in GI samples, surpassing the limitations of 16S rRNA gene sequences (Durazzi et al., 2021). Despite this progress, many researchers rely heavily on 16S rRNA analysis, resulting in a bias towards bacteria in the literature. Moreover, spatial variations in microbiome composition and development are often overlooked. While the caeca receive significant attention due to their bacterial abundance and diversity, this focus may not accurately represent conditions in the proximal gut or SI, where digestive functions predominate. The prevalent use of 16S rRNA sequencing for bacteria, without its fungal counterparts like 18S and 28S rRNA sequencing and ITS regions, has contributed to this bias.

There is a surprising abundance and diversity of bacteriophages within poultry intestinal tracts, but Archaea sequences are still limited (Gilroy et al., 2021). However, there is a growing understanding that microbiomes should be studied as complex ecosystems, encompassing microbiota from all life domains within their microecological niches.

1.4.3.1 *Fungi*

Bacteria dominate the poultry intestine microbiome, yet the fungal community remains understudied. While the literature extensively describes the successional changes in the broiler GI microbiome, little attention is given to the colonisation and development of the mycobiome. Fungal communities, representing less than 0.06% of the total microbial population in the GI microbiome (Robinson, 2019), exhibit temporal dynamics throughout the broiler's life (Robinson et al., 2022a), with no clear succession or maturation observed (Robinson, 2019). Initial colonisation is thought to occur in the hatchery environment, but diet significantly influences the mycobiome, with feed completely replacing the fungal community within just three days (Robinson, 2019). Fluctuations in the intestinal fungal composition underscore the importance of environmental factors, such as diet ingredients and their sources.

While studies have assessed fungi in bioaerosols within poultry housing and their presence in diets, investigations into the abundance of the mycobiome in the GI tract are scarce (Just et al., 2013). Mycotoxins found in the gizzard of poultry typically from the feed

due to poor quality raw materials (Murugesan et al., 2015), pose a significant risk, with occurrences of mycotoxicosis in flocks linked to toxins like aflatoxins, ochratoxin, trichothecenes, and fumonisins (Murugesan et al., 2015). Young poultry in adverse environments are particularly vulnerable to mycotoxins, which can cause immunosuppression and hinder nutritional absorption (Murthy et al., 2002). Despite the potential impact on health, broilers' intestinal or faecal mycobiome research remains limited, emphasising environmental aspects or external features like the beak and comb or the respiratory tract (Mulholland et al., 2021). However, recent findings suggest that broilers undergo successional changes in their mycobiome composition during development, akin to what has been observed with the bacteriome, with the greatest diversity observed in the upper GI tract (Robinson et al., 2020a).

Antibacterial antimicrobials target bacteria by disrupting their cell walls, protein synthesis, nucleic acid synthesis, metabolic pathways, or cell membranes (Garcia-Gutierrez et al., 2019). In contrast, antifungal antimicrobials target fungi by affecting their cell membranes, ergosterol synthesis, cell walls, nucleic acid synthesis, or microtubules (Fisher et al., 2022, Fisher et al., 2018). These differences are due to the distinct structures and metabolic processes of bacteria and fungi. The poultry industry has historically been more reactive than proactive in addressing fungal pathogens, with antimicrobial resistance (AMR) in pathogenic fungi emerging as a growing public health concern. However, this issue has not received the same priority as AMR in bacteria. Despite their potential threat to public health, Fungi have often been overlooked and excluded from AMR programs. This neglect of fungal pathogens in the context of AMR programs has been highlighted in a review by Fisher et al., 2022. Filamentous fungi threaten the poultry industry and have been attributed to feed contamination diseases (Sugiharto, 2019).

New resistant strains have emerged in the common fungus *Aspergillus fumigatus* (Verweij et al., 2020). *Aspergillus fumigatus* is known to inhabit various parts of poultry, including the skin, respiratory tract, and GI tract. While chronic aspergillosis typically affects older birds, particularly breeders, early detection of *A. fumigatus* can occur in poultry as early as the first week after hatching, leading to significant respiratory distress and lung damage (Vahsen et al., 2021). Compared to chickens, turkeys exhibit a slower anti-inflammatory, immune response, resulting in more severe clinical symptoms and higher mortality rates (Vahsen et al., 2021). Additionally, mycotoxins like aflatoxin, produced by *Aspergillus* fungi, are potent secondary metabolites that can inhibit the production and

pose significant health risks (Seyedmousavi et al., 2015). Furthermore, all pathogenic fungi have the potential to develop resistance through adaptation to drug selection pressure (Fisher et al., 2018).

However, not all aspects of filamentous fungi are detrimental. There exists a potential for filamentous fungi to serve as probiotics. For instance, probiotics based on Aspergillus awamori have improved growth performance, nutrient digestibility, and carcass weight (Saleh et al., 2012, Yamamoto, 2007, Saleh, 2011). Similarly, *Aspergillus niger* (Saleh, 2010) and *Aspergillus oryzae* (Lee, 2006) have been found to enhance broilers' growth performance, nutrient digestibility, and intestinal health.

The fungal component of the poultry gut microbiome exhibits lower density and diversity than bacteria. Within the GI tract, a limited number of fungal species, notably *Microascus*, *Trichosporon*, and *Aspergillus*, dominate, collectively constituting over 80% of the total fungal population across most GI segments (Robinson, 2019). Interestingly, the mycobiome demonstrates greater diversity in the upper GI tract than in the lower (Robinson et al., 2022a), partly attributed to the lower pH and higher acidity prevalent in this region, which is conducive to certain fungal species. Notably, this trend does not extend to caecal content, even though caeca harbour the highest density and diversity of bacteria. This observation underscores a discrepancy in fungal distribution within the GI microbiome compared to bacterial colonisation patterns. The tendency to focus research efforts on the caeca within poultry gut studies may have contributed to the relative neglect of fungi in microbiome investigations.

Fungi and yeast exhibit a greater biomass than bacteria despite being less abundant in terms of cell counts (Kurtzman et al., 2011, Huseyin et al., 2017). Notably, while multiple 16S genes are infrequent in bacteria, several fungal species possess multiple ITS regions when employing techniques like qPCR and sequencing methods targeting the 16S rRNA and internal transcribed spacer (ITS) regions to ascertain domain composition (Bradshaw et al., 2023). Moreover, unlike the relatively consistent lengths of the 16S rRNA variable regions (Baker et al., 2003), the size of the ITS rRNA gene varies, contributing to the complexity of bioinformatic analyses of the mycobiome (Bradshaw et al., 2023). There is a notable gap in the literature concerning the integration of both bacterial and fungal components of the gut microbiome by researchers.

The gut mycobiome exhibits a high degree of dynamism, characterised by limited diversity and no discernible patterns of successional changes (Robinson, 2019, Robinson et

al., 2020b, Davies et al., 2022, Robinson et al., 2022a). However, akin to bacterial communities, fungal composition displays spatial heterogeneity along the GI tract, with greater diversity observed in the upper GI regions (Robinson, 2019, Robinson et al., 2022b). Dietary fungi represent the primary source of mycobiome colonisation (Robinson et al., 2022b), underscoring the significance of fungal contaminants in the diet as potential threats. Although the hatchery environment plays a role in the initial colonisation of the GI tract in newly hatched chicks, fungal communities are swiftly replaced by dietary fungi within three days post-hatch (dph) (Robinson, 2019). Notably, a mere 26 taxa account for over 85% of the fungal population at each GI location (Robinson et al., 2022a). Absolute quantification (genome copy number/g digesta) of fungal and bacterial populations, measured as copy number per gram of digesta, reveals an average ratio of fungi to bacteria across the GI tract of 1:45,000. Despite fungi being one of the most biodiverse kingdoms, recent research highlights potential discrepancies in evaluating gut mycobiome abundance due to multiple ITS copies in fungal species (Bradshaw et al., 2023). For instance, a comprehensive analysis of 2414 fungal genomes revealed that approximately 65% of species contained multiple ITS copies, suggesting that estimates of gut mycobiome absolute abundance may be subject to distortion (Bradshaw et al., 2023).

1.4.3.2 *Viruses and Virus-like Particles*

Exploring the intestinal virome poses significant challenges due to the rapid mutations observed in viruses and the variability in nucleic acids, which can be single or double-stranded DNA or RNA (Day et al., 2015, Qiu et al., 2017). Within the chicken GI tract, various viral families, including *Adenoviridae, Caliciviridae, Circoviridae, Parvoviridae, Picobirnaviridae, Picornaviridae,* and *Reoviridae* have been identified in the chicken faecal virome (Lima et al., 2017). Despite advancements in analytical technologies, the extraction of viral material requires substantial quantities due to the abundance of viruses. Moreover, there has been limited research on the poultry intestinal phageome, and the diversity of bacteriophages remains largely unexplored. Sequencing and bioinformatics pose challenges for VLPs similar to viruses, as distinguishing between free bacteriophages and those integrated into the genome is challenging, limiting the utility of shotgun

metagenomic sequencing for this domain. Alternatively, genomic DNA extractions must be enriched or selected for bacteriophages, for instance, through filtration (Qiu et al., 2017).

One proposed alternative for managing the broiler microbiome involves the use of bacteriophages, which possess the ability to infect bacteria. In a study, applying phage therapy to broilers afflicted with multidrug-resistant avian pathogenic *E. coli* led to a swift reduction in infection rates. It enhanced growth and feed efficiency compared to antibiotic-treated birds (Eid et al., 2022). Additionally, administering a commercial Salmonella-phage cocktail to broilers was found to have no adverse effects on the typical microbiome structure and development. Instead, it led to increased diversity and a notable reduction in *Campylobacter* levels (Clavijo et al., 2022). Despite promising results observed in phage therapy applications on broiler farms, variability among animals and publications poses a challenge in determining specific effects. Given the potential of phage therapy as a viable alternative to antibiotics in farming, it is imperative to grasp the full scope and implications of viruses and virus-like-particles (VLPs) in the chicken GI microbiome, particularly considering their demonstrated efficacy in clinical contexts. A surprising diversity of bacteriophages was identified in the metagenomic sequences. Across 582 published metagenomic sequences of chicken faeces, 1,455 unclassified bacteriophage genomes were discovered (Gilroy et al., 2021).

1.4.3.3 *Protozoa*

Protozoa play a significant role in the health and welfare of poultry flocks, with coccidiosis being a prevalent disease affecting the GI tract caused by coccidian protozoa, specifically *Eimeria* (Williams, 2005). This condition is ubiquitous in poultry farming and manifests as clinical disease following the ingestion of a substantial number of sporulated oocytes, typically found in faeces, by susceptible birds (Hooks and O'Malley, 2019). Fortunately, these protozoa can be readily detected using conventional methods, as oocysts, merozoites, or schizonts are visible under microscopic examination (Laforest-Lapointe and Arrieta, 2018).

1.4.3.4 *Archaea*

Archaea, primarily *Methanobrevibacter*, have been predominantly detected within the caeca of chickens, with some presence observed in environmental bioaerosols (Just, 2012). Unlike other microbial domains, there is a notable absence of known pathogenic archaea, resulting in relatively limited research into this life domain and its correlation with, as well as prevalence in, the intestinal microbiome of poultry. *Methanobrevibacter* operates strictly under anaerobic conditions, generating methane while reducing carbon dioxide through hydrogen metabolism. Given contemporary environmental sustainability concerns, the methane produced from protein, particularly associated with poultry, serves as an attractive option for consumers, as methane emissions are substantially higher from mammalian livestock (Saengkerdsub et al., 2007).

1.5 THE POULTRY IMMUNE SYSTEM AND GUT HEALTH

The intestinal immune system operates with remarkable efficiency in combating pathogens while also orchestrating immunological tolerance, development, and activation (Broom, 2019). It could be argued that it surpasses the systemic immune system in functional complexity (Kogut, 2013), given its exposure to significant environmental challenges while continuously maintaining homeostasis (Kogut et al., 2018).

Immunological evaluations provide insights into the avian immune response, elucidating the interplay between the host's immune system and gut microbiota. The immunological status and resilience of chickens against pathogens and environmental stressors can be gauged by examining antibody responses. Histological analyses offer complementary information by visualising tissue architecture, cellular integrity, and inflammatory responses within the gut. This includes assessing villus height and crypt depth, which reflect gut health and functionality. Integrating immunological and histological approaches allows for a comprehensive understanding of the complex interactions between the host immune system, gut microbiota, and gut integrity. This is crucial for optimising chicken performance and promoting GI health in poultry production systems.

In contrast to mammalian livestock, avian species lack a lymphatic system characterised by encapsulated lymph nodes. Poultry species possess a spleen of mesenchymal origin and a thymus derived from epithelium. Additionally, they feature an extra lymphoid organ situated in the cloaca known as the bursa of Fabricius (Broom and Kogut, 2018). Functioning as a hollow dorsal diverticulum, the bursa of Fabricius plays a pivotal role in haematopoiesis and the development of B cells. The thymus and the bursa of Fabricius undergo physiological involution, with the former beginning around 4 months of age and the latter around 6 months. This involution process allows for the formation of secondary or peripheral lymphoid tissues (Kaspers and Schat, 2012).

During both embryonic and postnatal phases of development, diffused lymphoid tissues emerge at predetermined sites along the GI tract. However, the maturation of GALTs is driven by antigenic stimulation (Broom and Kogut, 2018, Lillehoj and Lee, 2012). T cells in secondary lymphoid tissues' intrafollicular space tend to aggregate, while B cells organise into follicles and germinal centres beneath specialised epithelial layers (Broom and Kogut, 2018). Apart from GALTs, the intestinal tract features individual lymphocyte aggregations within the epithelium and lamina propria (Broom, 2019). These lymphocytes primarily comprise T cells expressing immunosuppressive helper CD4 and TCRαβ1, with occasional scattered expressions of CD4+ TCRαβ2+ and cytotoxic CD8+ TCRγδ+ cells (Broom and Kogut, 2018).

The intestinal immune system employs various cellular and molecular mechanisms to promote tolerance towards the commensal microbiota, thereby reducing physiological damage and contributing to a balanced microbiome (Kogut, 2013, Kogut et al., 2017). Particularly during the first week after hatching, the intestinal immune system demonstrates a high degree of tolerance, with early host immune recognition prompting genetic alterations within immune cells to accommodate the initial colonising microbiota (Broom, 2019, Broom and Kogut, 2018). Notably, significant variances in T cell differentiation and recruitment are observable in the SI within the initial week post-hatching, while general metabolic processes may remain unstable for up to two weeks (Schokker et al., 2017).

Recent studies have further highlighted the role of microbiome-driven immune modulation in poultry. Zenner et al. (2021) demonstrated that early microbial exposure, particularly via maternal faeces, enhances gut bacterial diversity and significantly increases IgA and IgY levels compared to chickens raised under stringent hygienic conditions.

Additionally, the establishment of synthetic bacterial communities derived from cultured chicken gut bacteria has shown moderate potential to boost immunoglobulin levels when provided early in life. These findings emphasise the critical influence of microbial colonisation on immune system maturation and pathogen resistance, reinforcing the importance of microbiome-based interventions in poultry production.

1.5.1 NATURAL ANTIBODY PRODUCTION

Immunoglobulins are large Y-shaped glycoproteins that execute antibody activity, located in the blood and vascularised tissue of all jawed vertebrates (Kaspers and Schat, 2012). Comprised of four polypeptide chains, two heavy and two light, forming a monomeric unit, Ig can be secreted as soluble proteins or form membrane-bound antigen receptors (Lebacq-Verheyden et al., 1974). Three classes of avian Ig are known to have mammalian homologs: immunoglobulin M (IgM), A (IgA) and G (IgG). In avian species, IgG is interchangeably referred to as immunoglobulin Y (IgY), which appears to be the evolutionary predecessor of mammalian IgG and IgE (Broom, 2019).

Although the IgG and IgY isotypes share homology in function, the primary differentiating feature is that IgY consists of a single extra domain, increasing the heavy-chain length of the molecule. Interestingly, ducks produce two IgY isoforms, one containing a single domain less than mammalian IgG, resembling the structure of the F(ab')2 fragment of IgY (Kaspers and Schat, 2012). The chicken's immune system produces IgY in response to the presence of pathogens, and it functions to recognise and neutralise these foreign invaders. IgY is transferred from the hen to the egg yolk, providing passive immunity to the developing chick. IgG is another class of antibodies found in chicken serum. It plays a significant role in the adaptive immune response by specifically targeting and neutralising pathogens. IgG antibodies are involved in long-term immunity, as they are produced during the later stages of an immune response.

Unlike the more complex structures of IgM and IgA, IgG has a simple monomeric structure and is the most abundant isotype within serum (Lebacq-Verheyden et al., 1974). Avian IgG is the predominant isotype produced during the secondary immune response, while IgM is the most dominant B cell receptor produced during the primary response.

Correspondingly, after initial exposure to a novel antigen, a transient response of IgM is detectable (Kaspers and Schat, 2012). Small amounts of monomeric IgM have been reported, with free M chains present in bursectomised chickens, but the structure is more likely to be a pentameric or tetrameric configuration (Kaspers and Schat, 2012). In chicken bile and intestinal secretions, IgA has been identified as the most abundant Ig (Lebacq-Verheyden et al., 1974) making it particularly relevant to gut health and productivity. Secretory molecules integrated into IgA promote adhesion to the epithelial cell surface and protect against intrinsic cell degradation. When chicks received an adult caecal transplant on the day of hatching, despite slower growth, chicks had a firmer gut and increased serum IgM and IgA but not IgG (Richards-Rios et al., 2020b). The IgA complex is transported through epithelial cells before secretion into the lumen. Unlike the other Ig, the microbiome can directly induce IgA production (Pabst and Slack, 2019). Furthermore, IgG has been identified to bind to the surface of commensal microbiota members (Pabst and Slack, 2019). The complex interactions involving IgA and intestinal health are still being investigated in human and animal research in the context of the microbiome. When evaluating chronic stress in broiler chicks, serum IgA concentrations were increased in complex and low-density stocking environments, suggesting elevated levels are associated with less stressful environments (Campbell et al., 2023). Measurements of antibodies within the serum aid in elucidating the immunoglobin-microbiome network.

Probiotics have been shown to stimulate the production of natural antibodies in chickens through interactions with GALTs (Haghighi et al., 2006). Natural antibodies (NAbs) are a component of the host immune system that are present without external immune interventions, such as vaccination or prior exposure to pathogens (Berghof et al., 2015b). These antibodies exhibit broad specificity but low binding affinity. NAbs can be categorised into overt and cryptic types based on their binding to xenoantigens. Overt NAbs target non-self-antigens, aiding specific immunity and serving as an early defence mechanism against infections. On the other hand, cryptic NAbs recognise self- and neo-antigens, playing roles in maintaining homeostasis (Berghof et al., 2015b). The levels of NAbs typically increase with age, possibly due to environmental exposures (Kaspers and Schat, 2012). NAbs are heritable traits that contribute to disease resistance and overall survival in chickens, and their expression can be influenced by maternal factors, making them potentially valuable breeding traits (Berghof et al., 2019). Understanding the relationship between performance

metrics and NAbs could be of significant interest to the poultry industry, as NAbs have the potential to serve as markers of disease resistance and environmental susceptibility.

1.6 FACTORS INFLUENCING POULTRY GUT HEALTH

1.6.1 Host Factors

1.6.1.1 *Sex*

In mixed-sex broiler flocks, it is commonly observed that male broilers exhibit higher growth rates and improved FCR compared to female broilers (Kers et al., 2018). Although significant growth rate disparities between the sexes typically manifest around 21 dph, microbial variations discerned as early as 3 dph suggest that factors beyond growth may influence microbiome composition (Lumpkins et al., 2008). To mitigate the influence of fluctuating reproductive hormone levels, animal studies have traditionally favoured using male populations to establish a consistent baseline (Zucker and Beery, 2010). However, the existing literature may be subject to bias, potentially limiting our comprehension of the microbiome. Therefore, studies must report an equal distribution of sexes to ensure a comprehensive understanding of microbial dynamics in broiler populations.

1.6.1.2 *Breeding*

Poultry has undergone selective breeding to improve FCR and achieve rapid weight gain within a short timeframe. The "Chicken of Tomorrow" competition in America in 1940 played a significant role in shaping modern broiler genetics, aiming to breed chickens capable of providing sufficient meat for households (Wood-Gush, 1959). This objective persists in breeding contemporary commercial broilers, albeit with an increased emphasis on health and welfare. Modern poultry farming employs a tiered breeding system to maintain genetic consistency and ensure consistent performance metrics. Pedigree chickens with well-defined genetics serve as great-grandparent stock, while production breeds and final production birds exhibit less genetic uniformity (Díaz-Sánchez et al., 2019).

A paradox exists in broiler breeding, where traits favoured for broiler production can compromise performance. For instance, the emphasis on increased body weight in poultry farming may lead to increase weights putting too much pressure on the legs if not managed properly (Kapell et al., 2012b, Dawkins, 2017). In today's industry, balancing breeding for efficient production and ensuring good welfare is imperative. Commercial broiler breeds exhibit specific genetic, haematological, and immunological parameters, which can vary across different geographical locations (Bílková et al., 2017).

Historic extensive breeding efforts occurred during the widespread use of antimicrobial growth promoters (AGPs) in animal feed, however antimicrobials have not been used in primary breeding programs for many decades. While the precise mechanisms remained unclear, it was believed that the subtherapeutic administration of antibiotics suppressed the microbiome, thereby enhancing nutrient absorption by the host and consequently improving productivity and performance (Danzeisen et al., 2011). Until the 2006 ban on AGPs across the European Union amid global AMR concerns, they were of significant economic importance to the industry and predicted to maximise efficiency by 3-5% (Dahiya et al., 2006). Following the EU legislation, an initial surge in GI diseases, such as necrotic enteritis, was noted in commercial poultry (Dahiya et al., 2006, Bailey, 2010, Huyghebaert et al., 2011) }. This underscores the lack of consideration given to the microbiome during the early stages of breeding and the expansion of the commercial poultry industry. While genetics are recognised to influence the gut microbiome, environmental factors predominantly shape its structure (Rothschild et al., 2018). In poultry, *Campylobacter* colonisation exemplifies the interplay between genetic predisposition and non-genetic factors; while genetics play a minor role, the environment influences a significant portion of the variability (Bailey et al., 2018).

Continuous industrial innovation aims to develop broilers with modern characteristics, prioritising improvements in both intestinal health and production efficiency (De Verdal et al., 2010, Lv et al., 2018). Given current consumer demands and the rapidly increasing human population, slower-growing chickens are deemed less sustainable compared to modern commercial broilers (Dawkins, 2017, Ocejo et al., 2019b). Agrimprove, a brand under the Royal Agrifirm Group, is a European agriculture company that has recently proposed enhancing the physical size of the gizzard along with a system of refluxes to facilitate a longer digestion passage time. Despite the significant emphasis on feed consumption in poultry breeding, water intake is becoming an increasingly important

metric to consider (Jacobs et al., 2019)Global water shortages pose a major concern, significantly impacting agriculture. Consequently, growing interest is shown in breeding poultry species with greater water retention capabilities to reduce demand for this critical resource.

1.6.1.3 *Stress*

Chickens can demonstrate stress via a diversity of behavioural and physiological attributes associated with welfare (Scanes, 2016), with divergent caecal microbiome profiles related to specific stress behaviours (Birkl et al., 2018). During stress, noradrenaline is produced and systemically released. Both *Campylobacter* and *E. coli* have receptors for noradrenaline, stimulating their growth and colonisation (Aroori et al., 2014). Additionally, noradrenalin binds to lactoferrin and along with *E. coli*, activates auto-immunity via quorum sensing (Cogan et al., 2007, Aroori et al., 2014).

Individually restraining poultry, as in metabolic cages used for research purposes, induces more stress than open housing systems (Cotter, 2015). Factors such as the restraint method, duration, and body weight have all been shown to affect performance metrics negatively (Ismail et al., 2019). Contemporary open housing systems allow poultry to exhibit natural social behaviours, and monitoring behaviour in such environments aids in selecting desirable breed traits, including reduced aggression.

Assessing stress in poultry presents a challenge due to the stress induced by the data collection process itself. For instance, chasing and catching a bird for blood withdrawal can potentially cause more stress to the individual than it has experienced throughout its entire lifespan. Haematological changes, such as alterations in heterophil-to-lymphocyte ratios, reflect sustained stress (Cotter, 2015). However, it is important to note that different chicken breeds exhibit variations in baseline leukocyte parameters due to evolving immunological adaptations (Bílková et al., 2017).

1.6.2 ENVIRONMENTAL FACTORS

1.6.2.1 *Temperature*

Maintaining optimal temperature and humidity levels is crucial to support flock production and welfare. Poor climate management can lead to significant stress, discomfort, and even mortality due to conditions such as internal organ failure or hyperthermia. Higher environmental temperatures have been linked to increased tissue accretion at the expense of skeletal development (Nanto-Hara et al., 2019), underscoring the importance of considering heat response and susceptibility as hereditary factors in breeding programs.

Poultry primarily rely on convection, direct respiratory evaporation, and radiant cooling mechanisms for thermoregulation (Nanto-Hara et al., 2019, Liu et al., 2022). When a chicken's body temperature rises, its metabolic rate also increases. To counteract this physiological response and decrease metabolic heat production, adult chickens instinctively reduce their food and water intake (Donkoh, 1989). Consequently, this leads to diminished performance parameters.

Fortunately, poultry farmers in naturally cooler regions benefit from the collective grouping of birds, which is predicted to reduce heat loss efficiency by 40%. Broiler production typically involves gradually decreasing temperatures. Upon hatching, chicks are maintained at around 32°C to compensate for the radiant heat absorbed from their mother in nest-reared chicks (Ruff et al., 2019). As the chickens grow rapidly, they naturally increase the ambient temperature through radiation, necessitating a gradual reduction in housing temperature. By the broiler finishing stage at around 30-40 dph, the environmental temperature is approximately 24°C. These gradual temperature changes exert selection pressure on the colonization and growth of environmental microorganisms, potentially impacting the microbiome composition. Exposure to excessive heat (>32°C) elevates the internal body temperature from 42°C to 45°C, with a lethal limit of 47.2°C (Ruff et al., 2019). Acute and chronic heat stress disrupt the delicate epithelial cell barrier and tight junction proteins, leading to intestinal permeability (Ruff et al., 2019, Nanto-Hara et al., 2019).

1.6.2.2 *Lighting*

Lighting facilitates birds' visual perception and colour discrimination, stimulating various behavioural and physiological functions. Both innate instincts and learned behaviours influence the establishment of intrinsic rhythmic routines in poultry species. By

stimulating the secretion of hormones, light indirectly promotes growth and maturation, thereby enhancing overall performance (Olanrewaju et al., 2006). By carefully manipulating the environmental lighting conditions in commercial poultry settings, greater consistency in production can be achieved. Extensive research has investigated the circadian rhythms of diurnal poultry species, aiming to leverage metabolic activity for industrial benefits (Olanrewaju et al., 2006). Different lighting schedules, including near-continuous, intermittent, restrictive, and increasing photoperiods, have been explored to enhance poultry productivity, although their effects have proven multifaceted and complex (Schwean-Lardner et al., 2012a, Schwean-Lardner et al., 2012b).

The duration and frequency of lighting play a significant role in the growth of poultry species, with considerations also given to light intensity and source. Given the importance of artificial lighting in the agricultural sector, finding suitable alternatives to natural light sources is crucial. Studies have shown that mixed LED lighting, incorporating green and blue wavelengths, can significantly improve body weight and feed conversion rates (Yang et al., 2016). Vision is the primary sensory ability in birds, underscoring the importance of light regulation for their physiological and behavioural development. By employing light sources that align with the avian visual spectrum, chickens have demonstrated enhanced abilities in grain discrimination within their housing, ultimately leading to increased feed intake and associated weight gain.

1.6.2.3 *Flock Composition*

Flock uniformity plays a dual role as an economic driver and a performance gauge in poultry farming. Typically, broiler carcasses must conform to specific weight ranges, a criterion usually assessed only at the time of slaughter, thus making continuous monitoring of flock uniformity often neglected (RJ Hughes, 2017). Understanding the factors contributing to variation in feed efficiency is crucial for reducing flock discrepancies (Metzler-Zebeli et al., 2019, Gadde et al., 2017, Yadav and Jha, 2019) Modern processing facilities rely heavily on automated machinery operating within predefined parameters. Developing biomarkers capable of predicting feed efficiency would significantly benefit commercial operations.

Poultry farmers frequently face constraints in maximising profits without compromising animal welfare. One such example is the stocking density, as defined by the RSPCA in the UK, which stands at 38kg/m^2, allowing for approximately 2-4 Ross-308 broilers per square meter. While increasing stocking density may enhance financial returns, it has been associated with moderate and severe welfare issues due to overcrowding in poultry housing (Dawkins, 2017).

Partial depopulation of the flock involves removing birds once the average weight of the flock reaches the target they will just go in and remove a proportion of the flock. The negative impact is thought to be firstly due to potential biosecurity issues when the catching crews come in and potentially bring disease and secondly as it is stressful the release of noradrenaline can influence the microbiome (Aroori et al., 2014). This practice is additionally thought to negatively impact the microbiomes of the remaining flock because chickens naturally engage in coprophagy (Richards-Rios et al., 2020b, RJ Hughes, 2017).

Sentinel birds can be introduced into a flock to indicate intestinal health (Richards-Rios et al., 2020b, Chen et al., 2015), although this is not common practise in commercial farms and more relevant to experimental and research purposes. This practice is inefficient as sacrificing a separate group for assessing flock health and welfare contradicts efforts to reduce mortality. An alternative approach involves introducing birds with an established optimal commensal microbiome into the flock, facilitating natural microbial transfer (Wigley, 2015). Likewise, if a broiler flock consistently demonstrates strong performance, seeding samples of litter into the environment of a new flock may promote intestinal health benefits. Additionally, scoring the foot pad are typically from the feed due to poor quality raw materials health are typically from the feed due to poor quality raw materials of birds can offer insights into flock intestinal health (Jacobs et al., 2019). Wet litter, indicative of dysbiosis, visibly affects the skin on the birds' feet (Bailey, 2010). Unfortunately, by the time this is observed in birds, the flock may already have been affected.

1.6.2.4 *Diet and Feed Additives*

Feed constitutes approximately 80% of the costs in poultry production and significantly influences the development of the microbiome (Lebacq-Verheyden et al., 1974). The commercial poultry diet typically comprises wheat, maise, and soya,

supplemented with enzymes, amino acids, and vitamins (Acres, 2018). Modern broilers have an average lifespan of 35 days, with intervention times for microbial modulation identified as days 3-4 and 10-14 post-hatching, with day 14 being particularly optimal due to microbial dormancy (Ducatelle et al., 2018a, Gong et al., 2007). Broilers are transitioned through staged diets, with changes occurring at critical developmental stages to optimise performance (Gong et al., 2007, Jurburg et al., 2019, Schokker et al., 2021). The starter diet from hatch contains more additives to support early development. As the functionality of the proximal gut matures in the first one to two weeks, the physical form of the feed progresses from sieved crumbs to pellets (Acres, 2018). Once target body weights are reached, birds are switched to a grower diet with increased nutrient density to meet metabolic requirements for optimal biological performance (Ross, 2019). Finisher feeds, typically introduced after 25 days post-hatch, represent the highest feed intake and cost (Acres, 2018). Feed protection technologies employ a blend of carboxylic acids and phytochemicals to exert antimicrobial effects, albeit with variation depending on diet density and quantity. Heat treatment of feed is another strategy, although it may compromise nutrient integrity despite its effectiveness against pathogens.

A plethora of commercially available feed additives (Bortoluzzi et al., 2019, Ma et al., 2018, Sizova et al., 2019, Huyghebaert et al., 2011) are increasingly explored as alternatives to AGPs, yet their efficacy hinges significantly on management practices. While additives, often in combination and administered at specific developmental stages, offer benefits, their effectiveness may vary depending on delivery methods. No singular product is universally suitable throughout a bird's life, emphasising the importance of targeted support during feed transitions and veterinary interventions. Probiotics and prebiotics, intended to modulate or exploit the GI microenvironment, have shown limited efficacy in challenge models (Netherwood et al., 1999, Vuong et al., 2016). Mannan oligosaccharides, sometimes classified as prebiotics, are considered more of a modulator due to their ability to alter the functional capacity of a more complex microbiome (Corrigan et al., 2018). Moreover, nanoscale metals have been introduced as mineral feed additives (Sizova et al., 2019).

1.6.2.5 *Vaccinations and Veterinary Care*

Optimising vaccine response may be achievable through microbiome modulation (Broom and Kogut, 2018). Veterinarians routinely administer vaccines in commercial poultry management to prevent diseases (Kaspers and Schat, 2012). Vaccination and improved biosecurity measures have proven highly effective in reducing Salmonella infections and controlling Coccidiosis. However, this approach has shown limited efficacy against *Campylobacter* (Bailey et al., 2018). Additionally, vaccination programs may be less effective on multi-age poultry sites (Acres, 2018). It is crucial to consider the physiological effects of these immunisation programs; increases in body temperature and water consumption may occur, necessitating appropriate support for the flock (Abo et al., 2019). Alternative delivery systems have been explored to mitigate the risk of human contamination. Commercial *in ovo* gel or spray options for eggs or chicks offer an alternative to traditional vaccination methods (Bertocchi et al., 2019, Richards-Rios et al., 2020b). These systems are minimally invasive and eliminate the need for human intervention when the microbiome is particularly unstable. However, the effectiveness of *in-ovo treatment appears to be more pronounced on a small s*cale (Schokker et al., 2017). While Aviguard is widely used, it introduces an adult caecal microbiome (Gilroy et al., 2018, Huyghebaert et al., 2011) an alternative is to reuse litter from a healthy flock to mimic the microbial benefits observed in natural nest-hatching scenarios (Wigley, 2015).

1.6.3 ANTIBIOTICS IN POULTRY FARMING AND INTRINSIC RESISTANCE

To address concerns of AMR, there has been a global reduction in the use of antibiotics on poultry farms. Antibiotics remain a crucial medical recourse available to farmers and veterinarians for combating infections in poultry. Regulatory measures have led to a significant 80.2% reduction in total antibiotic usage in UK poultry since 2012 (Kaul, 2019). This reduction reflects a concerted effort to promote responsible antibiotic use in addressing farm animal health and welfare, coupled with extensive research into alternative growth-enhancing supplements. Historically, antibiotics have been pivotal in managing poultry health and bolstering productivity by inhibiting bacterial replication or causing bacterial death. They serve as vital treatments against pathogenic diseases.

Antibiotics induce alterations in mice's faecal metabolome, intestinal immunity, and microbiome composition (Yoon et al., 2022). In chickens, supplementation with therapeutic levels of antibiotics via diet or water significantly disrupts caecal microbial homeostasis at various stages of their life (Kairmi et al., 2022). A notable decrease in Lactobacillaceae abundance characterises this disruption, reduced microbial diversity and species richness, and decreased butyrate-producing bacteria levels. Despite their efficacy in controlling necrotic enteritis outbreaks, the detrimental effects of antibiotics on the gut microbiome can compromise poultry gut health and productivity (Dahiya et al., 2006, Williams, 2005). Therefore, restoring microbial richness and diversity in the gut microbiome is a more beneficial approach for managing poultry gut health and enhancing productivity.

1.7 SUMMARY

In the realm of poultry farming, the health and well-being of chickens are paramount considerations. Understanding the intricate dynamics of the chicken microbiome, including bacteria, fungi, viruses, and protozoa, is crucial for optimal health and biological efficiency. From the challenges of fungal pathogens to the potential benefits of probiotics, the poultry industry navigates a complex landscape of microbial interactions. Additionally, factors such as environmental conditions, lighting, and flock management play significant roles in shaping the health and performance of chicken flocks. Novel approaches, including phage therapy and alternative feed additives, offer promising avenues for mitigating disease and enhancing productivity while reducing reliance on antibiotics. As the industry evolves, ongoing research and innovation are essential for ensuring the sustainability and welfare of chickens, which remain dominant in the agricultural landscape.

Antibiotics, once pivotal in enhancing livestock productivity, now face regulatory scrutiny due to concerns over AMR. Alternative strategies, such as phage therapy, are emerging as promising alternatives to antibiotics, demonstrating efficacy in combating bacterial infections without disrupting the microbiome. Moreover, advancements in lighting management, feed additives, and vaccination programs underscore the multifaceted approach to poultry health and welfare. However, challenges persist, including the need for sustainable flock management practices and optimising interventions such as flock thinning and *in-ovo* treatments to minimise stress and maintain microbial balance. As

research progresses, a holistic understanding of the intricate interplay between genetics, environment, and microbiome dynamics will continue to drive innovations to enhance poultry health and productivity while ensuring animal welfare.

Enhancing the microbiome and promoting intestinal health have become pivotal priorities within the poultry sector. Identifying reliable markers indicative of gut health and performance would significantly advance our understanding and management of poultry health.

1.8 AIMS AND OBJECTIVES OF THE THESIS

Identifying biomarkers of intestinal health holds immense promise to commercial poultry, leveraging insights from the microbiome and immune system. With chickens, particularly broilers, constituting a substantial population and with a wealth of existing research, the initial focus will be on these commercial chickens. Emphasising non-invasive methodologies to minimise animal stress, the quest for a reliable biomarker molecule within faecal or caecal droppings emerges as a strategic priority. Alternatively, exploiting routine blood collection in veterinary practices offers another avenue for identifying detectable biomarkers within serum samples, presenting significant advantages for the poultry industry. Keeping these considerations in view, the primary objectives of this thesis are as follows:

1. Validate and refine methodologies suitable for assessing broiler gut health, including selecting sample types, genomic DNA extraction techniques, and sequencing methodologies.
2. Investigate the microbial landscape of broilers beyond bacteria, encompassing fungi, viruses, and protozoa, and explore the functional metabolome to elucidate their interrelationships across various life stages. Correlate these microbial components and performance metrics to uncover potential markers indicative of gut health.
3. Identify and establish suitable markers indicative of broiler gut health, aiming to facilitate their adoption within the poultry industry to elevate welfare standards and productivity levels.
4. Establish the optimal stage or age within the broiler life cycle for implementing markers of gut health and performance and interventions.

CHAPTER 2

GENERAL METHODS

2.1 STUDY ANIMALS

Recognising the use of study animals, such as chickens, is essential for ensuring transparency, scientific validity, and ethical compliance in research. Acknowledging the animals used helps establish the context of the study and allows for evaluation of the relevance and generalisability of the findings. It also ensures compliance with regulatory requirements and publication standards and prioritises the animals' welfare.

2.1.1 Animal Ethics Statement

All animals in this project were under the care of the Aviagen veterinary department and reared under the conditions set out in the European Broiler Directive (Council Directive 2007/43/EC). The farm is assessed annually by DEFRA to ensure compliance. No animals were researched or experimented with, and only surplus materials (e.g., faecal droppings and blood [if being used for another purpose]) were collected. Animals were only sacrificed as part of routine broiler processing or monitoring. Any animals sacrificed due to health concerns would be cervically dislocated, performed by trained personnel, authorised by Aviagen Veterinary and/or Welfare Director. None of the animals were reared or explicitly sacrificed for this project.

2.1.2 Sample Collection, Storage and Transportation

All broiler samples in this project originated from animals housed in a communal environment. Whenever possible, sample collection was personally conducted on a commercial broiler farm. Broilers of the same age were housed in pens according to their genotypes, separated by 4-ft barriers (Fig. 2.1). The chicken shed maintained a closed environment with a constant minimum temperature of 26°C. Environmental samples were obtained from the pens of the target broilers, ensuring a minimum of five samples were collected and pooled. Collection sites included the litter surrounding the diet feeder, water troughs and areas where chickens typically congregate, such as corners and pen entrances.

The chicken shed and litter consist primarily of wood, creating a distinctive selective environment for microorganisms.

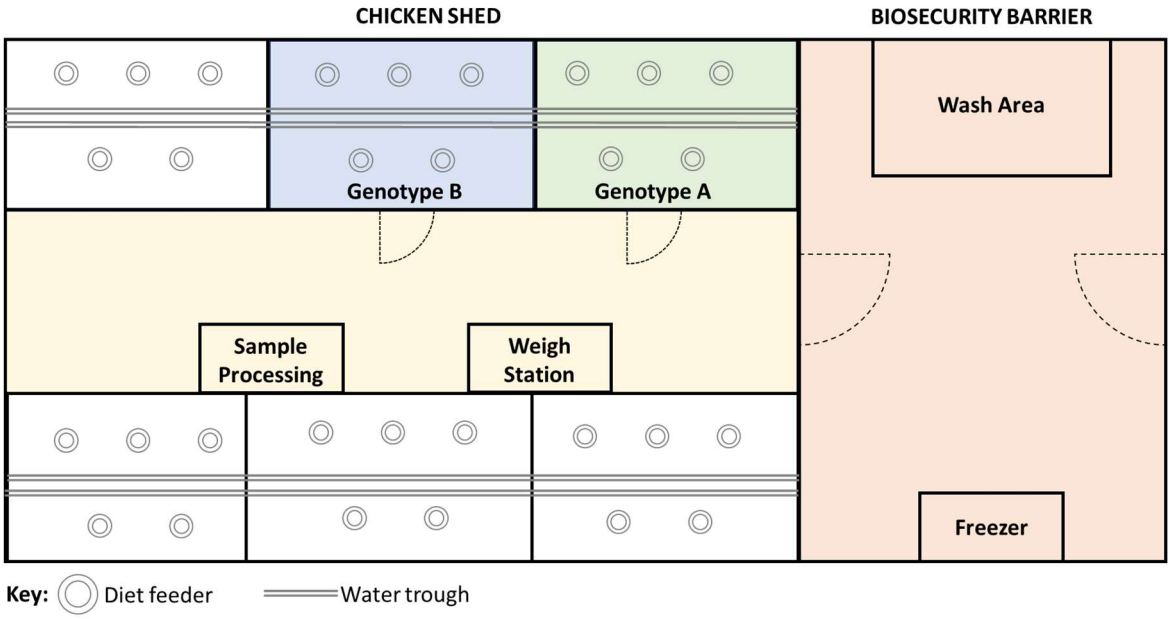

Figure 2.1 Diagram depicting the shared open-shed environment.

In this project, 385 broilers contributed samples, summarised in Table 2.1. Faecal collections and concurrent body weight measurements were conducted under the supervision of Dr Richard Bailey and Lauren Purves. Upon collection, samples were promptly divided into 200 mg aliquots and placed in sterile 2 mL freezer-safe tubes. Additionally, approximately 100 mg of faeces was preserved in 1 mL of 40% glycerol and immediately frozen for future isolation work. All samples were stored at -20°C before transportation to the Quadram Institute Bioscience (QIB) on dry ice. For long-term storage, faecal and GI content were kept at -70°C.

Throughout the avian influenza biosecurity restrictions mandated by the UK government from April 2020 to February 2022, Dr Richard Bailey and Lauren Purves sacrificed or collected chicken blood and organised the storage and transportation of samples from Aviagen, Edinburgh.

Each broiler cohort's specific details, such as performance metrics and collection dates, are outlined in the corresponding chapters. Performance parameters in this study pertain to broiler body weight or feed efficiency, quantified as the FCR. FCR is determined

by dividing the feed weight given by the broiler body weight, with lower FCR values indicating higher efficiency.

Table 2.1 Summary details for the birds used in the project.

Date	Number of birds	Age of birds	Metadata collected	Material collected	Chapter	Collected by
Nov-19	5	35 dph	Body weight, sex	Faecal, caecal, jejunal contents	3	Aviagen
Sep-20	20	28 dph	Sex	Serum	3	Aviagen
Sep-20	20	35 dph	Sex	Serum	3	Aviagen
Mar-20	50	7 dph	Body weight, sex, genotype	Faecal	4	Bushra Schuitemaker
Mar-22	50	7 dph	Body weight, sex, genotype	Faecal	4	Bushra Schuitemaker
Feb-21	10	13 dph	Body weight, sex	Faecal, jejunal, caecal, serum	5	Aviagen
Jan-22	200	35 dph	Body weight, sex, FCR	Serum	6	Aviagen
Oct-22	50	7 dph	Body weight, sex, genotype	Faecal	7	Bushra Schuitemaker
Oct-22		18 dph	Body weight, sex, genotype	Faecal, serum	7	Bushra Schuitemaker
Nov-22		27 dph	Body weight, sex, genotype	Faecal, serum	7	Bushra Schuitemaker
Nov-22		35 dph	Body weight, sex, genotype	Faecal, caecal, SI content and tissues, serum	7	Bushra Schuitemaker

2.1.2.1 *Faecal Water Retention*

Inadequate water absorption in chickens' large intestines can stem from several factors, including excessive water intake, intestinal inflammation, dysbacteriosis, infectious diseases like coccidiosis, dietary inadequacies, or environmental stress (Dawkins, 2017). Monitoring faecal water retention aids in the early detection and management of absorption-related concerns.

To assess faecal water retention, 22.1 and 273.5 mg of faeces were weighed into a sterile 1.7 mL Eppendorf tube within a microbial safety cabinet (MSC). Samples were centrifuged at 7,500 x g at room temperature for 5 min. Liquid supernatant was aspirated, and the volume was measured to calculate water retention as ml/g of faeces.

2.2. MICROBIOLOGY AND MOLECULAR BIOLOGY

All microbiology experiments were performed in a category II laboratory environment, adhering to standard microbial techniques and established safety protocols to contain potentially hazardous microorganisms.

All molecular biology experiments followed established techniques (Sambrook et al., 1989). Milli-Q water with a resistivity of 18 MΩ/cm was used, referred to hereafter at ultra-pure H_2O (upH_2O). All reagents and materials were high-quality PCR grade and sterile. Unless otherwise stated, chemicals and reagents were purchased from Sigma-Aldrich.

2.2.1 Bacterial and Fungal Isolation from Broiler Faeces

Chicken faecal and GI content stored at -70°C in 40% glycerol was thawed, and samples were streaked onto selective media agar plates using sterile toothpicks or disposable inoculation loops. For bacterial isolations, De Man–Rogosa–Sharpe (MRS), Brain Heart Infusion (BHI), and LB supplied by Oxoid Ltd. were utilised, while Rose-Bengal with Chloramphenicol (RB+C) and Yeast Peptone Dextrose (YPD) supplied by Sigma-Aldrich were employed for fungal isolations. The plates were then incubated overnight at 26°C to 42°C.

Morphologically distinct colonies were selected using a sterile toothpick and streaked three times to ensure purity before being sub-cultured onto the same medium in liquid format. Liquid cultures were subsequently incubated and monitored for growth at 24 and 48 hours. Dense liquid cultures with an OD_{600nm} of 0.8 – 1.0 were preserved in a 1:1 ratio with 40% glycerol and immediately frozen on dry ice for long-term storage at -70°C.

2.2.2 Growth Curves and Viable Counts

Bacterial cultures were grown aerobically overnight under preferred conditions. Before use, cultures derived from glycerol stocks underwent a minimum of two sub-cultures. Subsequently, the pre-warmed medium was inoculated with 4% (w/v) of the overnight culture and incubated at 42°C (representing chicken body temperature), 37°C (the isolation temperature), or 26°C (typical chicken housing temperature) unless specified otherwise. Optical density at 600 nm (OD_{600}) for rich medium or 540 nm (OD_{540}) for minimal media was measured using samples diluted by 1:10 in growth medium with the Jenway 6300 Spectrophotometer. Growth curve determination at different temperatures involved recording at 1-hour intervals.

To characterise the bacteria isolates, growth curves were conducted at 37°C and 42°C, 100 µl of the culture was diluted 1:10 in phosphate-buffered saline (PBS) at 3 hours and 8 hours to generate serial dilutions ranging from 10^{-2} to 10^{-7}. Three 10 µl aliquots were aseptically spotted from each serial dilution onto agar plates and incubated aerobically overnight at either preferred or experimental temperatures. Colonies were enumerated for each serial dilution at each time point to assess viability and quantify colony-forming units per ml (CFU/ml).

2.2.3 Colony PCR and 16S ribosomal RNA Gene Sequencing

Distinct colonies were selected and suspended in 10 µl upH_2O, subcultured onto agar using a sterile toothpick to avoid contamination, then the remaining suspended colony centrifuged at 7,500 x g at room temperature for 5 min, after which the supernatant was

aspirated, the pellet was washed with 150 µl of colony wash buffer (100 mM NaCl, 10 mM Tris pH 7, 1 mM EDTA) (Sambrook et al., 1989) and centrifuged under the same conditions. The final pellet was re-suspended in 15 µl upH_2O. The remaining sample was boiled at 95ºC for 5 min before proceeding with PCR

2.2.3.1 *Sequencing of 16S ribosomal RNA Gene for Bacterial Identification*

The 16S rRNA gene was amplified following the manufacturer's instructions using the GoTaq G2 Polymerase (Promega) kit. The PCR reaction mixture comprised 36.35 µl of upH_2O, 5x Buffer, 25 mM dNTPs (Bioline), 20 µM primers (Sigma Genosys, AMP_F 5'- GAG AGT TTG ATY CTG GCT CAG – T_m 60.5, AMP_R 5'- AAG GAG GTG ATC CAR CCG CA – T_m 68.9) (Baker et al., 2003), 1 µl template (either boiled colony sample or ten ng genomic DNA) and 0.25 µl GoTaq enzyme for a final reaction volume of 50 µl per sample. PCR amplification was carried out using a Thermo Scientific Hybaid HBSP02110 PCR Sprint thermal cycler under the following conditions: initial denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min per kilobase, and a final extension at 72°C for 5 min. Product size was 1.5 kb. A negative control containing no template was included for each experiment.

2.2.3.2 *Sequencing of ITS1-4 ribosomal RNA Gene for Fungal Identification*

The amplification of the ITS1-4 rRNA gene was conducted following the manufacturer's instructions using the KAPA2G Fast HotStart PCR Kit (Sigma-Aldrich) kit. The PCR reaction mixture comprised of 13.9 µl upH_2O, 5x KAPA2G GC Buffer, 10 mM dNTPs (Bioline), 20 µM primers (Sigma Genosys, ITS1_F 5'- CTT GGT CAT TTA GAG GAA GTA A – T_m 49.2, ITS4 5'- TCC TCC GCT TAT TGA TAT GC – T_m 68.9) (Xu et al., 2016), 5 µl template (either boiled colony sample or 10 ng genomic DNA) and 0.1 µl KAPA2G DNA polymerase (5U/µl) for a final reaction volume of 25 µl. The PCR amplification was carried out using a Thermo Scientific Hybaid HBSP02110 PCR Sprint thermal cycler under the following conditions: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1

min, annealing at 51°C for 1 min, extension at 72°C for 1 min per kilobase (52 s for ITS1-4 880 bp), and a final extension at 72°C for 8 min. A negative control containing no template was included for each experiment.

2.2.3.3 Gel Electrophoresis, Quantification and Purification of PCR Product

Agarose gel electrophoresis was performed as described (Sambrook et al 1989) using 0.9% agarose in 0.5 x TBE with detection by Midori XTRA (NIPPON Genetics). 10 µl of the PCR product with 1 µl loading buffer [0.15% bromophenol blue, 25 ml glycerol, 5X TBE (Sambrook et al., 1989)] was loaded into the wells; Hyperladder I (Bioline) was used as a size marker. After electrophoresis, the gel was visualised under blue light with an imager system (Syngene).

PCR products were purified using the QiagenQuick PCR Clean-up Kit, following the manufacturer's instructions. Briefly, the purification process involves DNA binding to the silica membrane in the presence of a high-salt buffer, followed by washing steps to remove impurities and elution of the purified DNA in a low-salt buffer or water. Purified samples were eluted in 50 µl of elution buffer (10mM Tris-HCl pH 8.5) and stored on ice before DNA quantification using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The instrument analyses the sample's absorbance at wavelengths typically used for nucleic acid quantification, such as 260 nm for DNA. The measured absorbance values are then used to calculate the concentration of the DNA sample in ng/µL and the A260/A280 ratio to assess purity. While NanoDrop provides a quick estimation of DNA concentration, it may not be accurate for samples containing impurities.

2.2.3.4 *Sanger Sequencing*

For bacterial 16S rDNA and fungal ITS1-4 identification, purified PCR-amplified products were subjected to Sanger sequencing using the Eurofins Genomics Mix2Seq Service. Sequence chromatograms were visually inspected using FinchTV. Alignments of forward and reverse reads were generated using Geneious Prime 7.0 (Biomatters), and the

aligned sequences were submitted to the Ribosomal Database Project (Maidak et al., 1996) for species identification, applying a minimum sequence match criterion of 97%.

2.3 METAGENOMIC SEQUENCING

2.3.1 Genomic DNA Extractions

In this project, 200 mg of faecal or intestinal content was prepared in aliquots for genomic DNA extractions stored at -70°C and then thawed overnight at 4°C. Three DNA extraction kits were selected based on their widespread use in microbiome research and their status as well-established, gold-standard methods for obtaining high-quality microbial DNA.

2.3.1.1 *Maxwell RSC PureFood GMO and Authentication Kit (Promega)*

The Maxwell RSC PureFood GMO and Authentication kit (Promega) provides a partially automated genomic DNA extraction method. 200 mg of faecal and GI content was prepared using components supplied in the kit: 1 ml of CTAB buffer was added to the sample, transferred to a Lysing Matrix E tube (MP Bio), and vortexed for 30 s. Samples were heated at 95^{o}C for 5 min and allowed to cool at room temperature for 2 min. Samples were vigorously vortexed before homogenisation using FastPrep-24™ Classic Instrument for three cycles of 1 min at 6.5 m/s, with a 5 min rest period on ice between cycles. For enzymatic digestion, 40 µl of proteinase K and 20 µl of RNase A were added to the samples and vortexed before incubating at 70 for 10 min. Samples were centrifuged for 5 min at 7,500 x g, and 100 µl of the supernatant was dispensed into the cartridge, per the manufacturer's instructions.

The Maxwell RSC Instrument was prepared with 100 µl of elution buffer and 300 µl of lysis buffer, and the PureFood GMO and Authentication program was applied. Eluted DNA was stored at -20^{o}C until required. A *kitome* negative control containing no faecal or GI material was used as a negative control for all experiments.

2.3.1.2 *Maxwell RSC Culture Cells DNA Kit (Promega)*

The Maxwell RSC Cultured Cells DNA Kit was used to extract DNA from 400 µl of dense bacterial cultures (OD_{600} 0.8 – 1.0). The cells were lysed using the Maxwell RSC Cartridge lysis buffer, followed by DNA binding to the solid phase within the cartridge. Subsequent steps involve washing away contaminants and eluting the purified DNA from the cartridge to 100 µl of elution buffer. The eluted DNA was quantified and assessed for quality before storage at -20^{o}C.

2.3.1.3 *FastDNA Spin Kit for Soil (MP Biomedicals)*

DNA was also extracted from faecal samples using the FastDNA Spin Kit for soil (MP Biomedicals). Following the manufacturer's instructions, 978 µl of sodium phosphate buffer and 122 µl of MT buffer were added to 200 mg of faecal material of GI content and vortexed thoroughly before incubating at 4oC for 1 hour. Samples were vortexed again, and 1 ml of the sample was transferred to Lysing Matrix E tubes and lysed using the FastPrep-24™ Classic homogeniser for three cycles of 1 min at 6.5 m/s, with a 5 min rest period on ice between cycles. Once lysed, samples were centrifuged for 1 min at 14,000 x g speed, and the supernatant was transferred to a clean tube. From the kit, 250 µl of PPS reagent was added, and the tube was vigorously inverted by hand before another centrifugation at 5 min, full speed, to precipitate the pellet. The supernatant was transferred into a fresh tube, and 1 ml of the Binding Matrix was added. The tubes were inverted by hand for 2 min and then stood for 3 min to allow the matrix to settle. Of the supernatant, 1 ml was discarded, and the remaining sample was re-suspended in the settled binding matrix. Of the mixture, 650 µl was transferred to a SPIN Filter Tube and centrifuged for 1 min at the maximum speed. Flow through was discarded, and 500 µl of SEWS-M was added before centrifugation for 1 min at the maximum speed. This step was repeated three times before centrifuging the sample ‘dry’ for 2 min to collect any final liquid. Samples were air-dried for 5 min at room temperature, and a SPIN tube was placed onto a fresh ‘catch tube’ to elute DNA in 100 µl of DNase/Pyrogen Free Water (DES), incubated for 1 min before a final 1 min maximum speed centrifuge. Eluted DNA was stored at -20^{o}C until required.

2.3.1.4 *QIAmp PowerFecal Pro DNA Kit (Qiagen)*

Initially, 200 mg of faecal material or GI content was homogenised and lysed with 750 µl of PowerBead Solution in PowerBead Tubes with 60 µl of InhibitEX Buffer to ensure efficient cell lysis. Tubes were heated for 10 min at 65^{o}C before being secured horizontally to a 24-holder vortex adapter. Samples were vortexed vigorously for 10 minutes, and then centrifuged at 7,500 x g for 1-min. 500 µl of the supernatant was loaded onto QIAamp Mini spin columns for DNA binding, followed by multiple washing steps to remove impurities. Finally, DNA was eluted from the columns using 100 µl of DNA Elution Buffer, yielding purified DNA suitable for metagenomic sequencing. Eluted DNA was stored at -20^{o}C until required.

2.3.1.5 *Quantification (Qubit) and Normalisation*

Quantification of genomic DNA yield was performed following the manufacturer's protocol for Qubit double-stranded DNA (dsDNA) Broad-Range (BR) and High-Sensitivity (HS) kits (Thermo Fisher).

The Qubit dsDNA Working Solution was prepared as needed by combining 199 µL of buffer per sample with 1 µL of the fluorescent reagent per sample. For sample preparation, 199 µL of the working solution was dispensed into the Qubit tubes, while 190 µL was dispensed for the standards. Subsequently, 1 µL of genomic DNA was added to the assay tubes for samples, or 10 µL of standards, resulting in a final volume of 200 µL. The tubes were then incubated for 2 min at room temperature to facilitate the binding of the fluorescent dye to the DNA. Following incubation, the fluorescence of each sample was measured using an Invitrogen™ Qubit™ 4.0 fluorometer. Samples with DNA concentrations exceeding the detection range were diluted at 1:10 and re-measured. In contrast, samples with DNA concentrations below the detection limit were reprocessed with up to 5 µL of genomic DNA per test.

For all metagenomic sequencing, genomic DNA was normalised to 5 ng/µ before library construction.

2.3.2 Library Construction

For ITS1-2 amplicon libraries, the initial PCR amplifying the gene with attached Illumina primers was performed using KAPA 2G Fast Hot Start Ready Mix (Merck Catalogue No. KK5601). For 16S amplicon metabarcoding and shotgun metagenomic sequencing, libraries were prepared by David Baker and his team at the QIB in-house Sequencing Facility, as detailed below. David Baker completed all amplicon sequencing in-house at QIB using the Illumina MiSeq® Reagent Kit v3 (600 cycle) (Illumina Catalogue FC-102-3003) following the Illumina recommended denaturation and loading recommendations which included a 20% PhiX spike in (PhiX Control v3 Illumina Catalogue FC-110-3001). The raw data was analysed locally on the MiSeq using MiSeq reporter. Shotgun metagenomic sequencing was outsourced to NovoGene (Cambridge, UK), Earlham Institute (Norwich, UK) and Source Bioscience (Nottingham, UK) using Illumina Hi-Seq sequencing platform prepared with Nextseq500 kit and run on an S4 lane at a depth of 5 Gb. A summary of sequencing platforms, strategies and who the work was carried out by is detailed in Table 2.2.

Table 2.2 Summary of libraries and sequencing strategies used in this project.

Date	***Number of samples in the library***	***Material Type***	***Sequencing Strategy***	***Sequencing Platform***	***Library Preparation by***	***Sequencing by***
Nov-19	10	Faecal	16S	Mi-Seq	QIB-in house	QIB in-house
Nov-19	10	Faecal	ITS1-2	Mi-Seq	QIB-in house	QIB in-house
Nov-19	15	Faecal	Shotgun	Hi-Seq	QIB-in house	QIB in-house
Mar-20	50	Faecal	Shotgun	Hi-Seq	QIB-in house	NovoGene
Mar-20	50	Faecal	ITS1-2	Mi-Seq	Bushra/QIB-in house	QIB in-house
Feb-21	30	Faecal, jejunal, caecal	Shotgun	Hi-Seq	QIB-in house	QIB in-house
Mar-22	50	Faecal	Shotgun	Hi-Seq	QIB-in house	Earlham Institute
Mar-22	50	Faecal	ITS1-2	Mi-Seq	Bushra/QIB-in house	QIB in-house

Oct-22	200	Faecal	Shotgun	Hi-Seq	QIB-in house	Source BioScience
Oct-22	200	Faecal	ITS1-2	Mi-Seq	Bushra/QIB-in house	QIB in-house
Nov-22	40	The SI, caecal	Shotgun	Hi-Seq	QIB-in house	Source Bioscience
Nov-22	40	The SI, caecal	ITS1-2	Mi-Seq	Bushra/QIB-in house	QIB in-house

2.3.2.1 *Amplicon Library Construction for 16S Ribosomal RNA Metagenomic Sequencing*

Genomic DNA was diluted to 5 ng/µl in elution buffer (10 mM Tris-HCl pH 8.5) and prepared for 16S rRNA paired-end sequencing at the QIB in-house facility. An amplification to yield 254 amplicon base-pairs from the V4 hypervariable region (515F/R806) was performed by David Baker, and the library constructed using HiFi Hot Start polymerase (KAPA) in a two-step PCR, modified from Caporaso et al. (Caporaso et al., 2011). The raw data was acquired locally on the Mi-Seq using a Mi-Seq reporter. The absence of Illumina adapters in raw Fastq files was checked with the FastQC tool (Babraham Bioinformaticians).

2.3.2.2 *Amplicon Library Construction ITS1-2 ribosomal RNA Metagenomic Sequencing*

Genomic DNA was diluted to 5 ng/µl in elution buffer (10 mM Tris-HCl pH 8.5) as a template. PCR amplification of the ITS1 rRNA gene was set up according to manufacturer's instructions using the KAPA2G robust PCR kit (Roche), applying 5x Buffer GC, ten mM dNTPs, 20 µM primers from Sigma Genosys (ITS1F_Illumina: 5' - TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CTT GGT CAT TTA GAG GAA GTA A – T_m 79°C, ITS2_Illumina: 5' - GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGC TGC GTT CTT CAT CGA TGC – T_m 82°C) (Huseyin et al., 2017), 10 µl of template and 5U/µl KAPA2G DNA polymerase. The PCR conditions were programmed into a Biometra Trio thermal cycler as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 52 s, and final extension at 72°C for 5 min.

Samples were visualised by gel electrophoresis then submitted to the QIB in-house sequencing team for the second PCR and final library construction. Pooled libraries were run on an Illumina Mi-Seq instrument using Mi-Seq® Reagent Kit v3 following the Illumina recommended denaturation and loading recommendations, including a 20% PhiX spike. The raw data was acquired locally on the Mi-Seq using a Mi-Seq reporter.

2.3.2.3 *Library Construction for Shotgun Metagenomic Sequencing*

Genomic DNA was normalised to 5 ng/µl in elution buffer (10 mM Tris-HCl pH 8.5) and prepared for shotgun metagenomic sequencing by David Baker at the QIB in-house facility using Tagment PCR library construction on a single S4 lane at 5 GB. Pooled libraries were run on an Illumina Nextseq500 instrument using a Mid Output Flowcell (NSQ® 500 Mid Output KT v2 (300 CYS) following the Illumina recommended denaturation and loading recommendations, which included a 1% PhiX spike-in (PhiX Control). Raw data was converted to 2 FASTQ files for each sample.

2.3.3 Whole Genome Sequencing of Bacterial Strains and Archiving

Bacteria isolated in this project underwent genome sequencing and were archived.

Sterile Nunc 2 ml screw-cap tubes were prepared for aerobic and facultative bacteria, each containing approximately 20 acid-washed beads. Bacterial cultures were cultivated overnight in 10-20 ml cultures until reaching the stationary phase, and fresh cultures were utilised. Harvesting involved centrifuging 2x 1.5 ml aliquots of the culture at 7,500 x g for 2 min (using two tubes), with an additional tube for DNA extraction. After removing the supernatant, the pellet was resuspended in 80ul of 40% glycerol by gentle pipetting with a filter tip. The entire liquid was then added to the tubes containing beads, mixed thoroughly, flash-frozen on dry ice and stored in a -80^{o}C freezer for long-term storage.

For DNA extraction, 1.5 ml was harvested by centrifugation for 2 min at 7,500 x g, and the pellet was resuspended in 1.5 ml of TE buffer pH 8. The centrifugation was

repeated, and the buffer removed before proceeding with the Maxwell RSC Culture Cells DNA Kit (Promega) in methods section 2.3.1.2.

Short-read sequencing was conducted in-house at QIB for whole genome sequencing. Following DNA extraction, David Baker oversaw the sequencing process. Genomic DNA was standardised to 5 ng/µl using EB buffer (10 mM Tris-HCl), and the sequencing was carried out at the QIB sequencing facility using an Illumina NextSeq 500 system, employing 2x150 bp paired-end reads. Libraries were prepared using Bead Linked Transposomes (BLT) (Illumina Catalogue No. 20018704) and P7 and P5 Illumina 9bp barcodes.

2.4 BIOINFORMATIC ANALYSIS

2.4.1 Sequent Variant Identification of 16S ribosomal RNA and ITS1-2 ribosomal RNA with Dadaist2 and QIIME2

Analysing bacterial and fungal datasets separately using rRNA amplicon sequence variant identification, Illumina data was acquired with a Mi-Seq sequencing platform. Sequences were analysed using QIIME2 core version 2019.10. Applying an adaptation of the method described by Bokulich (2013) (Bokulich et al., 2013), amplicon sequence variant assignments were completed with the DeBlur plugin, and a feature table was produced using the feature-table plugin. ITS amplicon sequence variants were further processed using Dadaist2 version 1.3.0 (Ansorge et al., 2021), which utilises Divisive Amplicon Denoising Algorithm 2 (DADA2). DADA2 is a bioinformatics software package used for the high-resolution inference of microbial community composition from amplicon sequencing data (Callahan et al., 2016). It employs a statistical approach to error correction and denoising, aiming to resolve amplicon sequence variants (ASVs) accurately without relying on traditional clustering-based methods. This allows correction for variability in the ITS gene length.

Alpha (α) and beta (β) diversity measurements were performed using the phylogeny plugin and rooted tree to determine the sampling depth for each dataset. Alpha diversity

describes community complexity, providing 'within sample' metrics, determining the species richness (the frequency of different operational taxonomic units (OTUs)) and species evenness (the proportional distribution of OTUs). Beta diversity provided 'between sample' metrics, describing the variance differences between features and a quantitative measurement of community dissimilarity incorporating phylogenetic relations. Distances between samples were tested within metadata columns using PERMANOVA, as defined by Anderson (2001) (Anderson, 2001).

The Shannon Index is a commonly used metric in microbiome research to quantify the α diversity of microbial communities within a sample. It measures species richness (the number of different microbial taxa present) and evenness (the relative abundance of each taxon). Shannon Index gives more weight to taxa that are more evenly distributed in the sample, resulting in higher index values for samples with greater diversity (Reese and Dunn, 2018). A higher Shannon Index value indicates greater diversity, with more evenly distributed abundances among the different taxa. In contrast, a lower Shannon Index value suggests lower diversity due to fewer taxa present or dominance by a few highly abundant taxa. Shannon Index facilitates assessments of community stability over time or in response to perturbations (Kers and Saccenti, 2022).

Bray-Curtis dissimilarity is a statistical measure commonly used in microbiome research to quantify the dissimilarity or similarity between pairs of microbial communities sampled from different environments or experimental conditions (Kers and Saccenti, 2022). It provides a way to compare the composition of microbial communities based on the abundance of other taxa, considering both the presence and relative abundance of taxa (Reese and Dunn, 2018). The resulting Bray-Curtis dissimilarity value ranges from zero to one, where zero indicates complete similarity (i.e., both samples have the same taxa present in the same abundances) and one indicates complete dissimilarity (i.e., no taxa are shared between the samples, or their abundances are entirely different). Bray-Curtis dissimilarity is often used to construct distance matrices, which can be used for various downstream analyses such as clustering and ordination (e.g., PCoA) for visualisation.

To assess the bacterial microbiome composition, taxonomy was assigned using the pre-trained Naïve Bayes classifier and q2-feature-classifier plugin, assigning a classification to the resulting FeatureTable sequences. Naïve Bayes classifier trimmed 16S sequences from the V4 region to 250 bp, bound by the 515F/806R primer pair. NaiveBayes classifier

based on the SILVA 132 database of the 515F/R806 region of the 16S rRNA gene was downloaded from www.arb-silva.de.

To assess the fungal microbiome composition, representative sequences were extracted from QIIME2 using the QAX tool (telatin.github.io/qax/) and transformed using the Dadaist2 package (quadram-institute-bioscience.github.io/dadaist2/) to enable customisation for the variable length of ITS1 amplicon. Sequences are consequently presented as ASVs. Individual ASVs were uploaded to the UNITE database (unite.ut.ee/) for taxonomic assignment.

2.4.2 Shotgun Metagenomic Analysis with Galaxy

Shotgun metagenomic sequences were processed for microbial community profiling in Galaxy using a workflow developed by QIB Bioinformatician Dr Rebecca Ansorge and subsequently adapted in this project for the broiler microbiome and to incorporate updated software.

The first workflow used the fast tool to create a multiQC HTML report detailing read-quality assessment. Host removal of *Gallus gallus* was applied to all samples (genome obtained from www.ncbi.nlm.nih.gov/genome/?term=gallus+gallus), followed by taxonomic profiling of filtered reads with *Kraken2* including Bayesian re-estimation of abundance with *Bracken*.

The second workflow determined taxonomic profiling from the high-quality trimmed reads using MetaPhlan 4.0. MetaPhlAn utilises metagenomics reads to identify microbes and their respective abundance. This process involved mapping the reads to the ChocoPhlAn database containing unique clade-specific marker genes. Clades represent groups of organisms, and clade-specific markers are coding sequences highly conserved within the genomes of a particular clade yet distinct enough from sequences outside the clade. These marker genes, found in the ChocoPhlAn database, were curated from over 17,000 reference genomes encompassing bacteria, archaea, viruses, and eukaryotes. Furthermore, the workflow performed functional profiling using HUMAnN3 to reveal gene family and pathway abundances.

HUMAnN3 (v3.0.0.alpha.3), or the HMP Unified Metabolic Analysis Network 3, is a tool for functional profiling microbial communities from metagenomic sequencing data. It employs a multi-step process to quantify the abundance of microbial pathways and functions within a sample. Firstly, HUMAnN3 aligns metagenomic reads against a comprehensive reference database of microbial genes and pathways. This database is compiled from diverse microbial genomes and includes information on metabolic pathways, enzyme functions, and associated gene families. Next, HUMAnN3 normalises the read abundances to account for variations in sequencing depth and genome sizes across different microbial taxa. This normalisation step ensures accurate comparisons of pathway abundances between samples. HUMAnN3 integrates the normalised read abundances to estimate the relative abundance of metabolic pathways and functions within the microbial community. This information provides valuable insights into the functional potential of the microbiome and its role in various biological processes. This comprehensively characterises microbial communities' potential metabolic capabilities, facilitating the study of their roles, interactions, and contributions to host health and microbiome.

Antibiotic Resistance Identification By Assembly (ARIBA) is a bioinformatics tool to detect AMR genes in bacterial genomes or shotgun metagenomic datasets. It employs a reference-based strategy, aligning sequencing reads against curated databases such as the Comprehensive Antibiotic Resistance Database (CARD) and ResFinder. CARD offers a rich compilation of AMR genes alongside detailed annotations, covering diverse bacterial pathogens and resistance mechanisms. ResFinder, maintained by the Center for Genomic Epidemiology, is another prominent database providing curated sequences of known resistance genes, facilitating accurate identification and characterisation of AMR.

2.4.3 Whole Genome Assembly in Galaxy

For genome assembly, a Galaxy workflow created by QIB Bioinformaticians called Assembly and Annotation Pipeline was applied, where reads were trimmed and assembled with Shovill (v 1.1.0). The completeness and contamination of the assemblies were checked using QUAST (v 5.1.0). Genomes were annotated using Prokka (v 1.14.6), and contig alignment was assessed in GeneiousPrime 7.0 (Biomatters). Nucleotide alignment was also

assessed in GeniousPrime 7.0. The RefSeqMasher Pipeline was run simultaneously to find what NCBI RefSeq genomes most closely match or are contained in the sample sequences.

2.5 STATISTICAL AND GRAPHICAL ANALYSIS

Unless otherwise stated, statistical analyses including t-tests, ANOVA, regression analysis, and non-parametric tests, were performed in GraphPad Prism (version 5.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Statistically significant thresholds are P >0.05, FDR >0.05 and R^2 >0.8. Analyses of metagenomic sequencing were performed in RStudio (R version 4.3.3)

2.5.1 16S and ITS1-2 ribosomal RNA Analysis with Microbiome Analyst 2.0 (R package)

Amplicon metagenomic data was analysed using MicrobiomeAnalyst 2.0 and R (Lu et al., 2023). Uploaded data files containing microbial abundance information were obtained through QIIME2 (and Dadaist2 for ITS). Low-abundance taxa may prove a valuable marker for gut health therefore preprocessing filters were not applied. Data was normalised using total sum scaling, where the abundance values of microbial taxa within each sample are divided by the total sum of counts (or reads) in that sample. This effectively scales the abundance values to represent the proportion of each taxon relative to the total microbial community size within the sample, mitigating differences in sequencing depth and allowing more accurate comparisons of microbial abundance across samples.

In this project, the visualisation of both α and β diversity was facilitated by Exploratory Data Analysis tools. Alpha diversity, which assesses within-sample diversity, was evaluated using the Shannon Index. Meanwhile, β diversity, which measures between-sample diversity, was quantified using Bray-Curtis dissimilarity.

Based on the experimental design and data distribution, statistical analysis was then performed to identify differentially abundant taxa or functional features between groups (e.g., t-test, ANOVA, PERMANOVA). Statistical significance was when P = >0.05, adjusted for

multiple tests using false discovery rate (FDR) correction methods where necessary. FDR is a statistical measure used to control the rate of false positives in hypothesis testing, particularly in multiple testing scenarios where many hypotheses are being tested simultaneously. A lower FDR indicates better control over false positives, while a higher FDR suggests a higher likelihood of false positives. An FDR threshold of 0.05 (or 5%) was used as the standard cutoff for significance.

Results were presented via MetaboAnalyst, utilising stacked bar graphs to depict relative abundances, whisker plots to visualise α diversity, and PCoA for β diversity assessment, generated in R.

2.5.2 Shotgun Metagenomic Analysis with Phyloseq (R package)

Analysing microbiome shotgun metagenomic data from MetaPhlAn 4.0 in R encompasses several sequential steps, which include data preprocessing, taxonomic profiling, differential abundance analysis, and visualisation. Initially, the MetaPhlAn output file, containing taxonomic abundance information for each sample, is imported into R, ensuring uniformity in sample names and taxonomic annotations. Subsequently, a phyloseq object is constructed in R, utilising the sample metadata, taxonomy table, and abundance matrix.

Using the phyloseq R package (McMurdie and Holmes, 2013) a taxonomic abundance table is generated from the MetaPhlAn output, with data normalisation applied through total sum scaling to mitigate discrepancies in sequencing depths across samples. This is followed by an initial exploratory data analysis to visualise relative abundance at different taxonomic levels. The examination includes an assessment of domain composition (bacteria, eukaryotes, viruses) and identifying the top 10 most abundant genera and species, which are visualised using stacked bar plots.

Further analysis entails differential abundance assessment using DESeq2 to discern significantly different taxa between various groups, such as broiler age, sex, genotype, or GI location. Adjustment for multiple testing methods was performed, with a significant false discovery rate (FDR) cutoff set at >0.05.

The outcomes of taxonomic profiling and differential abundance analysis are then depicted using diverse plotting functions in R, such as bar plots, heatmaps, volcano plots, or scatter plots, to elucidate taxonomic composition, differential abundance, and associations between taxa and metadata variables. ggplot2 was employed for biological interpretation and analysis of these visualisations.

2.6 METABOLOMICS

2.6.1 Preparation for ^{1}H-NMR and Data Acquisition with NEO600 MHz (Bruker)

For an untargeted investigation of the broiler serum metabolome, samples were processed for ^{1}H-NMR following NEO600 protocol IconNMR for Metabolomics, developed by Sergey Nepogodiev at the John Innes Centre NMR facility. Serum samples were thawed on ice, followed by centrifugation at 1,700 x g for 1 min. The supernatant was diluted 0.2% (w/v) with NMR buffer (1.9 mM Na_2HPO_4, 8.1 mM NaH_2PO_4, 150 mM NaCl, 1 mM sodium trimethylsilyl propionate (TSP) in D_2O), 550 µl of the sample was transferred to 5 mm o.d. NMR tubes (Wilmand) for acquisition.

For an untargeted investigation of the broiler faecal, caecal and jejunal metabolomes, samples were processed for ^{1}H-NMR following the same protocol as stated above. Preprepared aliquots containing 200mg of chicken faecal material were thawed on wet ice or overnight at 4^{o}C. Each sample was diluted 0.2% (w/v) with NMR buffer and mechanically homogenised with sterile pellet pestles. Following homogenisation, samples were centrifuged at 7,500 x g for 1 min. The supernatant was stored at -20°C, then thawed overnight at 4^{o}C and 550 µL was transferred to 5 mm o.d. NMR tubes (Wilmand) for acquisition.

Spectra were recorded on a 600 MHz Bruker Advance spectrometer fitted with a 5 mm cryoprobe and 24-slot autosampler. Unless otherwise stated, each serum spectrum consisted of 32 scans. Each faecal spectrum consisted of 16 scans unless otherwise stated, with a comparison of 16 and 32 scans made on the duplicate sample for each experiment to confirm consistent sensitivity.

For all samples, spectral width was 14 ppm, and the *noesypr1d* pre-saturation sequence was used to suppress the residual water signal. Spectra were transformed with 1 Hz line broadening, manually phased, and baseline corrected using the TOPSPIN 2.0 software. Metabolites were identified using the information in the literature or assigned with the Chenomx 600Hz software library, curated by Dr Gwenaelle Le Gall (UEA). To determine the signal-to-noise ratio, the signal was acquired between 0.7 ppm and 0.6 ppm (width 2.00 ppm/1202.09 Hz) for all serum samples, and the noise was acquired between 11.041 ppm and 9.0417 ppm (width 0.1 ppm/60.98 Hz). The signal-to-noise ratio (sino) was calculated using the Bruker TopSpin 4.0 software.

2.6.2 Analysis of ^{1}H-NMR Metabolomics Data

Chenomx vs 9.0 was used to analyse NMR data in metabolomics studies. Metabolites were additionally identified using the information found in the literature or assigned with the Chenomx 600Hz software library with additional curation contributed by Dr Gwenaelle Le Gall (UEA). Statistical analysis was conducted using MetaboAnalyst 5.0, which is accessible via both a web interface and an R package (Xia et al., 2009). Raw NMR data underwent normalisation through global normalisation, involving mean centring and division by the standard deviation of each variable. MetaboAnalyst provides a comprehensive array of statistical methods, encompassing t-tests and ANOVA for comparing compound concentrations, fold-change analysis to assess relative differences between variables, and multivariate techniques such as partial least squares-discriminant analysis (PLS-DA) to unveil metabolomic patterns associated with biological factors, metadata, or experimental variables. Significance was determined at a threshold of $P > 0.05$.

2.7 IMMUNOLOGY AND HISTOLOGY

2.7.1 Detection of Natural Antibodies in Serum with Indirect Two-Step Enzyme-Linked Immunosorbent Assay (ELISA)

The method described by Berghof research groups (Berghof et al., 2015a, Van Der Klein et al., 2015, Berghof et al., 2018, Berghof et al., 2019) was adapted to look at the titres of natural antibodies (immunoglobin (Ig) heavy and light chains (IgY H+L) and isotypes IgG, IgM and IgA) binding to the xenoantigen keyhole limpet hemocyanin (KLH) in the serum of broilers. The indirect two-step ELISA uses horseradish peroxidase-conjugated antibodies and measurement of colourimetric extinction at 450 nm with a 96-well plate microspectrophotometer. The final protocol was adapted to use prediluted serum and an increased number of washes to 3 between steps, and final washes increased to 5.

Clear flat-bottom immuno nonsterile 96-well plates (MediSorp, Cat# 467320) were coated with 2 µg/mL of KLH in 100 µL coating buffer (5.3 g/L Na_2CO_3 and 4.2 g/L $NaHCO_3$; pH 9.6) to allow 12 h of binding. Serum samples were pre-diluted and prepared in dilution buffer (PBS supplemented with 0.5% normal horse serum, and 0.05% Tween 20) with IgY (H+L), IgM and IgG diluted 1:10 and IgA 1:5. Pre-dilutions were stored at 4°C until use the next day. Pre-diluted serum was further diluted with dilution buffer to non-binding flat-bottom 96-well plates so serum for IgY (H+L), IgM and IgG were 1:40, 1:160, 1:640, 1:2560 and serum for IgA 1:10, 1:20, 1:40, 1:80. Experimental set up was technically duplicated on a plate, samples were randomised to avoid plate effect, and there was one plate per antibody. Pooled serum samples were diluted for the internal standard, as well as serum dilutions. As with the serum, pools were pre-diluted to 1:10 for IgY (H+L) rabbit-anti-chicken IgY heavy and light chain labelled with horse radish peroxidase (Abcam, Cat# A30-107P, Lot# RRID:AB_67386), goat-anti-chicken IgM labelled with horse radish peroxidase (Abcam, Cat# A30-102P, Lot #RRID:AB_66857), goat-anti-chicken IgG(Fc) labelled with horse radish peroxidase (Abcam, Cat# A30-104P, Lot# RRID:AB_6684) and 1:5 for goat-anti-chicken IgA labelled with horse radish peroxidase (Abcam, Cat# A30-103P, Lot# RRID:AB_66833). Pooled standards were used for a 7-point, 10-fold dilution starting at 1:10 with dilution buffer. All serum dilutions (samples and pooled-standard) of 200 µl in duplicate in a standard 96-well plate were stored at 4°C overnight, using a multichannel pipette the following morning to quickly transfer the serum to the treated ELISA plates. For example, for the plate layout, see **Fig 2.2**.

KLH-treated plates were washed with wash buffer (upH_2O, 0.05% Tween 20) and tapped dry three times before diluted serum was transferred directly to the KLH-treated plates. Plates were incubated for 1.5 hours at room temperature to allow the serum natural

antibodies to bind to the xenoantigen. Following incubation, the wash step was repeated and 200 µl of either 1:20,000-diluted rabbit-anti-chicken IgY (H+L), 1:40,000-diluted goat-anti-chicken IgG, 1:20,000-diluted goat-anti-chicken IgM or 1:7,500-diluted goat-anti-chicken IgA, all labelled with horseradish peroxidase. Plates were incubated for another 1.5 hours at room temperature.

Following the second incubation, the wash step was repeated and binding of antibodies to KLH was visualised by adding 100 µL substrate buffer (containing reverse osmosis purified water, 10% tetramethylbenzidine buffer [15 g/L sodium acetate, and 1.43 g/L hydrogen peroxide; pH 5.5], and 1% tetramethylbenzidine [8 g/L TMB in DMSO]) and incubated at room temperature. For IgY (H+L), IgG and IgM, the reaction was quenched after 15 min with 50 µl of 1.25 M H_2SO_4. For IgA, the reaction was quenched after 30 minutes. Absorbance was immediately measured using the BMG Labtech CLARIOStar Plus microplate spectrophotometer at 450 nm.

To calculate antibody titres, a linear regression of pooled standards for each plate (absorbance on Y axis, dilutions (log10) on the x-axis) was used to calculate R^2 (where the threshold is >0.8) and *y values*. For samples, relative antibody titre is first corrected with absorbance values – blank values + *y value* and multiplied by the dilution factor. Furthermore, averages, standard deviation (SD), and coefficient of variance (CV=STD/average) are calculated. For ELISAs, CVs between plates should be less than 15, and CVs within a plate (e.g. between replicates) should be less than 10. Final NAb titres were calculated with $Yij = \mu + Pi$ where *Yij* is IgM, IgG or IgA titre or IgT (all immunoglobins and IgY (H+L)), μ is mean and *Pi* is plate variation using coefficient of variance (CV=populations standard deviation/population mean).

IgY (1:10) x10 samples x50 birds = 5 plates											
Blank	1:10	1:100	1:1000	1:10000	1:100,000	1:1,000,00	1:10,000,0	1:40	1:160	1:640	1:2560
Blank	1:10	1:100	1:1000	1:10000	1:100,000	1:1,000,00	1:10,000,0	1:40	1:160	1:640	1:2560
1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560
1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560
1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560
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1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560
1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560
	Serum										
	20ul -->	*20ul -->*	*20ul -->*	*20ul -->*	*20ul -->*	*20ul -->*	*20ul*				
200ul	180ul	180ul	180ul	180ul	180ul	180ul	180ul	*Serum*			
200ul	180ul	180ul	180ul	180ul	180ul	180ul	180ul	*5ul*	*50ul -->*	*50ul -->*	*50ul -->*
Serum				*Serum*				195ul	150ul	150ul	150ul
5ul	*50ul -->*	*50ul -->*	*50ul -->*	*5ul*	*50ul -->*	*50ul -->*	*50ul -->*	195ul	150ul	150ul	150ul
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195ul	150ul	150ul	150ul	195ul	150ul	150ul	150ul	195ul	150ul	150ul	150ul

Volume (µl) for serial dilution

Volume (µl) of prediluted serum

Volume (µl) of dilution buffer

Figure 2.2 Plate layout for two-step indirect NAb ELISA against xenoantigen KLH.

2.7.2 Acquisition of Microscopy Images

Measurements of villus height and crypt depth in chickens are an established method for evaluating intestinal morphology and development. Histological examinations were performed on formalin-fixed broiler SI sections at 35 dph. Tissues were sectioned to approx. 5 mm and routinely processed with wax embedding at the University of Edinburgh Easter Bush Pathology Unit. Embedded sections were sliced at 4-6 µm to produce three slides per tissue sample. Slides were stained with haematoxylin and eosin (H&E). Slides stained with H&E were captured at QIB Bioimaging Facility using the ZEISS Axioscan 7 Microscope Slide Scanner at x20 to x40 magnification, fitted with automatic stitching of images greater than the microscope's field of view. Villus height and crypt depth measurements were obtained using ZEN Microscopy Software. At least ten measurements per section were collected, totalling 30 measurements per chicken.

CHAPTER 3

DEVELOPING AND VALIDATING METHODS TO DEFINE BROILER PERFORMANCE AND GUT HEALTH

3.1 INTRODUCTION

The validation of broiler gut health and performance encompasses assessing factors including gut morphology, microbiota composition, immune function, and overall bird performance.

The chicken GI microbiome is integral to gut health and performance (Stanley et al., 2016). Next-generation sequencing techniques, such as 16S and ITS1 rRNA amplicon and shotgun metagenomic sequencing, are used to analyse the composition and diversity of the gut microbiota (Díaz-Sánchez et al., 2019). However, weighing each technique's advantages and limitations is essential to ascertain their suitability for specific experimental conditions (Durazzi et al., 2021, Laudadio et al., 2018).

Metagenomic sequencing methods are indispensable tools in microbiome research due to their ability to comprehensively characterise microbial communities and their functional potential. Shotgun metagenomic sequencing provides an unbiased and holistic view of microbial genomes within a sample, allowing for the identification of novel microbes, functional genes, and metabolic pathways (Durazzi et al., 2021, Laudadio et al., 2018). This approach enables the investigation of entire microbial communities' genetic diversity and metabolic capabilities, offering insights into their ecological roles and interactions. On the other hand, amplicon sequencing strategies, such as 16S rRNA gene sequencing for bacteria (Baker et al., 2003) and ITS rRNA sequencing for fungi (Xu et al., 2016), offer a targeted approach to assess microbial diversity and community composition. These methods provide high-resolution taxonomic profiling, allowing researchers to identify specific microbial taxa in complex communities and explore diversity patterns across different environments or conditions.

Extracting genomic DNA from chicken faeces or GI content involves a series of steps to release and purify DNA for metagenomic sequencing. Initially, the material is mechanically homogenised in a lysis buffer containing detergents and proteases to break down cells and degrade proteins enzymatically. Employing mechanical and enzymatic homogenisation enhances the disruption of cell walls in bacteria, fungi, and archaea (Rothrock Jr et al., 2014). Subsequently, DNA is isolated by precipitation using alcohol or

binding to a solid phase, such as silica or magnetic beads, while contaminants are removed. The purified DNA is then eluted in a storage-compatible buffer or used immediately.

Monitoring growth performance parameters such as BWG, feed intake, FCR, and mortality rate offers a comprehensive evaluation of broiler performance. These metrics serve as indicators of overall health and functionality, providing a means to assess gut efficiency quantitatively. Additionally, they facilitate comparisons across studies, as FCR and body weight are standardised measurements known to be influenced by gut health (Akbarian et al., 2014, Dehghani-Tafti and Jahanian, 2016, Dittoe et al., 2022, Stanley et al., 2016).

Analysing intestinal tissue samples collected during necropsy offers valuable insights into gut health. Techniques such as measuring the villus height-to-crypt depth ratio and histological staining provide reliable assessments of gut morphology (Wilson et al., 2018). However, since this approach necessitates animal sacrifice, it primarily applies as an endpoint method.

Assessing immune-related parameters such as Ig concentrations and the expression of immune-related genes can offer valuable insights into the gut immune response (Borowska, 2016). Standard techniques employed for immune function assessment include ELISA, flow cytometry, and gene expression analysis (e.g., RT-qPCR). Given the importance of modulating and managing the immune system to reduce reliance on antibiotics, the GI microbiome emerges as a significant host factor influencing immunity (Lillehoj and Lee, 2012, Broom and Kogut, 2018, Kogut, 2013, Schokker et al., 2017, Yoon et al., 2022). Therefore, investigating immunological and serum parameters to develop and validate methods for assessing gut health holds considerable value for research and commercial poultry applications.

Assessing serum biomarkers such as acute phase proteins can yield insights into systemic inflammation and indirectly indicate gut health status (O'Reilly, 2016, Omar et al., 2021). Likewise, evaluating intestinal permeability through techniques like the FITC assay enables the assessment of gut barrier integrity and mucosal permeability (De Meyer et al., 2019, Ducatelle et al., 2018b).

Observing behavioural cues like feeding patterns, drinking habits, and activity levels offers valuable clues about the welfare and comfort of broilers, indirectly indicating their

gut health and performance. Stress has been shown to affect gut health and immunity, as evidenced by studies on feather-pecking layers (van der Eijk et al., 2019, Desbruslais et al., 2021).

Developing and validating these methodologies will facilitate a comprehensive assessment of broiler gut health and performance in research and commercial environments. The most effective approach for delineating broiler performance and gut health likely involves integrating multiple techniques (Ducatelle et al., 2018b). Moreover, refining and implementing these methodologies may spur the development of novel approaches for characterising gut health and performance in broilers.

3.1.2 Aims & Objectives

The primary aims and objectives of this thesis chapter are as follows:

1. Evaluate three genomic DNA extraction methods to identify the most effective approach for capturing the GI microbiome present in broiler faecal samples.
2. Validate a sequencing strategy to characterise the microbiome composition within broiler faecal samples effectively.
3. Refine and validate metabolomics methodologies to enable comprehensive analysis of metabolite profiles throughout the developmental stages of broilers, spanning from 7- to 35 dph.
4. Assess the feasibility and relevance of quantifying serum NAb titres as a potential marker for immune response in broiler chickens.

3.2 METHODS

3.2.1 Sample Collection and Storage

Jejunal, caecal and colon content of five adult broilers of mixed sex and body weight were received from Aviagen in November 2019 on dry ice from Aviagen, Edinburgh. The GI content of these five birds was stored at -70^{o}C. Samples were aliquoted to 200 mg under sterile conditions and defrosted overnight at 4^{o}C as required.

Eight randomly selected broiler faecal samples at 7 dph from a commercial broiler farm in Ayrshire, Scotland, were collected in March 2020. Samples were immediately aliquoted to 200 mg and stored at -20^{o}C whilst on the farm before being transported on dry ice to QIB and stored at -70^{o}C.

Aviagen collected the serum of 20 adult birds (10 male and ten female) at 4 and 5 weeks of age in September 2020 to develop and optimise an ELISA to detect serum immunoglobins.

3..2.2 Faecal Preparation for Targeted Hydrophilic Interaction Liquid Chromatography Spectrometry (HILIC-MS) for Taurine Detection

For targeted metabolomics analysis, taurine concentration was quantified in chicken faecal samples using the HILIC-MS amino acid AccQ-Tag Ultra Derivatization Kit. Seven faecal samples were selected from chicks at seven dph: four females and one male used in the ^{1}H-NMR analysis, and an additional two males from the same cohort. Samples were thawed on wet ice, and 10 mg, 50 mg or 100 mg of faeces were weighed into 1.7 mL Eppendorf, duplicated for technical replicates, in a microbiological safety cabinet. Taurine standards were prepared in 0.1 M HCl by Mark Philo (QIB) at 5, 2.5, 1, 0.25 and 0 µg/ml. Each sample was vortexed thoroughly with 1 mL 0.1 M HCl and centrifuged at maximum speed for 3 min. The supernatant (400 µl) was transferred to Whatman Autovial syringeless 0.2 uM filters before a 15-h run on HILIC-MS. Taurine concentrations were determined using area-under-curve analysis, and subsequent data analysis involved linear regression and graphical representation using GraphPad Prism.

3.3 RESULTS

3.3.1 Comparison of Genomic DNA Extraction Using Three Different Methods

3.3.1.1 *Genomic Yield and Quantification*

The three genomic DNA extraction methods being compared all use mechanical disruption and enzymatic degradation to lyse the cell walls of bacteria and fungi. The FastDNA Spin kit for Soil from MP Biomedicals, hereafter referred to as MP Bio, and Maxwell RSC PureFood GMO Authentication, hereafter referred to as Promega, utilise the FastPrep-24™ Classic (MP Bio) for bead-beating lysis. The QIAamp PowerFecal Pro DNA extraction method, referred to as Qiagen, has bead beating with a 24-holder vortex adapter. This method was recommended for fungal genomic DNA extraction but requires more handling. The MP Bio and Qiagen methods use spin columns and ethanol precipitation, whereas Promega uses a robot and magnetic beads.

No significant differences were observed between the three extraction methods (ANOVA, $P > 0.05$). Comparable DNA yields were attained across all genomic DNA extraction methods, with Qiagen yielding the highest mean genomic DNA. The average genomic DNA yield from the Promega method was 25.87 ng/µl (SD ± 28.49), and MP Bio was 36 ng/µl (SD ± 21.03). Qiagen was 74.20 ng/µl (SD ± 34.51) (Fig. 3.1). For the construction of metagenomic libraries, DNA extractions needed to reach a minimum concentration of 5 ng/µl, which all samples achieved.

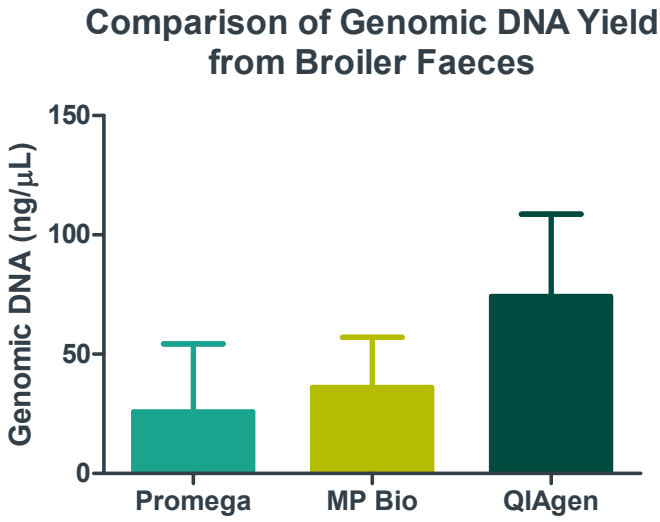

Figure 3.1 Concentration of genomic DNA yielded from broiler faeces comparing different extraction methods, quantified with Qubit Broad Range kit assay.

3.3.1.2 *Metabarcoding 16S ribosomal RNA Sequencing*

The quality of 16S rRNA amplicon metagenomic sequences obtained from the Promega and MP Bio methods was compared. No significant differences were observed in the total number of reads (**Fig. 3.2A**) or the number of reads that passed Illumina Mi-Seq intrinsic quality control filtering. Likewise, there were no significant differences in the number of OTUs observed in broiler faeces between these two methods, although the Promega method exhibited a slightly higher mean (Fig. 3.2B).

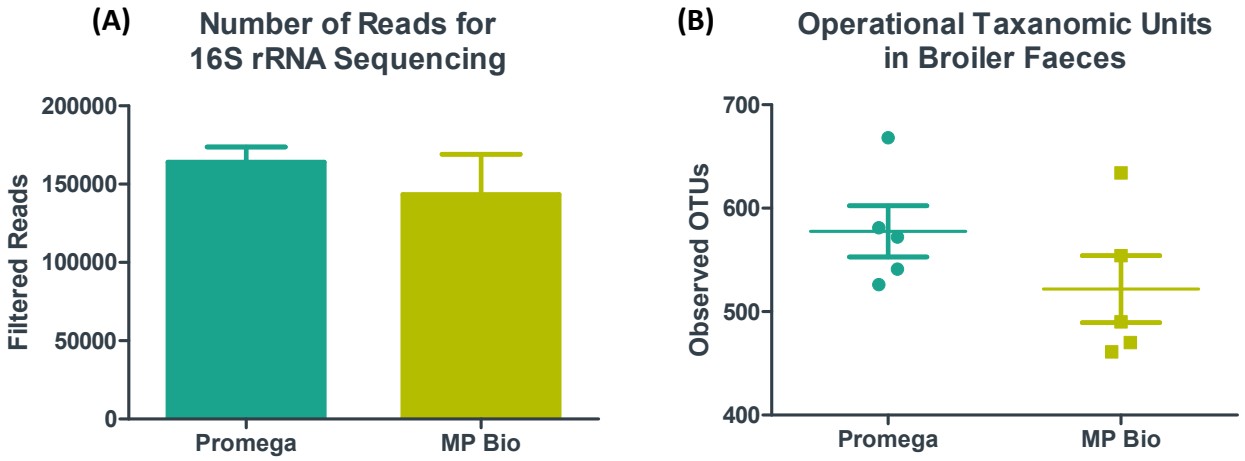

Figure 3.2 (A) The total number of 16S rRNA amplicon reads as determined by the Illumina Mi-Seq instrument and (B) the number of 16S rRNA OTUs identified with each extraction method.

Assessing α- and β-diversity is crucial for understanding microbiome composition and structure. Decreased diversity is often associated with compromised gut health (Rodrigues et al., 2020). No significant differences were observed in α- and β-diversity between the Promega and MP Bio methods in 16S rRNA amplicon sequencing. However, as with the number of reads and OTUs, the Promega method exhibited a higher mean Shannon Index than the MP Bio method (Fig. 3.3A). There was also more variability between faecal α-diversities with the MP Bio method. Conversely, the comparative compositions assessed by Bray-Curtis were similar (Fig. 3.3B).

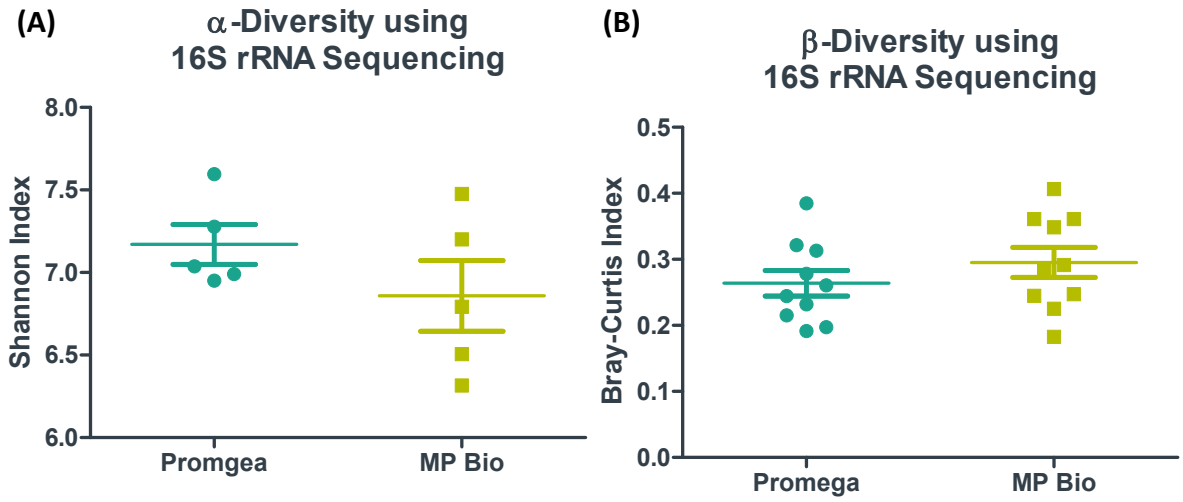

Figure 3.3 Broiler faecal bacterial α-diversity measure by Shannon Index (A) and β-diversity measure by Bray-Curtis similarities (B), comparing two extraction methods for 16S rRNA sequencing.

There were no significant differences between bacterial communities when comparing Promega and MP Bio. The same most abundant genera were identified, but with MP Bio, they accounted for over 80% relative abundance in just one of the five samples (Fig. 3.4B) suggesting more low abundant taxa are being captured with MP Bio. In contrast, they accounted for >80% of the extracted with the Promega kit (Fig. 3.4A). The dominant bacteria were as would be expected in the chicken GI microbiome, with *Alistipes* an indicator of a mature chicken microbiome (Richards et al., 2019).

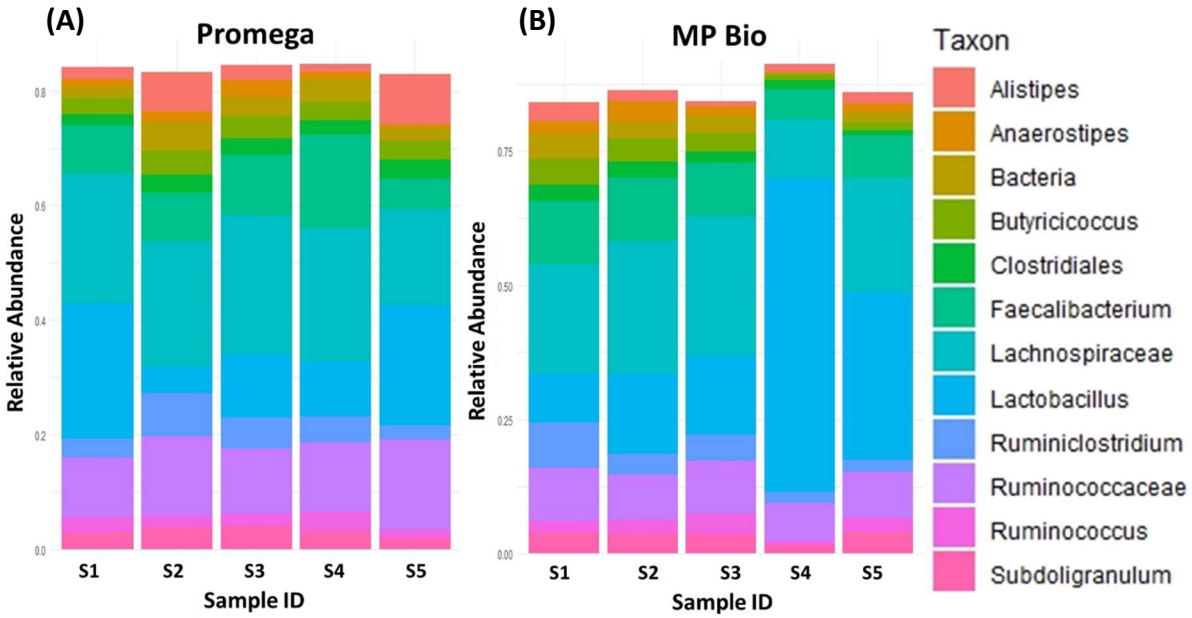

Figure 3.4 Bacterial composition in broiler faeces using 16S rRNA amplicon sequencing, comparing MP Bio and Promega extraction methods.

No differences were identified between the Promega and MP Bio DNA extraction methods for 16S rRNA amplicon sequencing, as evidenced by comparing read counts and composition. Hence, either method is deemed suitable.

3.3.1.2 *Metabarcoding ITS1 ribosomal RNA Sequencing*

The quality of fungal ITS1 rRNA amplicon metagenomic sequences obtained from the Promega and Qiagen genomic DNA extraction methods was compared. Despite the absence of significant differences in the total number of reads (Fig. 3.5A) or the number of reads that passed Illumina Mi-Seq intrinsic quality control filtering, significantly more ASVs were observed in sequences where DNA was extracted with the Promega method (ANOVA, P=0.045) (Fig. 3.5B).

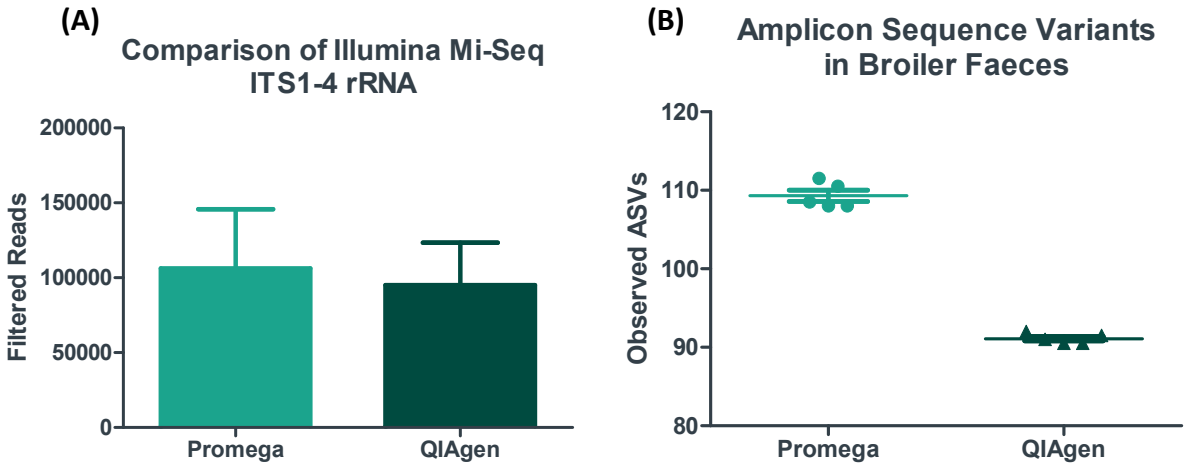

Figure 3.5 Total ITS1 rRNA amplicon reads determined by the Illumina Mi-Seq instrument (A) and 16S rRNA OTU identified with each extraction method (B).

Considering the difference in observed ASVs between the Promega and Qiagen methods, it is unsurprising that the α-diversity from Promega is significantly higher ($P < 0.0001$) (**Fig. 3.6A**), demonstrating a greater species richness detected. However, the β-diversity demonstrates high similarity between DNA extraction methods (**Fig. 3.6B**). This indicates that while the fungal communities are diverse within each faecal sample, the overall composition of the communities is similar across all samples.

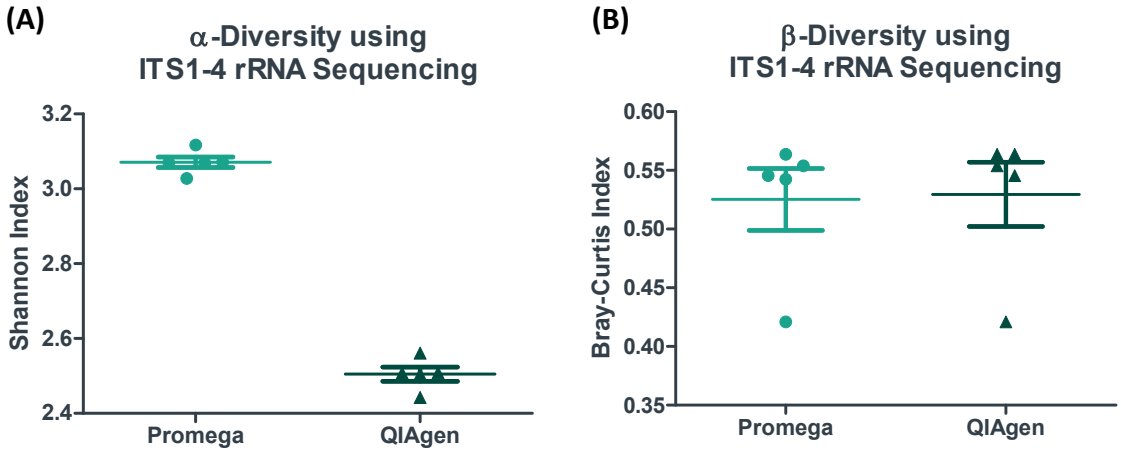

Figure 3.6 Broiler faecal fungal α-diversity measured by Shannon Index (A) and β-diversity measured by Bray-Curtis similarities (B), comparing two extraction methods for ITS1 rRNA amplicon sequencing.

No significant differences in ITS1 rRNA composition were identified between Promega and Qiagen samples. However, *Alternaria* dominates both Promega samples (Fig. 3.7), whereas *Saccharomycopsis* is dominant in one Qiagen sample (Fig. 3.7). *Alternaria*, characterised by its filamentous nature, possesses a complex cell wall (Frau et al., 2019). In contrast, *Saccharomycopsis* is a yeast species known for simpler cell walls than filamentous fungi (Kurtzman et al., 2011). Additionally, while *Saccharomycopsis* can produce secondary metabolites, it may not produce the same diversity or quantity of toxins as *Alternaria*, an opportunistic plant pathogen. Consequently, the potential interference from secondary metabolites during DNA extraction might be lower with *Saccharomycopsis*. Extraction methods optimised for filamentous fungi, such as *Alternaria*, often involve mechanical disruption to break down the cell wall and release DNA. Using the Fast-Prep in the Promega method likely contributes to the observable difference in the most abundant genera compared to the extraction method in Qiagen.

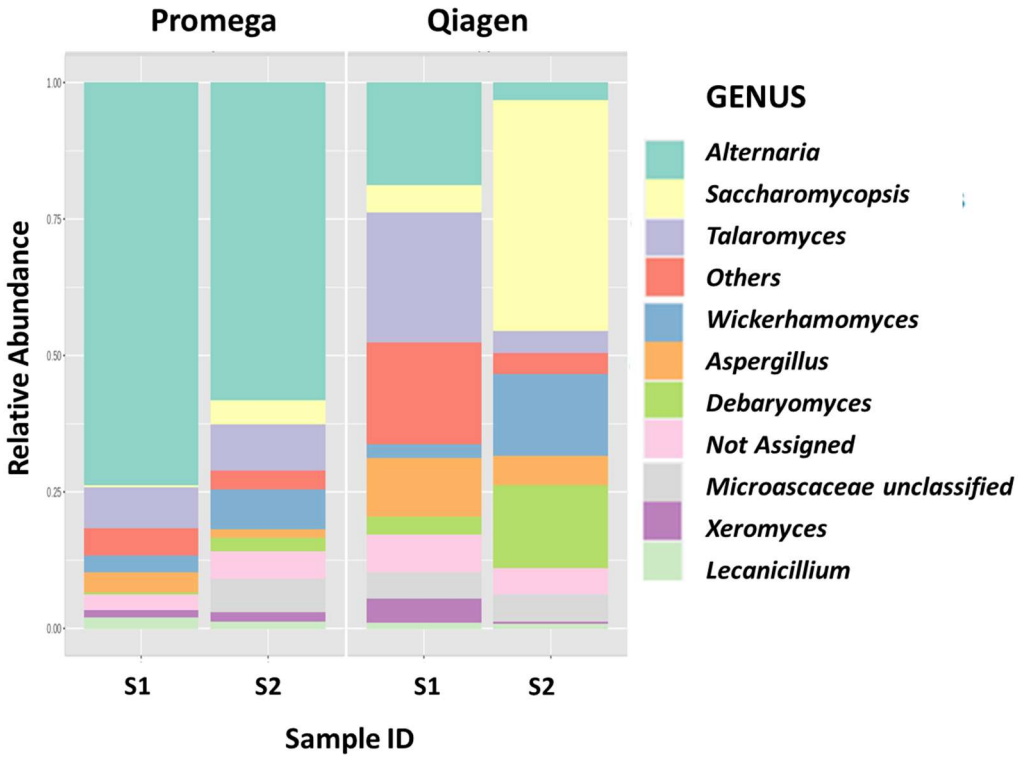

Figure 3.7 Top 10 most abundant fungal genera in broiler faeces identified with ITS1 rRNA amplicon sequencing.

No significant differences were observed in β-diversity or abundance between Promega and Qiagen methods for ITS1 rRNA amplicon sequencing. However, the Promega method yielded significantly higher numbers of observed ASVs and α-diversity measures. This indicates that the Promega method is more suitable for extracting genomic DNA when investigating fungal communities in broiler faeces. Additionally, the Promega method demonstrated efficacy in extracting DNA from filamentous fungal species, which typically possess more complex cell walls (Frau et al., 2019). This suggests that the Promega method may better capture the full spectrum of fungi in broiler faeces.

3.3.1.2 *Shotgun Metagenomic Sequencing*

Genomic DNA extracted using three different methods was assessed through shotgun metagenomic sequencing. The Promega extraction method yielded an average of 3,332,286 quality-filtered reads, MP Bio yielded 6,837,800 reads, and Qiagen yielded 3,505,514 reads (Fig. 3.8A). Although MP Bio resulted in the highest mean number of filtered reads, no significant differences were observed between the three extraction methods. Since shotgun metagenomic sequencing offers a comprehensive view of the microbial community without reliance on specific marker genes, the number of species in each sample was measured instead of using OTUs or ASVs. Notably, no significant difference was observed in the number of species identified across the samples (Fig. 3.8B).

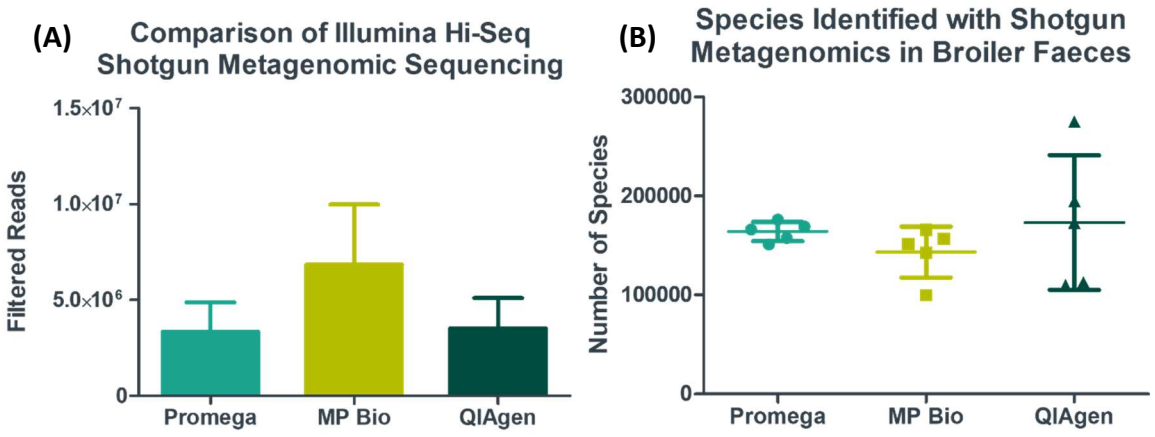

Figure 3.8 Total number of reads from Illumina Hi-Seq (A) and species identified with shotgun metagenomic sequencing (B), comparing three genomic DNA extraction methods.

The Shannon index revealed that the Promega extraction method exhibited significantly higher α-diversity than the MP Bio and Qiagen methods (t-test, P=0.02) (Fig. 3.9A). This suggests that the Promega method captures a greater richness and evenness of species in broiler faeces. Additionally, a significant difference was observed in β-diversity between Promega and MP Bio (t-test, P=0.01) (Fig. 3.9B), indicating that DNA extracted using the Promega method shows greater similarity in community composition. However, no significant differences were found between Qiagen and the other extraction methods. Furthermore, the standard deviation of Bray-Curtis values in faecal samples extracted with MP Bio and Qiagen methods was higher, suggesting greater variability.

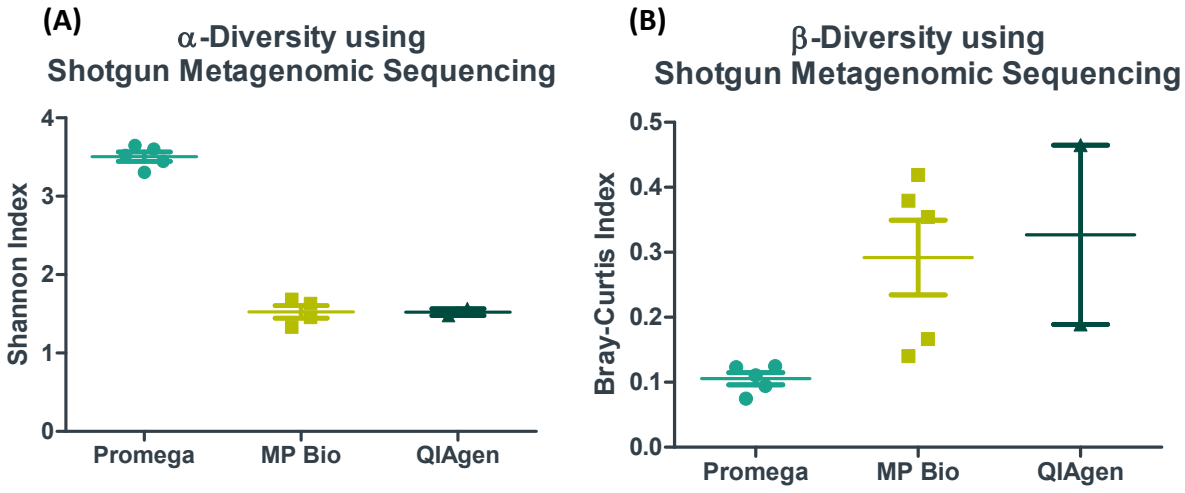

Figure 3.9 Broiler faecal fungal α-diversity measured by Shannon Index (A) and β-diversity measured by Bray-Curtis similarities (B), comparing two extraction methods for I1-4TS rRNA sequencing.

The top ten most abundant species were consistent between MP Bio (Fig. 3.10B) and Qiagen (Fig. 3.10C) extraction methods. This coherence aligns with the similarity observed in both the Shannon index and Bray-Curtis measurements for these two methods. Across all extraction methods, *Lactobacilli* predominated, representing six of the most abundant species. In the case of MP Bio and Qiagen, *E. coli*, *Escherichia* unclassified, *Bacteroides fragilis*, and *Butyricicoccus pullicaecorum* rounded out the top species in broiler faeces. In the Promega-extracted samples (Fig. 3.10A), *E. coli* remained dominant, yet uniquely, *Ruminococcus torques* and *Enterococcus hirae* were among the most abundant

species. Notably, one Promega sample exhibited avian endogenous retrovirus within the top ten species, marking the sole non-bacterial taxon in this analysis. However, it is important to acknowledge potential inaccuracies due to the shared genomic regions with similar viruses (Sacco.). Across all extraction methods, the top ten most abundant species collectively accounted for over 95% of the relative abundance.

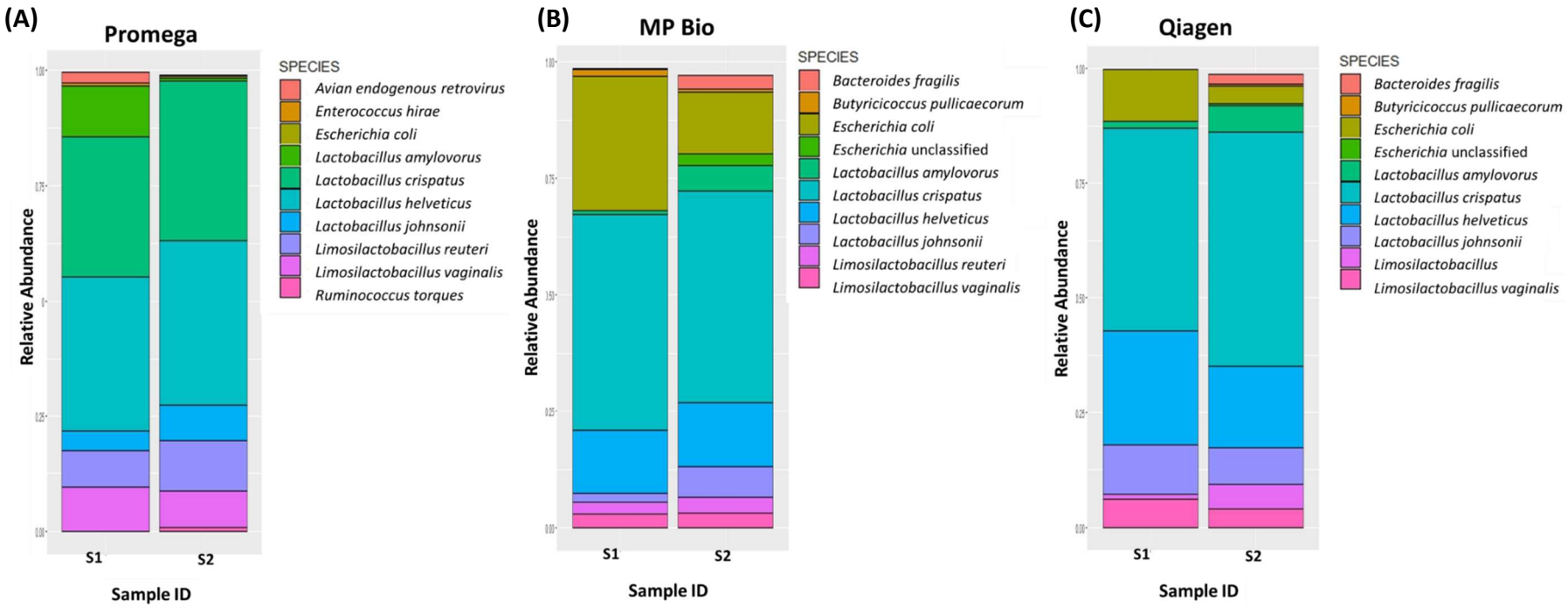

Figure 3.10 Comparison of the Top 10 species in broiler faeces using three genomic DNA extraction methods: (A) Promega, (B) Qiagen, and (C) MP Bio.

Despite generating more reads with MP Bio, minimal significant differences were observed in the Illumina Hi-Seq shotgun metagenomic sequences of broiler faeces when comparing three genomic DNA extraction methods. The Promega method exhibited higher α-diversity and captured more similar microbiomes than those obtained with MP Bio and Qiagen. Considering its superior performance in ITS1 rRNA amplicon sequencing and the lack of significant differences between Promega and MP Bio kits for 16S rRNA amplicon sequencing, Promega emerged as the optimal choice for genomic DNA extraction in this project.

3.3.2 Comparison of Sequencing Strategies

Shotgun metagenomic sequencing is conducted using the Illumina Hi-Seq platform, while 16S rRNA and ITS1 amplicon sequencing utilise the Illumina Mi-Seq platform. Consequently, shotgun metagenomic sequencing was expected to yield significantly more reads (ANOVA, P=0.002). Indeed, shotgun metagenomic sequencing produced an average of 3,332,286 reads per sample, whereas 16S rRNA amplicon sequencing yielded an average of 164,029 reads, and ITS1 rRNA amplicon sequencing resulted in an average of 106,259 reads (Fig. 3.11A).

One advantage of shotgun metagenomic sequencing over amplicon sequencing is its capability to examine microorganisms from multiple domains (Fig. 3.11B). In the case of bacteria, shotgun metagenomic sequences yielded 3,330,969 reads, representing 99.9% of all faecal shotgun reads, while 16S rRNA amplicon sequencing identified an average of 577 OTUs in broiler faeces. Conversely, eukaryotes were not detected in faecal samples using shotgun metagenomic sequencing, whereas ITS1 rRNA amplicon sequencing observed an average of 109 ASVs in broiler faeces. Additionally, only shotgun metagenomic sequencing detected viruses, averaging 1,311 reads per faecal sample. This highlights the superiority of shotgun metagenomic sequencing in identifying bacteria in broiler faeces, although current databases are insufficient for assigning eukaryotic taxonomy. Therefore, ITS1 rRNA amplicon sequencing should be conducted alongside shotgun metagenomic sequencing to capture the fungal component of the broiler GI microbiome.

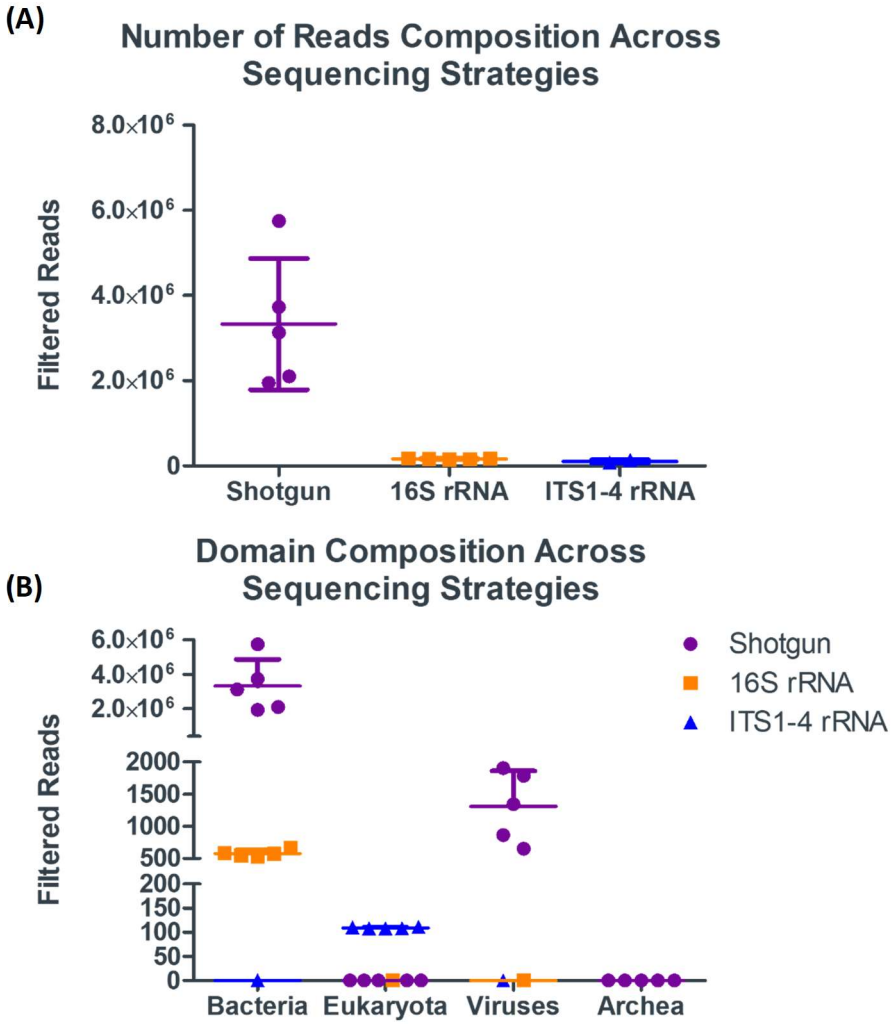

Figure 3.11 Number of filtered reads across sequencing strategies (A) and domain composition (B).

Comparing the diversity obtained from shotgun metagenomic and amplicon sequencing strategies allows for a comprehensive assessment of microbial communities; however, shotgun metagenomic sequencing provides higher taxonomic resolution at the species or strain level than rRNA amplicon sequencing. As a result, some taxa identified by shotgun metagenomic sequencing may not be accurately classified at the same level by amplicon sequencing.

The Shannon Index obtained from shotgun metagenomic sequencing significantly surpassed that from 16S rRNA (t-test, P=0.0019) and ITS1 rRNA (t-test, P=0.0079) sequencing, indicating a greater species richness and evenness, hence higher diversity, with the shotgun metagenomic approach (Fig. 3.12A). This outcome was expected, considering the larger number of sequences generated by shotgun metagenomic sequencing covering

both bacterial and viral domains. The Bray-Curtis analysis demonstrated a significant difference between shotgun metagenomic and ITS1 rRNA amplicon sequencing (ANOVA, P=0.0019), but no differences were observed between 16S rRNA amplicon sequencing and other sequencing strategies (Fig. 3.12A). This indicates a high degree of similarity in the faecal microbiome composition sequenced with shotgun metagenomic sequencing. Moreover, the absence of a significant difference between shotgun and 16S rRNA Bray-Curtis analyses suggests a similar composition. This aligns with both sequencing strategies targeting the bacterial component of the faecal microbiome. In contrast, ITS1 rRNA amplicon sequencing, focusing on fungi, which are less abundant than bacteria, yielded a composition dissimilar to other sequencing strategies.

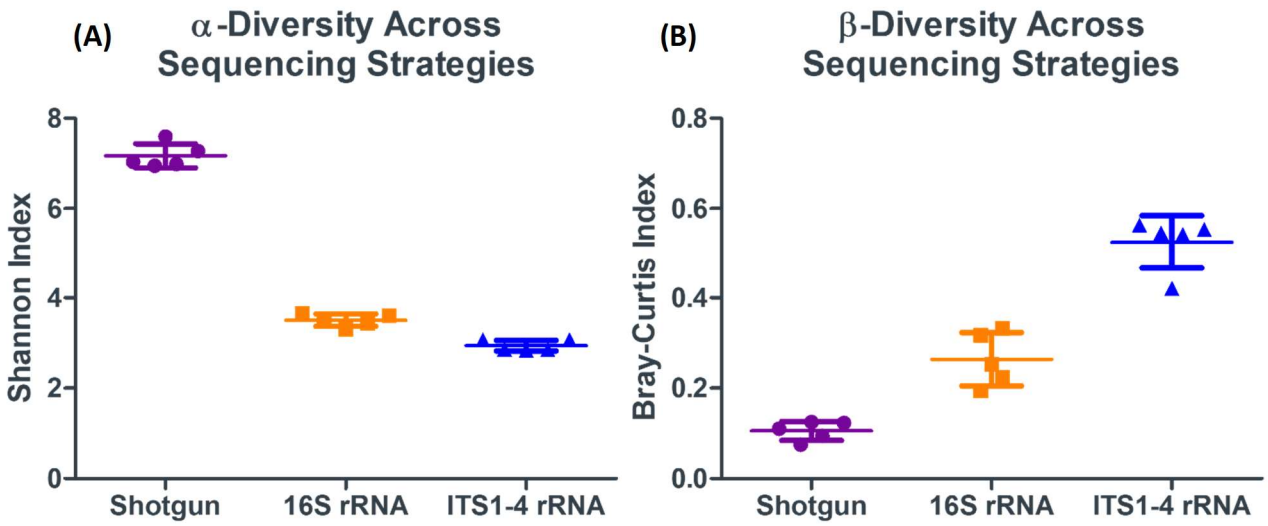

Figure 3.12 Broiler faeces α-diversity measured by Shannon Index **(A)** and β-diversity measured by Bray-Curtis similarities **(B),** comparing two extraction methods for I1-4TS rRNA sequencing.

The 16S and ITS1 amplicon sequence data permits genus taxonomic assignment at best, whereas shotgun metagenomic sequencing enables strain level, giving greater specificity. Both shotgun metagenomic and 16S rRNA capture *Lactobacilli* and the *Ruminococcus* genus. Still, *Lactobacilli* dominate only in the shotgun metagenomic sequencing (Fig. 3.13A) and not in the 16S rRNA amplicon composition (Fig. 3.13B). *E. coli* and *E. hirae* are unique to the bacterial composition from shotgun metagenomic data,

whereas 16S rRNA additionally captured *Alistipes*, *Anaerostipes*, *Butyricicoccus*, *Faecalibacterium*, *Ruminiclostridium* and *Subdoligranulum* genera as well as *Clostridiales* (order), *Lachnospiraceae* (family), *Ruminococcaceae* (family) as part of the most abundant taxa in broiler faeces. These taxa were all identified in the shotgun metagenomic data but were slightly less abundant. Whereas all genera identified through ITS1 amplicon sequencing were unique, comprehensively capturing the faecal mycobiome (Fig. 3.13C).

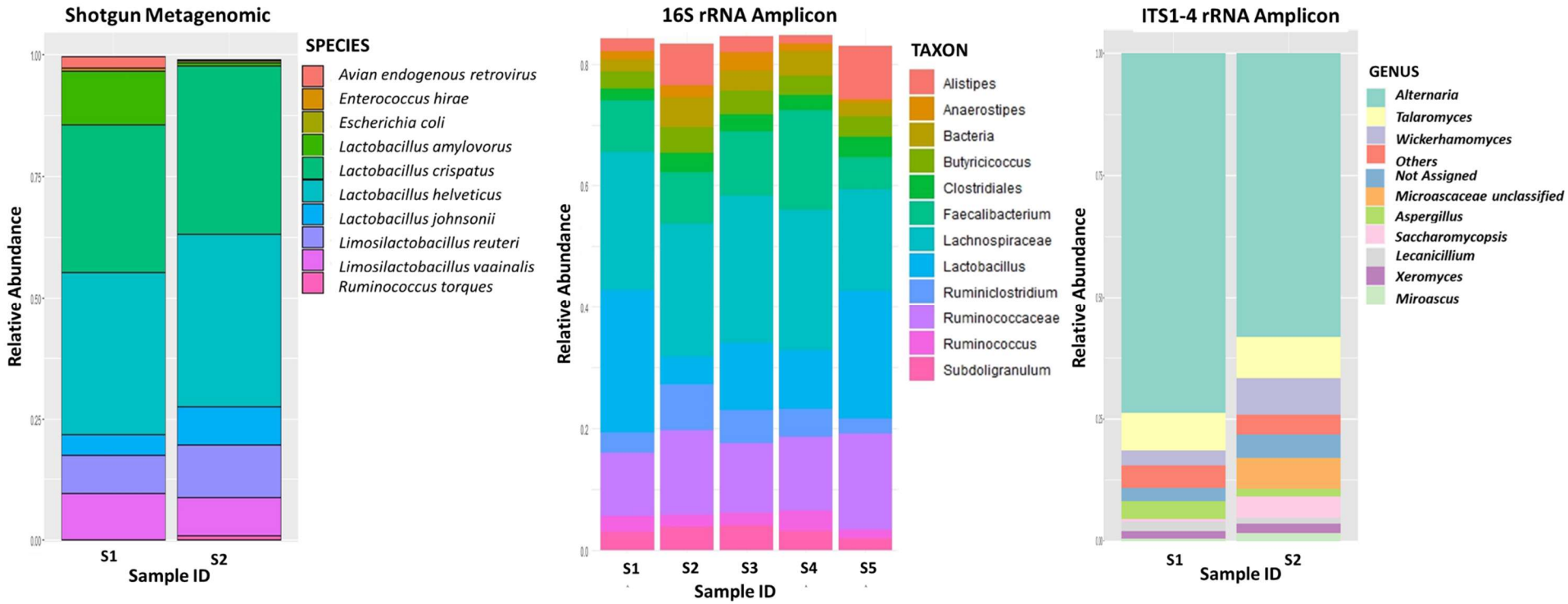

Figure 3.13 Analysis of the most abundant **(A)** shotgun metagenomic species (visualised with phyloseq), **(B)** 16S rRNA taxa (visualised with QIIME2) and **(C)** ITS1 rRNA genera (visualised with MetaboAnalyst 2.0 R package) from samples extracted with the Promega method.

The shotgun metagenomic data allows one to explore gene family pathways, functional genes, and AMR and virulence factors. The ResFinder database detected an average of three AMR genes, while CARD identified an average of 19; however, due to variance, the difference is not statistically significant (**Fig. 3.14**). Specifically, the ResFinder database identified only five AMR genes in the broiler faeces. Among these, four were resistant to tetracycline; the remaining one, *InuC*, confers resistance to lincosamide. Interestingly, the *InuC* gene in this dataset has been associated with pig farm seepage pollution, suggesting its potential as a biomarker (Matjuda and Aiyegoro, 2019). CARD identified 52 AMR genes, with over half having antibiotic efflux resistance. Three AMR genes were associated with resistance to tetracycline. Only one gene was associated with resistance to macrolides, carbapenems, beta-lactams and aminoglycosides. The CARD database captured a more comprehensive range and number of AMR genes compared to ResFinder.

Five virulence factors were identified in the broiler's faeces, with two samples having none identified. Two very close gene clusters were annotated as *E. coli* lysis genes. Similarly, another two close gene clusters were annotated as *E. coli* chromosomal genes. The final virulence factor was a gene cluster annotated as an *Enterococcus faecalis* plasmid. Virulence factors offer an insight into pathogenicity; however, the genes identified in this analysis are for survival, not toxicity, and therefore not of concern with respect to animal health.

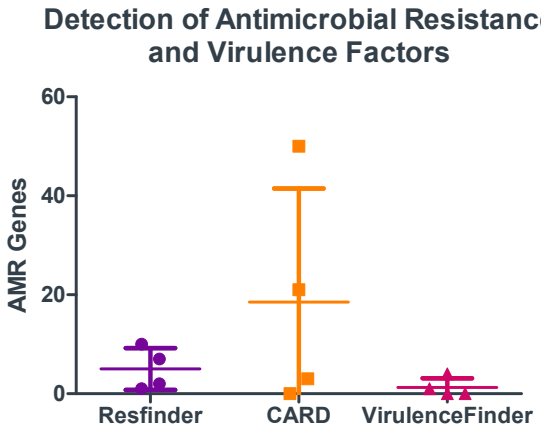

Figure 3.14 Number of AMR genes referencing the CARD and ResFinder databases and virulence factors (using VirulenceFinder) identified within chicken faeces.

3.3.2 Metabolomics

3.3.2.1 *Untargeted Faecal and Serum Metabolomics Using ^{1}H-NMR*

The focus is on developing methods using faeces and serum whenever possible, as neither requires the sacrifice of animals. Faeces are easily accessible, while blood is routinely collected to check genetic integrity and health status.

The signal-to-noise ratio is an important criterion when acquiring spectral data to determine sensitivity and ensure accurate integration of peaks. A signal-to-noise ratio greater than ten is considered of sufficient quality for publication. Both faecal and serum samples exhibited an adequate signal-to-noise ratio at 16 scans, with improvements observed with increased scans (Fig. 3.15A). A comparison between adult and chick faeces further demonstrated no differences in signal-to-noise ratio, supporting the suitability of this method for the entire broiler life cycle.

There was no difference in metabolite concentration between 32 and 64 scans in serum at 35 dph (Fig. 3.15B), faeces at 35 dph (Fig. 3.15C) or faeces at 7 dph (Fig. 3.15D). There was no difference between metabolite concentration at 16 scans in faeces at either age. This demonstrates that although increasing the number of scans increases the signal-to-noise ratio of the spectra and sensitivity, it does not affect the measurement of concentrations. However, fewer scans appear to cause greater variability between replicates, as shown by the SD (Fig. 3.15B-D).

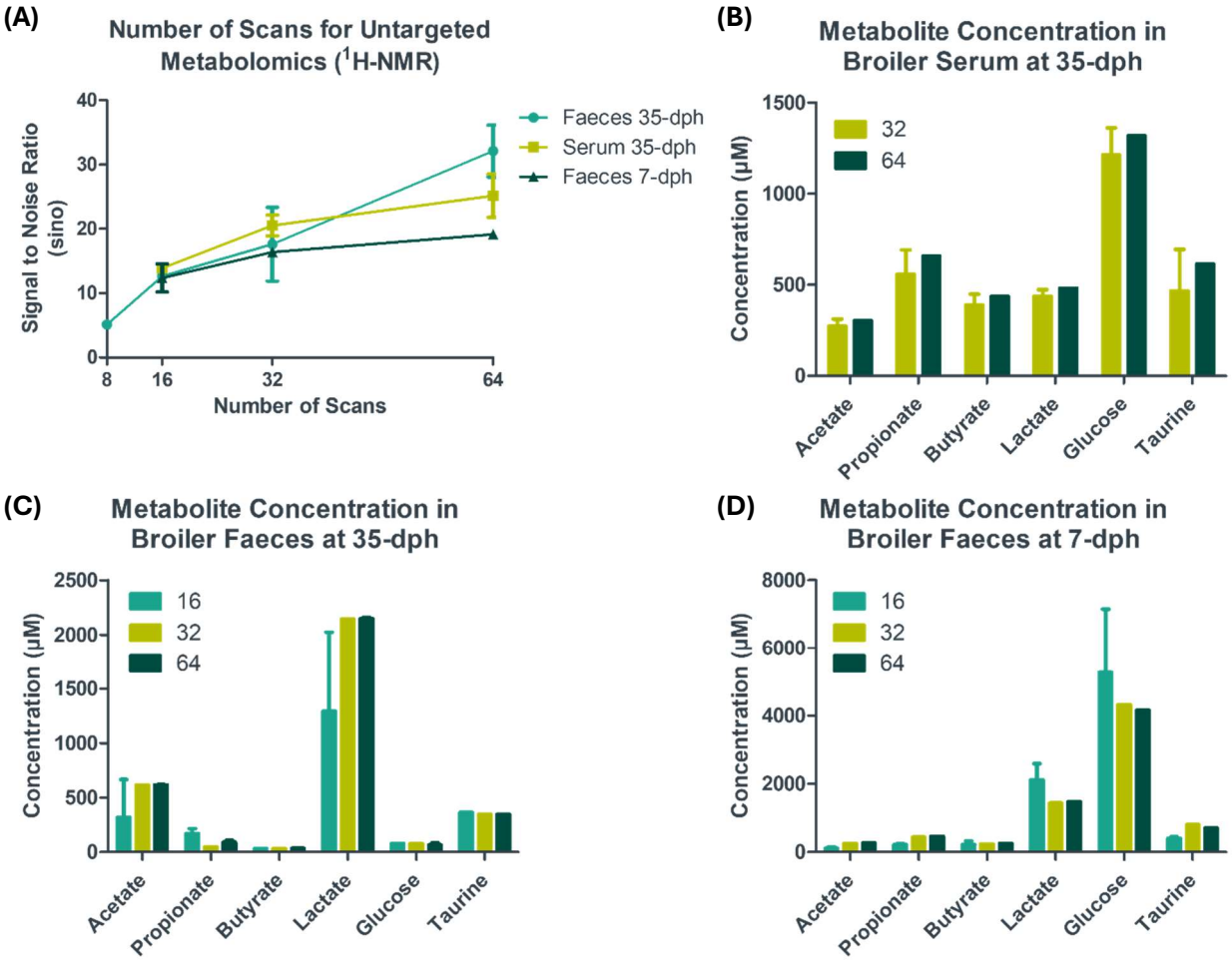

Figure 3.15 Measurements of the signal-to-noise ratio of broiler faeces and serum against the number of scans used to acquire the spectra (A) and effect of scan number on metabolite concentrations in serum (B), faeces at 35 dph (C) and faeces at 7 dph (D).

Visualising the full spectrum assists in identifying inconsistencies between samples (Fig. 3.16A). The most intense peak is at 0 ppm, with the TSP serving as an internal standard for variations in experimental conditions. Trimethylsilylpropanoic (TSP) in the NMR buffer acts as a reference peak that appears at a consistent chemical shift in the spectrum, allowing for accurate determination of the chemical shifts of other peaks in the spectrum. The second most intense peak on the spectra is a doublet (1:1) at 1.334 ppm, which is lactate (Le Roy et al., 2016). The differences in peak positions on the spectrum and peak intensity can easily identify differences between serum and faeces. The intensity of the peak in faeces is higher than in serum, demonstrating a greater concentration (Fig. 3.16B). Quantifying the integrated peaks revealed significantly more lactate in faeces than serum in adult broilers (ANOVA, P=0.02).

At 35 dph, lactate exhibits a higher concentration in faeces compared to serum (Fig. 3.16C) (ANOVA, $P < 0.001$). Conversely, glucose concentrations were significantly higher in serum than in faeces (ANOVA, $P < 0.01$). Propionate and butyrate play pivotal roles as SCFA in modulating the broiler microbiome and metabolome (Liao et al., 2020), underscoring their significance in this analysis. While an untargeted approach will be adopted, detecting anticipated compounds of interest remains crucial. SCFA are predominantly synthesised in the caeca and are detectable in lower concentrations in the large intestine and faeces (Cao et al., 2020). Glucose emerged as the most prevalent metabolite in serum, crucial for cellular functions (Rezende et al., 2017). Taurine, the predominant bile acid conjugate in chickens, is a proposed marker for dysbacteriosis when detected in faeces or serum, indicative of irregular bile acid deconjugation by the gut microbiome (Bailey, 2010). Utilising these six metabolites as reference points for method development ensures the comprehensive capture of essential metabolites for validation as markers in either faecal or serum samples.

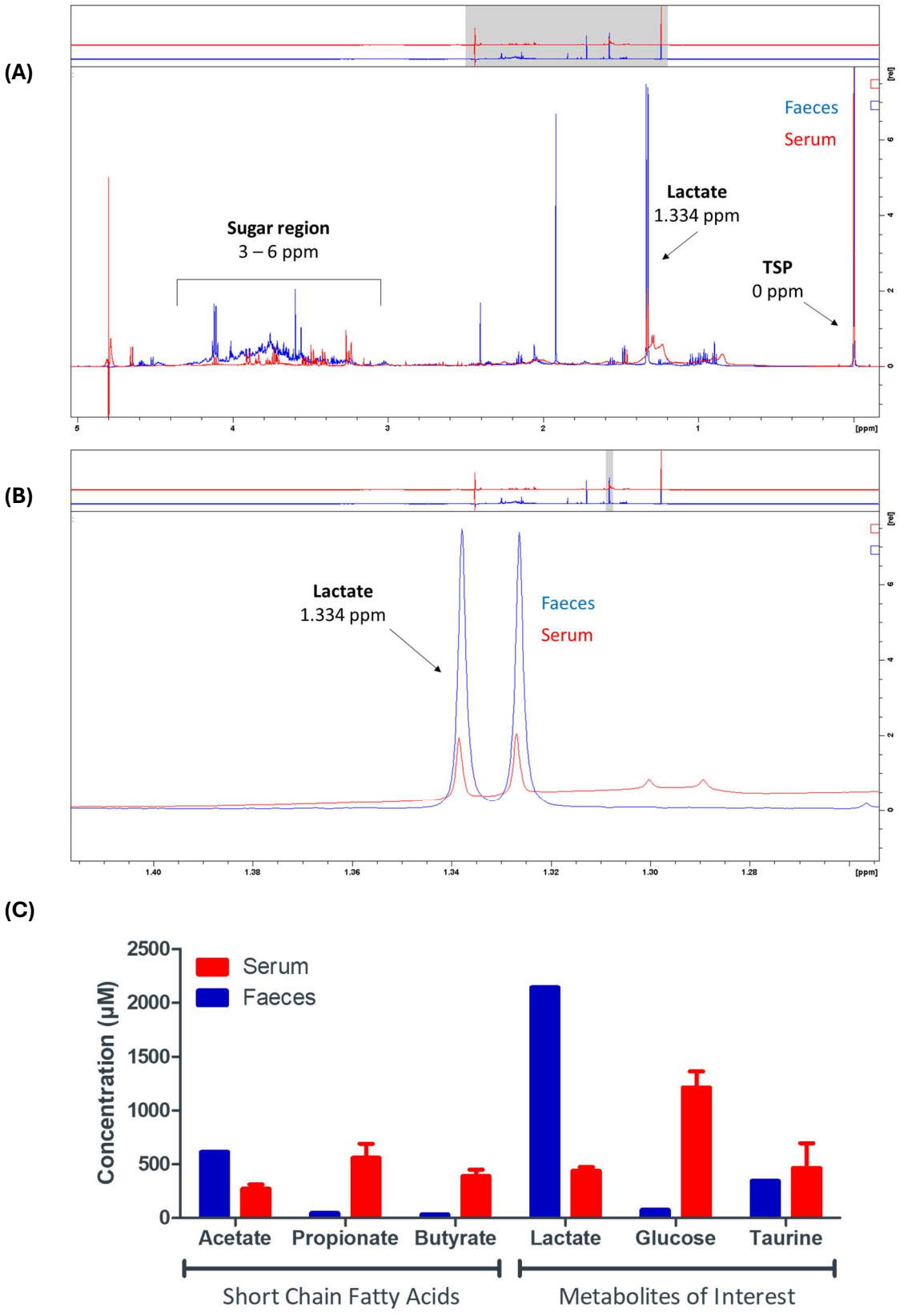

Figure 3.16 Full ^{1}H-NMR spectra of broiler faeces and serum **(A)**, lactate **(B)** and the quantified concentrations of six metabolites at 32 scans **(C)**.

The spectra from the 7 dph faeces exhibited higher peaks at 1.334 ppm (Fig. 3.17A), suggesting a higher concentration of lactate in these samples compared to the 35 dph faeces, consistent with the predominance of *Lactobacilli* in the chick faecal composition (Angelakis and Raoult, 2010, Jurburg et al., 2019, Schokker et al., 2021). Quantification of these peaks confirmed a significantly higher faecal lactate concentration at 7 dph compared to 35 dph ($P < 0.05$) (Fig. 3.17B). In contrast, there was a significantly higher acetate concentration at 35 dph than at 7 dph ($P < 0.01$), possibly indicating microbiome maturation attributed to SCFA production capability. However, consistent concentrations of butyrate, propionate, glucose, and taurine were detected in both adult and chick faeces, further affirming the method's suitability for monitoring broiler faeces for markers throughout their life cycle.

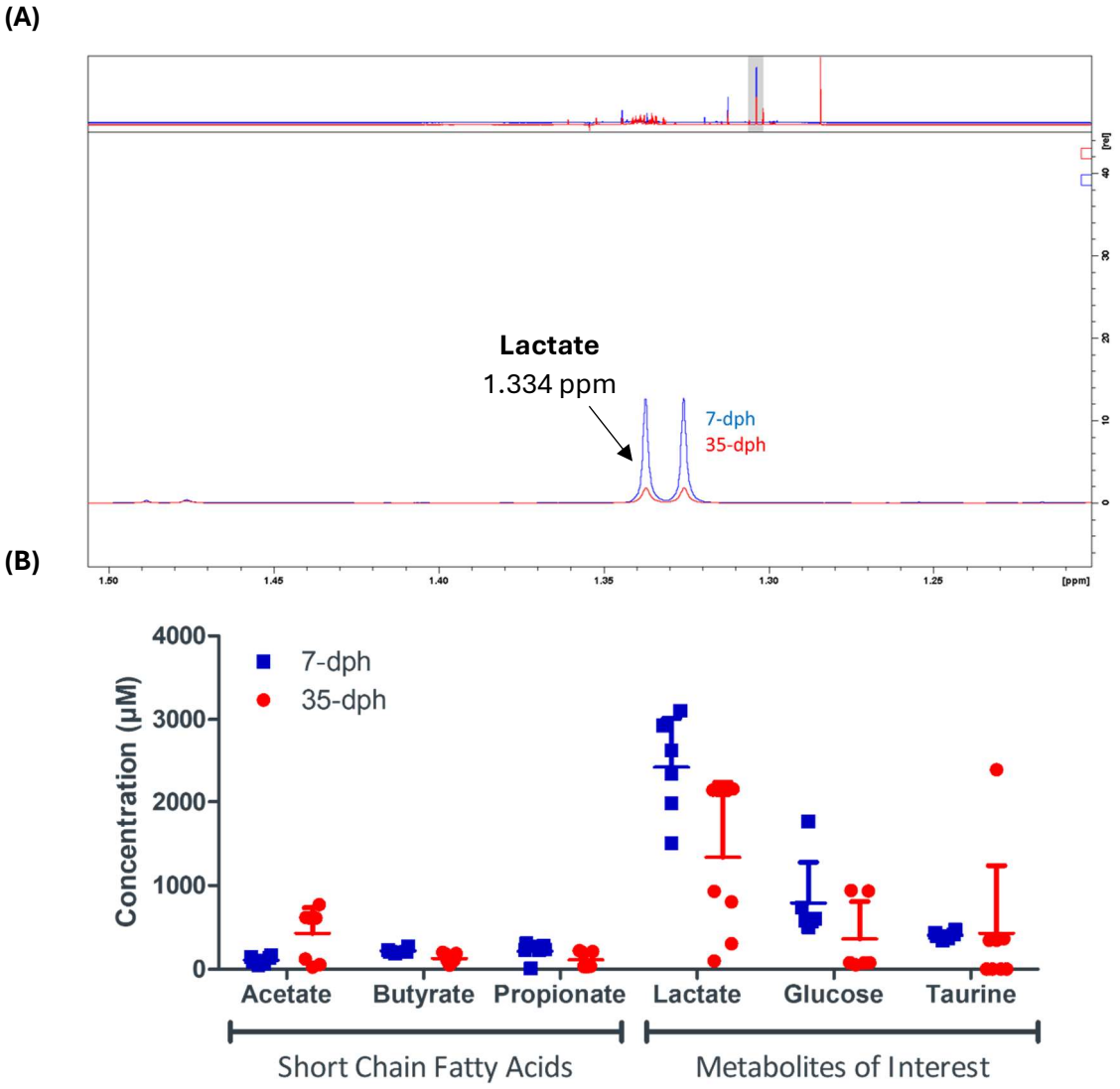

Figure 3.17 shows an example of stacked spectra for broiler faeces at 7—and 35 dph, with the identification of lactate (doublet) at 1.334ppm (A) and concentrations of six metabolites (B).

3.3.2.2 *Detection of Taurine in Targeted Faecal Metabolomics Using HILIC-MS*

The faecal metabolome of broiler chicks was chosen to validate targeted metabolomics, focusing particularly on taurine. This decision was based on the observation from untargeted ^{1}H-NMR analysis that faecal samples from broiler chicks at 7 dph exhibited

greater consistency than those at 35 dph. This consistency renders the 7 dph samples more suitable for method development and validation.

In contrast to untargeted metabolomics, which can identify a broad range of metabolites, targeted metabolomics focuses on precisely measuring specific compounds. Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry (HILIC-MS) is a potent analytical technique for separating, identifying, and quantifying polar and hydrophilic compounds in complex mixtures, often employed in faecal metabolomics studies. Markers of performance or gut health identified through untargeted analysis can be further validated with targeted HILIC-MS. Establishing a targeted approach for routine testing could significantly enhance industry accessibility.

Taurine concentration was determined with linear regression of control standards, with an R^2 score of 0.9985 (Fig. 3.18A). There was no significant difference in taurine concentration in broiler chick faeces between males or females or between the individual animals (data not shown), with high consistency between technical replicates.

Both targeted and untargeted analyses of the faecal metabolome revealed an average taurine concentration of 190 µM (1.25 µg/mL) (**Fig. 3.18B**). While the HILIC-MS method proved accurate and held promise for future applications with low-concentration faecal compounds, untargeted metabolomic analysis using ^{1}H-NMR is deemed adequate for detecting potential metabolome biomarkers.

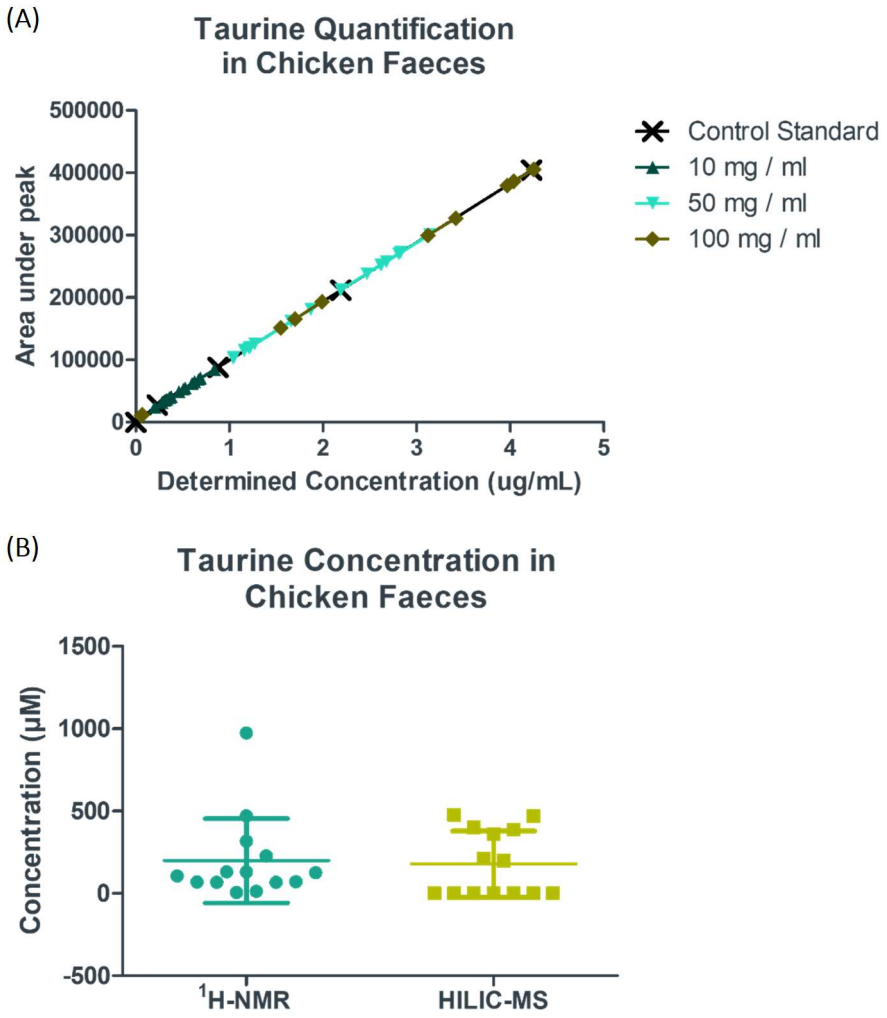

Figure 3.18 Quantification of the HILIC-MS with taurine control standards **(A)** and a comparison of the detected taurine concentrations with untargeted ^{1}H-NMR metabolomics and targeted HILIC-MS **(B)**.

Proton NMR-based untargeted metabolomic analyses offer a comprehensive exploration of compounds, aiding in discovering potential faecal or serum markers indicative of broiler performance and gut health across various experimental conditions. Upon marker discovery, targeted HILIC-MS metabolomics will be used to validate these compounds' association with broiler performance and gut health.

3.3.3 Detection of Natural Antibodies in Broiler Serum Using Indirect 2-Step ELISA Against Unknown Antigen KLH

The two-step indirect ELISA designed to detect natural antibodies binding to the unknown antigen KLH was shown to be a fitting assay for exploring layer immunity. In adapting this method for broilers, 18 surplus serum samples were procured from Aviagen's 4- and 5-week breeding stock, ensuring an equal distribution across sexes. This adaptation draws from the work of Berghof et al. (Berghof et al., 2018, Berghof et al., 2019, Berghof et al., 2015b, Van Der Klein et al., 2015), who initially assessed NAb levels in established layers, all of which were female and aged between 13 and 14 weeks. Given the advanced age of layers, they have had more time for immune system maturation than the abbreviated 35- to 40-day life cycle typical of commercial broilers. Hence, to gauge the method's applicability, the intermediary 4- and 5-week-old broiler breeding stock is a suitable sample time point for method development, representing adult broilers aged 28 to 35 dph.

Antibody titres were determined with pooled pre-diluted serum samples as control standards, using a 7-point 10-fold dilution starting at 1:10 (Fig. 3.19). Values were determined using linear regression with an R^2 greater than 0.8 (as described in CHAPTER 2 2.1 General Methods). Relative NAb titres were calculated with $Yij = \mu + Pi$ where Yij is IgM, IgG or IgA titre or IgT (all immunoglobins and IgY), μ is mean, and Pi is plate variation using coefficient of variance (CV=populations standard deviation/population mean).

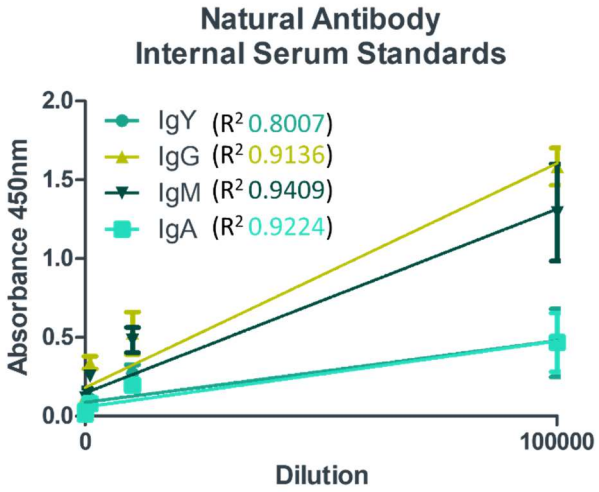

Figure 3.19 Linear regression of internal standards used to calculate the serum NAb titres.

The coefficients of variance among technical replicates, where the same sample was repeated on different plates, remained below 10%, meeting the minimum requirement for a consistent and reliable ELISA (Fig 3.20A). Conversely, the variance observed among biological replicates representing individual birds was between 47% and 192%, indicating variability between individual animals (Fig. 3.20B). Notably, there was no significant disparity in the coefficients of the variance observed for natural antibodies between either technical or biological replicates, likely due to individual bird variability.

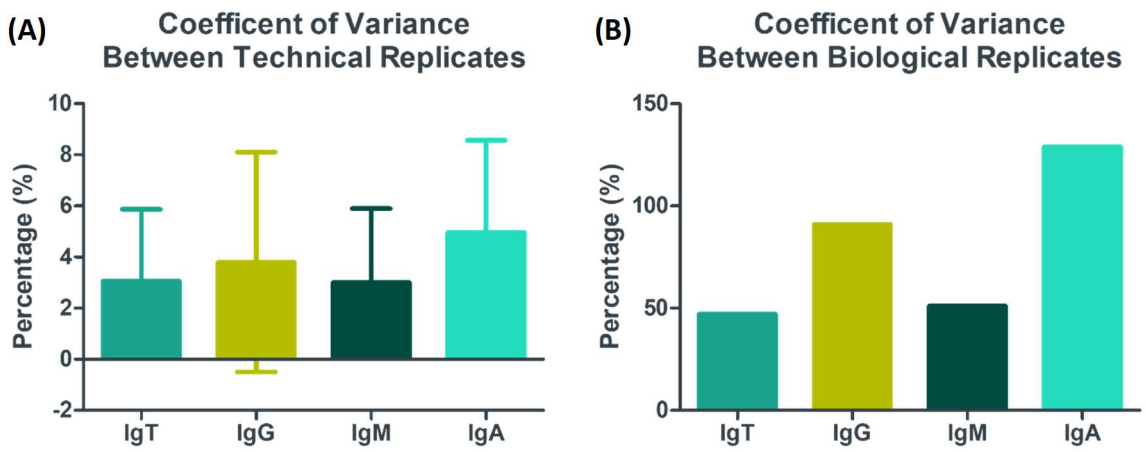

Figure 3.20 The coefficients of variance between technical replicates (A) and biological replicates (B).

No significant difference was observed between male, and female NAb titres (Fig. 3.21A), nor between antibody titres at four and five weeks (Fig. 3.21B). The average NAb titres were as follows: 5.5 (SD ± 2.6) for IgT, 1.8 (SD ± 1.6) for IgG, 1.9 (SD ± 1.0) for IgM, and 0.4 (SD ± 0.6) for IgA. Total Ig, encompassing total serum NAb titres, exhibited the highest values, incorporating all Ig and thus surpassing individual Ig levels significantly. Although IgG was anticipated to be the predominant individual Ig in broiler serum, its levels were comparable to IgM. While there was no significant difference between IgG and IgM antibody titres, both were significantly higher than IgA, demonstrating the lowest serum NAb titres, aligning with expectations (Lebacq-Verheyden et al., 1974, Pabst and Slack, 2019).

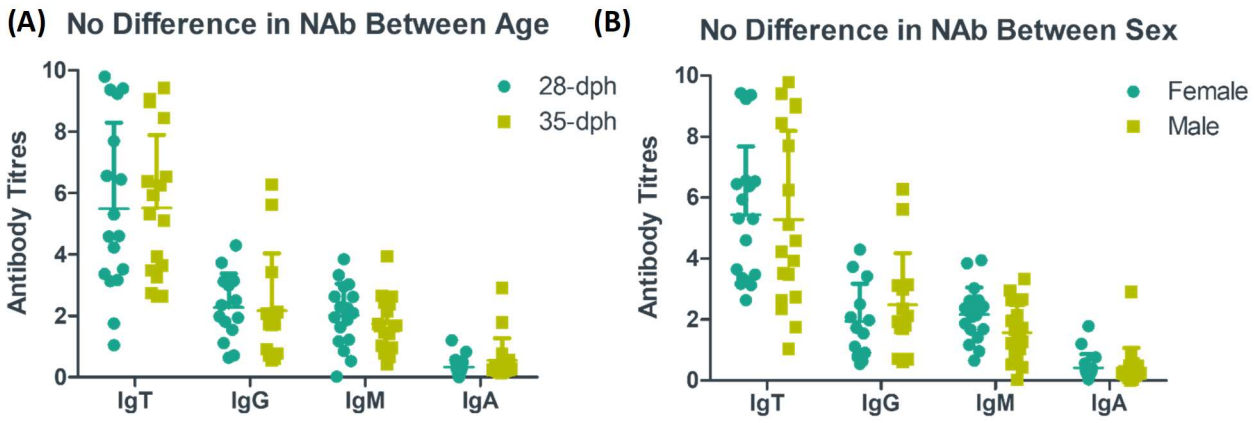

Figure 3.21 Vertical scatter graph displaying NAb titres in 20 breeding boilers where no significant difference was found between age **(A)** or sex **(B)**.

A power model was developed with Dr George Savva, utilising variance coefficients derived from technical and biological replicates. This model aimed to ascertain future sample size requirements for achieving statistical significance. To achieve this, a simulation model in R was constructed to evaluate natural antibody titres and discern any variances in broiler performance parameters or gut health status.

The analysis revealed minimal deviation among technical replicates but considerable variation among biological replicates. Hence, prioritising an increase in biological replicates holds greater significance over technical replicates. The simulation model underscores that the efficacy of employing three technical replicates alongside 40 biological replicates is notably inferior compared to a single measurement of NAb titres across 120 broilers (Fig. 3.22). However, maintaining scientific rigour necessitates at least two technical replicates. Moreover, randomly chosen samples will undergo a minimum of three technical replicates to ensure assay sensitivity and consistency, which will then be cross-referenced with the initial data set to validate results.

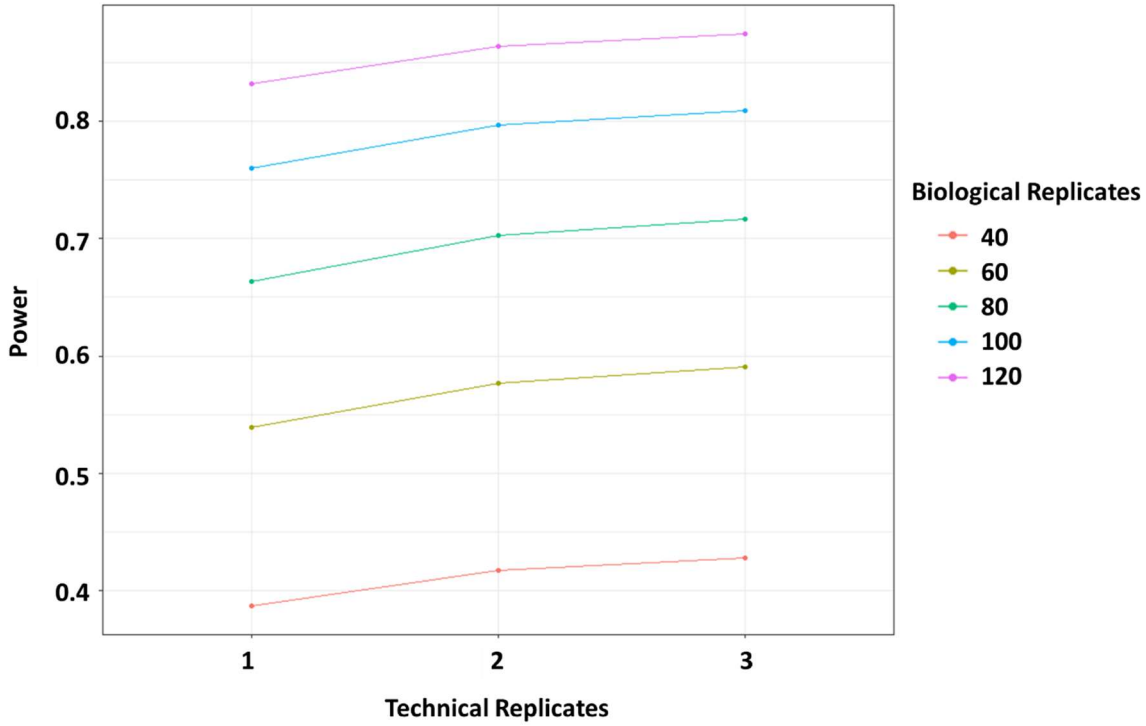

Figure 3.22 Power model simulation for the experimental design to measure broiler serum NAb titres.

3.4 DISCUSSION

3.4.1 Methods for Defining the Broiler Faecal Microbiome

This study modified the Promega method to incorporate mechanical homogenisation using bead beating with the FastPrep-24™ Classic (MP Bio), a step adapted from the FastDNA Spin kit for Soil by MP Biomedicals. The FastDNA Spin Kit for Soil is a molecular biology kit designed to rapidly and efficiently extract high-quality DNA from soil samples. Soil samples typically contain a complex mixture of organic and inorganic materials and diverse microbial communities, making this method suitable for extracting DNA from chicken faecal and GI content. The QIAamp PowerFecal Pro DNA Kit offers a comprehensive method for extracting high-quality DNA, specifically from faecal samples.

Unlike Qiagen and MP Bio, which utilise spin columns and ethanol precipitation, the Promega method employs an automated robot for lysing and can process up to 48 samples simultaneously, enhancing throughput. Implementing automation in the Promega method facilitates higher sample processing capacity, which is particularly beneficial for large-scale animal studies involving broiler faecal samples. Automation minimises human errors and ensures greater consistency across extractions, thus enhancing the reliability and efficiency of genomic DNA extraction. Overall, the Promega method streamlines DNA extraction workflows, saving time and labour while improving the quality and reliability of extracted DNA.

Qiagen employs a 24-holder vortex adapter for bead-beating mechanical lysis. While the MP Bio method is regularly utilised for extracting genomic DNA in faecal microbiome analysis (Pankoke et al., 2019), the Qiagen method has been suggested for fungal DNA extraction from faecal samples. Fungal cell walls pose a more significant challenge for DNA extraction than bacterial cell walls due to their composition. Fungal cell walls primarily consist of chitin, glucans, and other complex polysaccharides, providing structural rigidity that can resist standard DNA extraction methods (Frau et al., 2019). In contrast, bacterial cell walls mainly comprise peptidoglycan, which is more susceptible to breakdown using enzymatic or chemical methods commonly employed in DNA extraction protocols (Rothrock Jr et al., 2014). Thus, effective lysis of fungal cell walls necessitates the inclusion of mechanical disruption to release genomic DNA.

The Promega and Qiagen DNA extraction methods evaluated were subjected to assessment by the World Health Organization (WHO) for the creation of the 1st Reference Reagent for DNA extraction of the gut microbiome, aiming to standardise genomic DNA extraction methods (WHO/BS/2023.2455). Using a similar bioinformatic workflow, the WHO analysed the shotgun metagenomic data with MetaPhlAn 4.0. Results revealed that the Promega method outperformed Qiagen, achieving 95% sensitivity and 62% similarity to the reference reagent, thus surpassing the minimum quality criteria of 89% and 62%, respectively. The Qiagen method attained 89% sensitivity and 66% similarity. Consequently, the first objective of this chapter was fulfilled, and Promega was designated as the preferred method for genomic DNA extraction from chicken faeces and GI content.

This Hi-Seq platform is designed for high-throughput sequencing and can generate large volumes of data in a single run. It offers high scalability and can process many samples simultaneously. (de Muinck et al., 2017). Hi-Seq systems are suitable for large-scale projects such as whole-genome and shotgun metagenomic sequencing. Hi-Seq instruments typically produce longer reads than Mi-Seq, which can be advantageous for applications such as de novo genome assembly and variant detection (Clooney et al., 2016). The Mi-Seq platform is more compact and designed for lower throughput sequencing than Hi-Seq systems. It is suitable for smaller-scale projects, such as amplicon sequencing (Na et al., 2020). While Mi-Seq produces fewer reads per run than Hi-Seq, it still provides high-quality data with shorter turnaround times and lower costs (Clooney et al., 2016).

Shotgun metagenomic sequencing offers a broader perspective on the faecal microbiome and provides deeper insights into the intestinal ecosystem than 16S rRNA gene amplicon sequencing (Gilroy et al., 2021). Similarly, when applied to the chicken gut, shotgun metagenomic sequencing retrieves more information on low-abundance taxa than 16S sequencing, with the latter recovering less than 31% of the genera (Durazzi et al., 2021). Additionally, existing literature indicates that shotgun metagenomic sequencing enables higher-resolution taxonomic analyses (Durazzi et al., 2021, Campanaro et al., 2018, Laudadio et al., 2018). The similarity observed in shotgun metagenomic composition between Qiagen and MP Bio methods could be attributed to their utilisation of spin column methods, unlike the Promega method, which utilises automation and magnetic beads.

Despite using the same genomic DNA, the discrepancy in microbial composition observed between shotgun metagenomic sequencing and 16S rRNA amplicon sequencing can be attributed to several factors. While 16S rRNA amplicon sequencing targets a specific region of the bacterial 16S rRNA gene, providing high sensitivity and taxonomic resolution at the genus or species level, shotgun metagenomic sequencing offers a broader perspective by encompassing the entire microbial community, including bacteria, archaea, viruses, and fungi, albeit with potentially lower sensitivity for detecting specific taxa. Disparities between the two methods may arise from primer specificity, PCR amplification biases, and taxonomic resolution differences. Moreover, inherent biases in DNA extraction, library preparation, and sequencing technology can also impact the outcomes of both sequencing approaches. Integrating data from multiple sequencing methods can enhance our understanding of microbial community composition and diversity, providing a more comprehensive perspective.

Minimal differences were observed between extraction methods, indicating that any of the three methods could suffice for constructing genomic metabarcoding amplicon libraries. Despite the small sample sizes used in method development and validation, there were no discernible differences in composition. However, noteworthy distinctions included increased α-diversity observed with ITS1 rRNA amplicon and shotgun metagenomic sequencing using the Promega method. Additionally, β-diversity was significantly higher with MP Bio for shotgun, highlighting dissimilarities between samples. While low microbiome diversity in early life is typically associated with poor gut health (Díaz-Sánchez et al., 2019, Ducatelle et al., 2018a), the inability to quantify bacterial numbers, rather than relative abundance, may influence this interpretation. Species richness and evenness play crucial roles in determining microbiome stability, yet current literature predominantly focuses on bacteria, overlooking contributions from fungi and viruses to GI microbiome stability.

Analysing the shotgun metagenomic data to detect AMR and virulence genes offers valuable insights into pathogenicity within the broiler microbiome. Among poultry's most concerning AMR genes is blaCTX-M, known for encoding extended-spectrum beta-lactamase enzymes. These enzymes confer resistance to many beta-lactam antibiotics, including penicillin and cephalosporins, commonly used in human and veterinary medicine. Additionally, other AMR genes of significance in the poultry industry, such as those

conferring resistance to tetracyclines and macrolides, were identified using the CARD database. These antibiotics are frequently utilised in poultry production to prevent and treat bacterial infections, and the emergence of resistance to these drugs can severely limit treatment options, posing risks to animal health and welfare. Encouragingly, the data revealed only one gene associated with macrolides and three related to tetracycline.

Utilising shotgun metagenomic and ITS1 rRNA amplicon sequencing enables comprehensive profiling of bacteria, viruses, and fungi, with the ability to explore functional genes such as AMR and virulence factors. This approach fulfils the second objective of the chapter by identifying an appropriate sequencing strategy for uncovering markers associated with broiler performance and gut health.

3.4.2 Methods for Defining the Broiler Faecal &and Serum Metabolome

Untargeted metabolomics analysis of faeces and serum enables examining hundreds of compounds, which can later be validated as potential indicators of broiler performance and gut health. In identifying markers of performance or gut health in broiler faeces or serum, a metabolite should be easily detectable in all animals; therefore, the project does not aim to implement compounds that are scarcely measurable and require increased scans for detection. Employing 32 scans for untargeted ^{1}H-NMR analysis of both faeces and serum metabolomes allows for a high-throughput method and generates extensive data. The abundance of metabolites in both faeces and serum aids in avoiding bias in investigations. Future experimental designs can explore the broad spectrum of faecal and serum metabolites and their relationship with gut health or performance metrics, such as body weight and feed efficiency.

Targeted metabolomics using HILIC-MS presents a viable method with comparable sensitivity to ^{1}H-NMR, simplifying the validation of potential markers. Identifying a compound or metabolite as a marker for performance or gut health is highly desirable. While exploratory metabolomics can be costly, the feasibility of adapting an on-farm test for specific compounds offers a more straightforward application of markers for performance or gut health compared to the requirements of sequencing technologies.

The third objective of the chapter has been accomplished through the optimisation of untargeted metabolomics for faecal samples aged 7 to 35 dph, allowing for the utilisation of this method throughout the entire broiler life cycle, as well as for adult serum samples. Owing to welfare considerations, serum samples cannot be obtained before 13 dph, alleviating concerns about applying this method to chicks.

3.4.3 Methods for Detecting Broiler Serum NAb Titres

The average antibody titres fell below those reported by Berghof et al. (Berghof et al., 2018). However, it is essential to consider that their methods were applied to layers at 13-14 weeks of age so that the disparity could stem from differences in animal age or production objectives. Additionally, the referenced literature (Berghof et al., 2018, Berghof et al., 2015a, Berghof et al., 2019) does not include SD or CV values, despite studies involving upwards of 1000 birds.

Utilising the two-step indirect ELISA for detecting natural antibodies, which bind to the unknown antigen KLH, proves to be a suitable assay when exploring broiler immunity, fulfilling this chapter's final objective. Validating what constitutes 'high' and 'low' natural antibody titres could provide insights for future breeding strategies. Furthermore, examining the development of natural antibody titres throughout the broiler life cycle can ascertain their consistency and determine the age at which they can be detected, facilitating their introduction as a marker. Introducing such a marker at an earlier stage is advantageous given the approximately 35-day broiler life cycle.

Modulating and managing NAb titres, such as through selective breeding, holds promise for enhancing broiler stock immunity, thus providing increased protection against pathogens and diseases (Berghof et al., 2018), (Berghof et al., 2019). Furthermore, lower levels of IgM in plasma have been linked to stress, while birds exhibiting high-feather pecking behaviour demonstrate significantly lower antibody titres compared to control and low-feather pecking counterparts (van der Eijk et al., 2019). Notably, the highest CV is observed with IgA antibody titres, indicating the lowest concentration of this Ig isotype in

the serum of these broilers. Additionally, IgA plays a pivotal role in modulating the microbiome, thereby serving as a crucial link between immunity and gut health.

3.4.4 Summary & Conclusion

The methodologies established and validated within this chapter represent a comprehensive suite of analytical tools encompassing genomic DNA extraction, amplicon, shotgun metagenomic sequencing, and targeted and untargeted metabolomics. By examining faecal samples from both adult birds and chicks, along with serum samples from adult broilers, these techniques offer a holistic approach to investigating gut health and performance in poultry. By introducing experimental conditions, these methods allow for a nuanced exploration of various molecular pathways and biological processes underlying broiler health and productivity. Moving forward, these validated methodologies will be leveraged in large-scale animal studies aimed at delineating and characterising markers of broiler performance and gut health across different stages of their life cycle. This comprehensive approach holds promise for uncovering novel insights into the complex interplay between genetic, environmental, and physiological factors influencing poultry health and productivity, ultimately contributing to advancements in poultry science and production practices.

CHAPTER 4

SNAPSHOT OF BROILER CHICK FAECES AT 7-DAYS POST HATCHING

4.1 INTRODUCTION

The early stages of a broiler chick's life, particularly the first week post-hatching, are of paramount importance in establishing and developing its microbiome. Successional changes in the broiler microbiome are strongly influenced by the composition of the initial microbial community, leading to variations in the mature microbiome (Glendinning et al., 2022). Lactic acid bacteria have been shown to promote greater colonisation of symbiotic populations in young broiler ilea (Rodrigues et al., 2020).

Inoculating freshly hatched chicks with an adult caecal microbiome results in fewer shifts in microbial communities compared to chicks inoculated at a later stage, underscoring the critical role of early microbiome development in broiler chicks (Ramírez et al., 2020). Despite differences in the structural composition of the microbiome between chicks receiving caecal transplants and control groups, there was no difference in body weight at 14 dph (Ramírez et al., 2020). However, the benefits of the caecal transplants are evident in their role in pathogen control. Interestingly, the relatively stable microbiome observed in transplanted chicks provided added protection when challenged with *Salmonella typhimurium*; however, chicks that received an environmental culture inoculum obtained from commercial poultry litter exhibited the highest body weights after the pathogen challenge (Ramírez et al., 2020). This environmental culture inoculum was primarily comprised of *Firmicutes*, which have been linked to beneficial effects on immunomodulation (van Staaveren et al., 2020).

The administration of live microbial feed supplements, commonly referred to as direct-fed microbials, has been proven to be highly effective when introduced early in life (Richards-Rios et al., 2020b). These therapies not only influence the microbiome composition but also contribute to improving gut development and immune system maturation, offering a promising avenue for improving chick health (Jurburg et al., 2019). These supplements are often supplied through water or feed but can alternatively be administrated as a spray onto eggs before hatching or as a gel directly administered to the feathers of newly hatched chicks.

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The caecum is crucial in the GI tract, facilitating fermentation and SCFA production. Due to its dense microbial diversity, it is often studied to understand the intestinal microbiome. However, caecal droppings or content may not fully represent digestive function or microbial composition throughout the entire tract (Andreani et al., 2020). Additionally, the bird empties the caecum approximately every 18-22 hours, typically in the morning, suggesting that the timing of caecal collections could influence the determined microbial composition. The faecal microbiome, although recognised for its variability, stands out as the least invasive and most efficient sampling method, avoiding the need to sacrifice any birds. Influential factors shaping faecal microbiome composition include age, diet, and environmental conditions (Schokker et al., 2021). By encompassing resident microbiota from both the ileum and cecum, faecal samples offer a comprehensive snapshot of the entire GI microbiome (Schokker et al., 2021). A recent investigation highlighted that in adult broilers (aged 19-31 dph), caecal communities exhibit greater variability compared to those in the cloaca (Andreani et al., 2020). It was estimated that cloacal swabs capture approximately 60% of the taxa identified within the caeca of broilers (Andreani et al., 2020). However, rare taxa present in the caeca may be underrepresented in cloacal samples, though their functional significance was not evaluated. This underscores the potential of faeces for high-throughput studies but suggests limitations for precise taxonomic estimates.

As shotgun metagenomic sequencing costs decline, researchers increasingly recognise the importance of exploring non-bacterial components within poultry research (Mulholland et al., 2021, Gilroy et al., 2021). Shotgun metagenomic sequences offer the unique advantage of examining higher taxonomic resolution and functional genes and pathways within the GI microbiome, surpassing the limitations of traditional 16S rRNA gene sequencing (Durazzi et al., 2021). Recent studies have indicated that broilers do not undergo successional changes in mycobiome composition during development as observed in the microbiome (Robinson, 2019, Davies et al., 2022, Robinson et al., 2022b). Instead, the mycobiome has been described as transient and highly variable (Robinson et al., 2022a), suggesting that negative health and performance are not likely related to the development of fungal populations (Davies et al., 2022). Unlike the microbiome, which exhibits maximum diversity in the caeca, the mycobiome is reported to display its highest diversity in the upper GI tract (Robinson et al., 2020a).

Knowing that the broiler microbiome can be manipulated to improve pathogen control (Ramírez et al., 2020) and that initial microbiome colonisation has a lasting effect on its structure (Rodrigues et al., 2020), the faecal metabolome at 7 dph may be a valuable source of biomarkers. Integrated microbiome and metabolomics analyses have been proposed to differentiate broilers with metabolic tibial-tarsal disorder, characterised by differences in caecal microbiome diversity and enriched purine, vitamin, and amino acid metabolism in faeces at 11 dph (Huang et al., 2022). Studies have demonstrated high reproducibility of the faecal metabolome via GC-MS and LC-HRMS at 12 dph, highlighting persistent differences in energy metabolism and microbiota composition in chicks in response to negative postnatal experiences, such as deprivation of feed and water, transportation under irregular movement, and variable room temperatures (Beauclercq et al., 2019b).

In addition to being a potential source of biomarkers due to its reported reproducibility, the broiler chick faecal metabolome can provide insights into microbiome function. Identifying these functional differences may explain variations in biological efficiency at 7 dph. Such differences could indicate opportunities for nutritional interventions or dietary modifications, such as the inclusion of specific feed additives. One example is phytase, a common feed additive which was absent from the farms used in this study. The complete breakdown of phytate liberates phosphorus and myo-inositol, both of which are accessible for absorption, thereby aiding in phosphorus nutrition (Selle and Ravindran, 2007), enhancing metabolic performance (Sommerfeld et al., 2018) and potentially improving feed conversion rates (Walk et al., 2018). Phytate solubility is facilitated in the upper chicken GI tract by a relatively low pH (Sommerfeld et al., 2019). The hydrolysis of phytate leads to the detection of myo-inositol in faecal matter (Walk et al., 2018, Sommerfeld et al., 2018). However myo-inositol may also be detected in the faeces if it is not being effectively absorbed in the gut. This could suggest issues with gut health or function, impacting the overall efficiency of nutrient absorption.

Through shotgun metagenomic sequencing for microbiome analysis, ITS1 gene sequencing for mycobiome characterisation, and untargeted ^{1}H-NMR for faecal metabolome profiling, microbial and metabolic factors associated with performance traits in early-stage broiler chicks are anticipated to be uncovered.

4.1.2 Aims & Objectives

The primary objectives to address in this chapter are as follows:

1. Assess differences and consistencies in broiler faecal microbiome, mycobiome, and metabolome at 7 dph to determine if this age is suitable for performance marker identification. Examine the similarity of faecal profiles across three years of collections in birds with the same environment and genotype.
2. Explore the impact of genotype on broilers' faecal microbiome, mycobiome, and metabolome at 7 dph and total effects on performance. .
3. Investigate the broiler faecal microbiome and metabolome at 7 dph to determine the presence of myo-inositol and evaluate its potential as a marker for performance.

Hypotheses

This study hypothesises that differences in broiler genotype and body weight of body weight growth will influence the faecal microbiome, mycobiome, and metabolome of broilers at 7 dph. It is predicted that distinct faecal compositions will be observed between genotypes, with significant variations in biological efficiency from 7 to 35 dph, reflecting diverse gut microbial and fungal colonization dynamics associated with performance.

4.2 METHODS

4.2.1 Sample Collection and Storage

As described in Chapter 2: Methods, shotgun metagenomic sequencing (Methods 2.3–2.4) and statistical analyses (Methods 2.5) were used for microbial and metabolomic analyses.

Faeces were collected at a commercial broiler farm in Scotland from 25 broiler chicks of mixed sex from two broiler genotypes at 7 days post-hatch (dph) (total 50 birds), housed in the same open shed environment (Methods 2.1.2). The shed housed approximately 1,000 birds of the same age, with separate pens for each broiler genotype. Wood chippings/sawdust was used as bedding, which was cleaned and replaced between flocks.

Sample collection took place in the morning, ensuring consistency across birds. Chicks were randomly selected and temporarily placed in individual sterile containers (one bird per container) to allow natural excretion. Fresh faecal samples were collected immediately after excretion using sterile scoops and transferred into sterile tubes to minimise environmental contamination. The birds were housed indoors in a controlled environment, with a recorded shed temperature of 28°C at the time of collection. Humidity was not measured.

For each bird, four replicates of 200 mg faecal samples were collected and stored at -20°C on the farm before being transported on dry ice to QIB, where they were stored at -70°C until further analysis. Faecal samples were not pooled; each aliquot was derived from a single bird.

4.2.2 Screening and Quantification for Phytase Activity

The screening process can be seen in Fig. 4.1. Assays screening for phytase activity used defined minimal media (yeast nitrogen base (YNB: $(NH_4)_2SO_4$, $MgSO_4$, NaCl, $CaCl_2$) without amino acids or KH_2PO_4 (Formedium Ltd)) with and without phytic acid (dipotassium salt 98%, Sigma-Aldrich), adapted from (Olstorpe et al., 2009).

Analysis of the annotated whole genome sequences of species documented to produce phytase in the QIB culture collection provided three potential candidates as positive controls: *Bacillus subtilis* (ATCC 6633) grown in Lysogeny Broth (LB, Oxoid) at 30°C, *Chryseobacterium bovis* FI11139 also grown in LB at 30°C and *Enterobacter cloacae* FI11317 grown in MRS at 30°C. All screens were performed once from a direct isolate sub-culture and once from the isolate's glycerol stock, which was sub-cultured three times before screening. Liquid cultures for analysis were enriched with 0.5% phytic acid (Pa) to induce phytase activity. Liquid cultures were incubated at 42°C and checked for growth at 24 h and 48 h. Stocks of dense liquid cultures were preserved at 1:1 in 40% glycerol, frozen, and stored at -70°C. Remaining liquid cultures were used to screen for phytase activity.

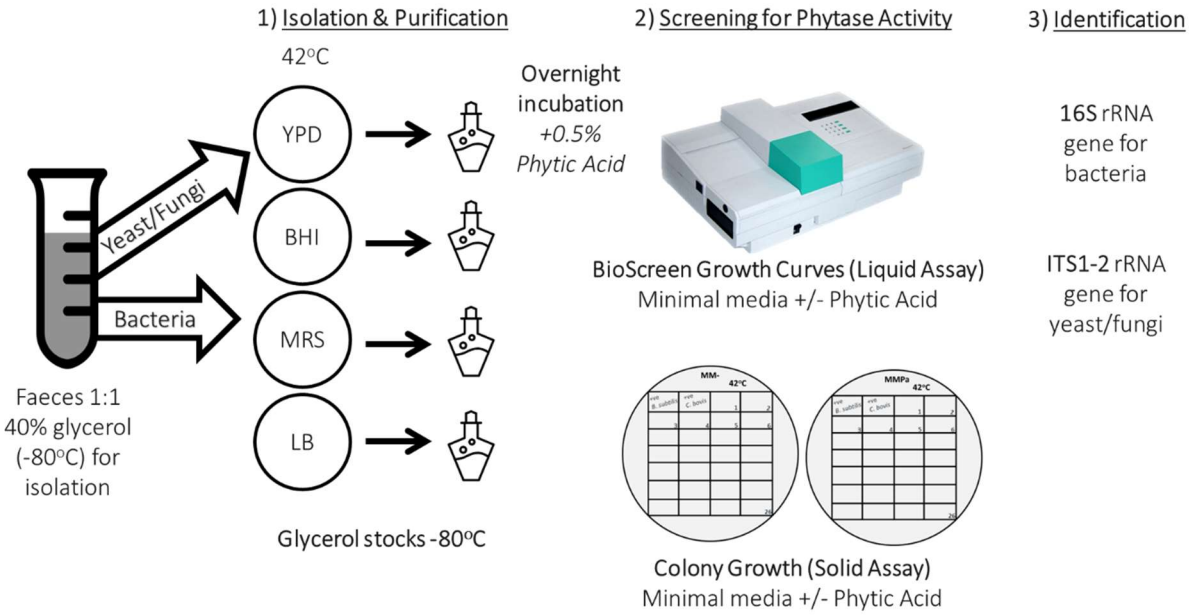


Figure 4.1 Schematic of screening process from broiler faeces stored in 1:1 40% glycerol.

4.2.2.1 *Assay for Possible Phytase Activity Using Growth in Liquid Minimal Medium*

Adapting a published protocol (Olstorpe et al., 2009), isolates were grown in 10 mL liquid media with 0.5% Pa at 42°C (positive controls incubated at a preferred temperature of 30°C) and checked at 24 h and 48 h. Once optical density (OD) at 600 nm reached 0.9, isolates and positive controls were prepared in triplicate at a 1:10 dilution in defined minimal media without (MM-) or with (MMPa) Pa. Diluted samples were transferred to 100-well Honeycomb 2 plates (ThermoFisher) for BioScreen C (Oy Growth Curves Ab Ltd) growth curve analysis. Media blanks were included in triplicate as negative controls. The BioScreen C was formatted to take OD measurements at 540 nm (wavelength for minimal media bacterial growth) every 15 m for 24 h, with 10 s shaking before reading.

4.2.2.2 *Assay for Possible Phytase Activity Using Growth in Solid Medium*

Adapting a published protocol (Olstorpe et al., 2009) the same dense cultures used for the liquid screening assay were simultaneously used to set up the colony growth screening assay. Minimal medium (MM- and MMPa) was prepared with 1.5% agar. A sterile toothpick was used to streak dense cultures diagonally to the plates within a numbered grid space. Plates were prepared in duplicate to observe colony growth at 30°C and 42°C. Plates were photographed after 24 and 48 hours to compare colony growth.

4.2.2.3 *Quantification of Phytase Activity (Megazyme)*

Phytase activity was measured using a phytase assay kit (Megazyme) following the manufacturer's instructions. Briefly, Pa standard solutions were prepared according to the kit instructions to create a standard curve by plotting the absorbance against concentration. A blank was prepared for each sample by adding 100 µl of stop solution (reagent B) to 200 µl of Pa solution and 200 µl of the diluted enzyme. The blanks would not be expected to demonstrate phytase activity as they would not be heat-activated.

The samples containing phytase enzymes were pre-incubated at 40^{o}C for 5 min. 200 µl of each standard solution and samples were pipetted into separate 1 mL cuvettes. 200 µl of buffer solution (Reagent A) was thoroughly mixed into each tube. Tubes were incubated for 10 min at 40^{o}C to enable substrate reaction. After incubation, 100 µl of stop solution (Reagent B) was added to each tube to terminate the enzyme reaction. 500 µl of colour regent (Reagent C) was added to each tube and mixed via inversion for 1 min. Tubes were incubated for 20 min at room temperature. The absorbance of each solution was measured at 360 nm using a Jenway Spectrophotometer. The Pa concentration in each sample was determined using the standard curve, and phytase enzymatic activity was calculated based on the amount of Pa released in the specified conditions.

4.3 RESULTS

4.3.1 Performance Differences between Genotypes of Three Flocks at 7-days post-hatching

There are significant differences between genotypes and performance with some overlaps, but a few distinctions stand out. Specifically, at 7 dph (Fig. 4.2A), body weight of both genotypes from March 2022 and September 2022 indicates slightly higher values compared to March 2020. At 35 dph (Fig. 4.2B), body weight from March 2022 was slightly higher in genotype B compared to genotype A, while September 2022 demonstrates slightly higher body weight compared to March 2020 and genotype A from March 2022. These body weight measurements at 35 dph were provided by Aviagen at the time of broiler end-of-life processing to allow for growth calculations across the production cycle. This data was included to contextualise early-life body weight trends in relation to final growth performance. Body weight gain (Fig. 4.2C) was notably greater in genotype B than in genotype A in March 2020 and March 2022, although this difference is not observed in September 2022. Additionally, there appears to be no discernible difference in faecal water retention (Fig. 4.2C).

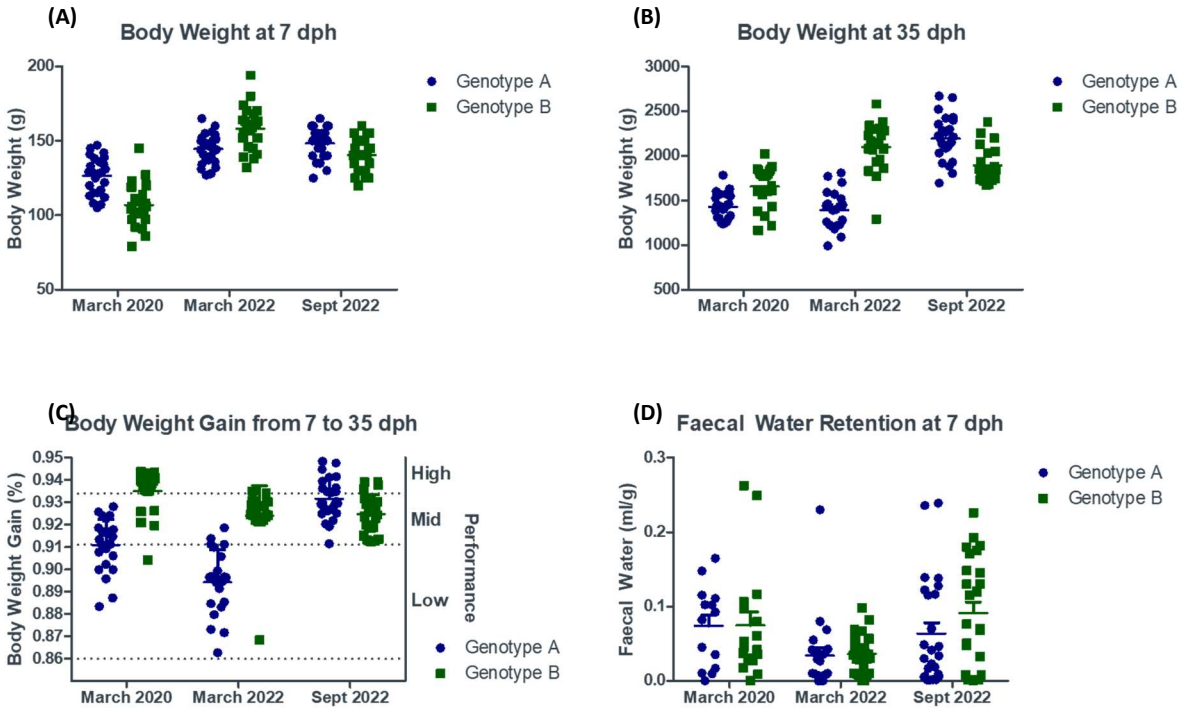


Figure 4.2 Performance distribution between genotypes at three collection points at 7 dph (A), 35 dph (B), growth from 7 to 35 dph (C) and faecal water retention at 7 dph (D). Lines on (C) Y-axis indicate interquartile boundaries for broilers within top 25% and bottom 25% of BWG from 7 to 35 dph.

Using the BWG interquartile ranges (IQR), broilers with a BWG of more than 92.4% were classified as higher performers, while those with a BWG of less than 91.1% were classified as lower performers. Broilers with BWG between 91.1% and 92.4% were classified as neutral performers. Notably, no broilers from September 2022 met the criteria for low performers (Fig. 4.3A). Additionally, there was no difference in performance between sexes (Fig. 4.3B).

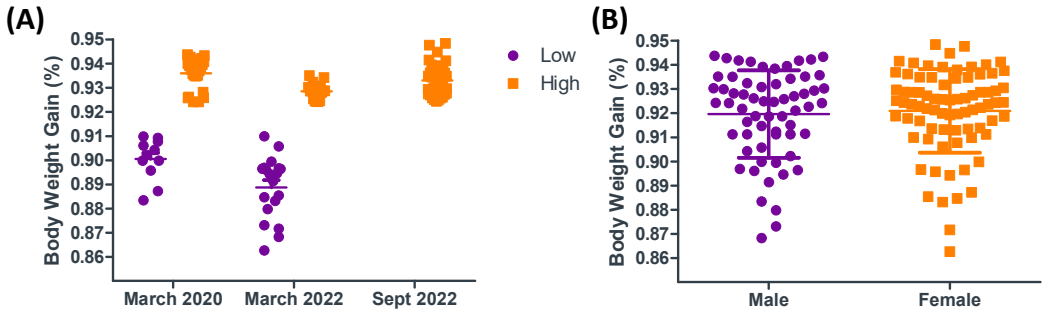


Figure 4.3 The distribution between lower and higher performing boilers (A), and distribution of growth between males and females (B).

4.3.3 Faecal Microbiome of Broiler Chicks

Three broiler faecal samples failed sequencing quality control. There were 544 species of bacteria identified across 147 broiler faecal samples at 7 dph. One sample failed quality control in March 2020, and two failed in March 2022. Species richness was similar between March 2020 (192 taxa) and March 2022 (196 taxa) but lower in September 2022 with 166 taxa. As expected from the literature (Jurburg et al., 2019, Schokker et al., 2021), the *Lactobacillaceae* family was dominant across all collection points, representing an average of 89% of the broiler chick faecal microbiome (Fig. 4.4).

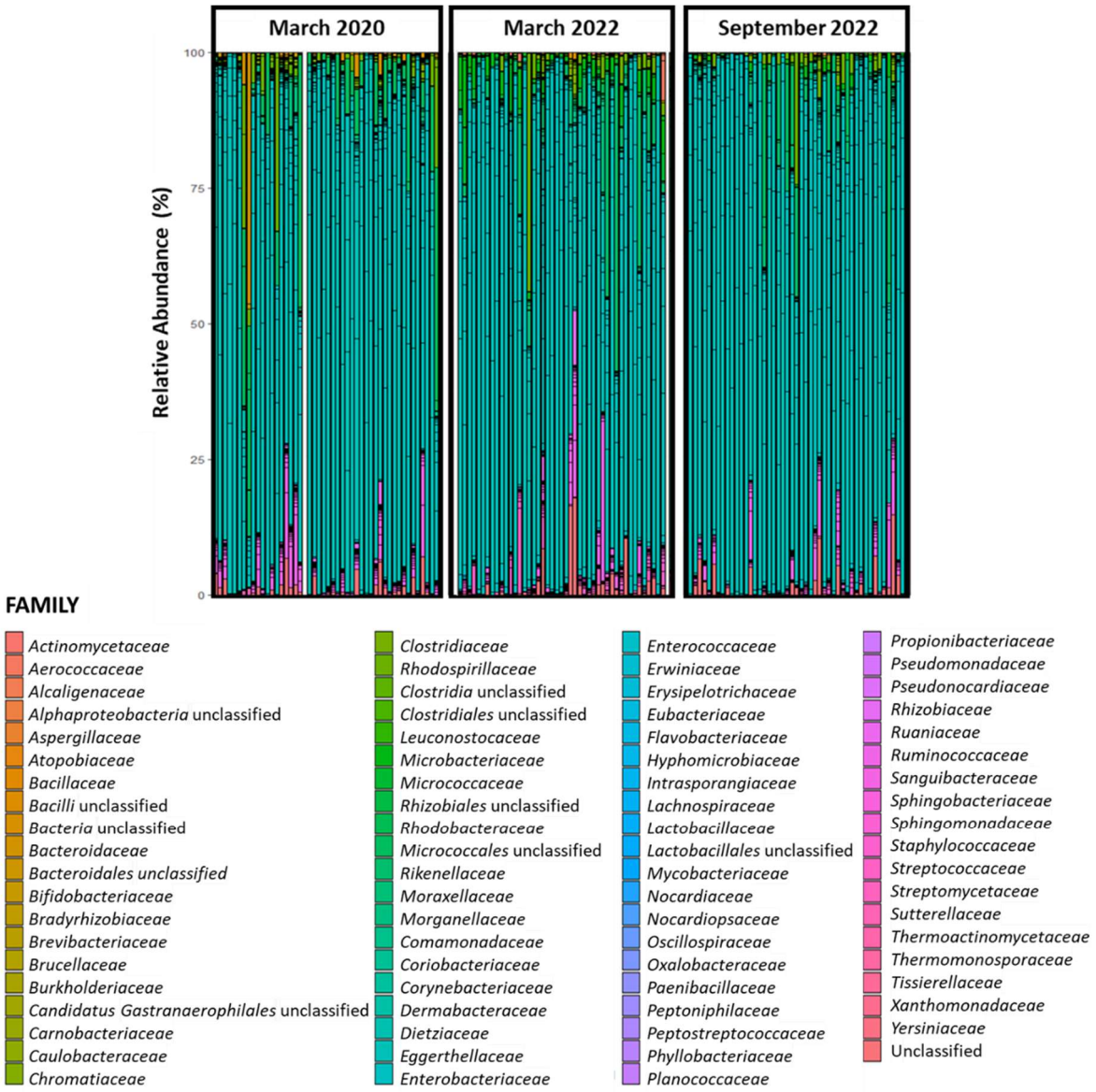


Figure 4.4 Relative abundance of all families in broiler faeces at 7 dph, across three collection points.

Twenty-one genera were consistently present across all collection dates (Fig. 4.5), including *Acinetobacter*, *Alistipes*, *Brachybacterium*, *Clostridium, Corynebacterium, Enterococcus, Eubacterium, Faecalibacterium, Flavonifractor, Gordonibacter, Lachnoclostridium, Lactobacillus, Lactococcus, Leuconostoc, Ligilactobacillus, Limosilactobacillus, Paracoccus, Pseudoflavonifractor, Pseudomonas*, and *Staphylococcus*. However, a unique addition was made to this list in the March 2022 dataset: a single fungal genus, *Aspergillus*. This finding is particularly noteworthy as it is the only non-bacterial genus taxonomically classified using MetaPhlAn 4 in this study.

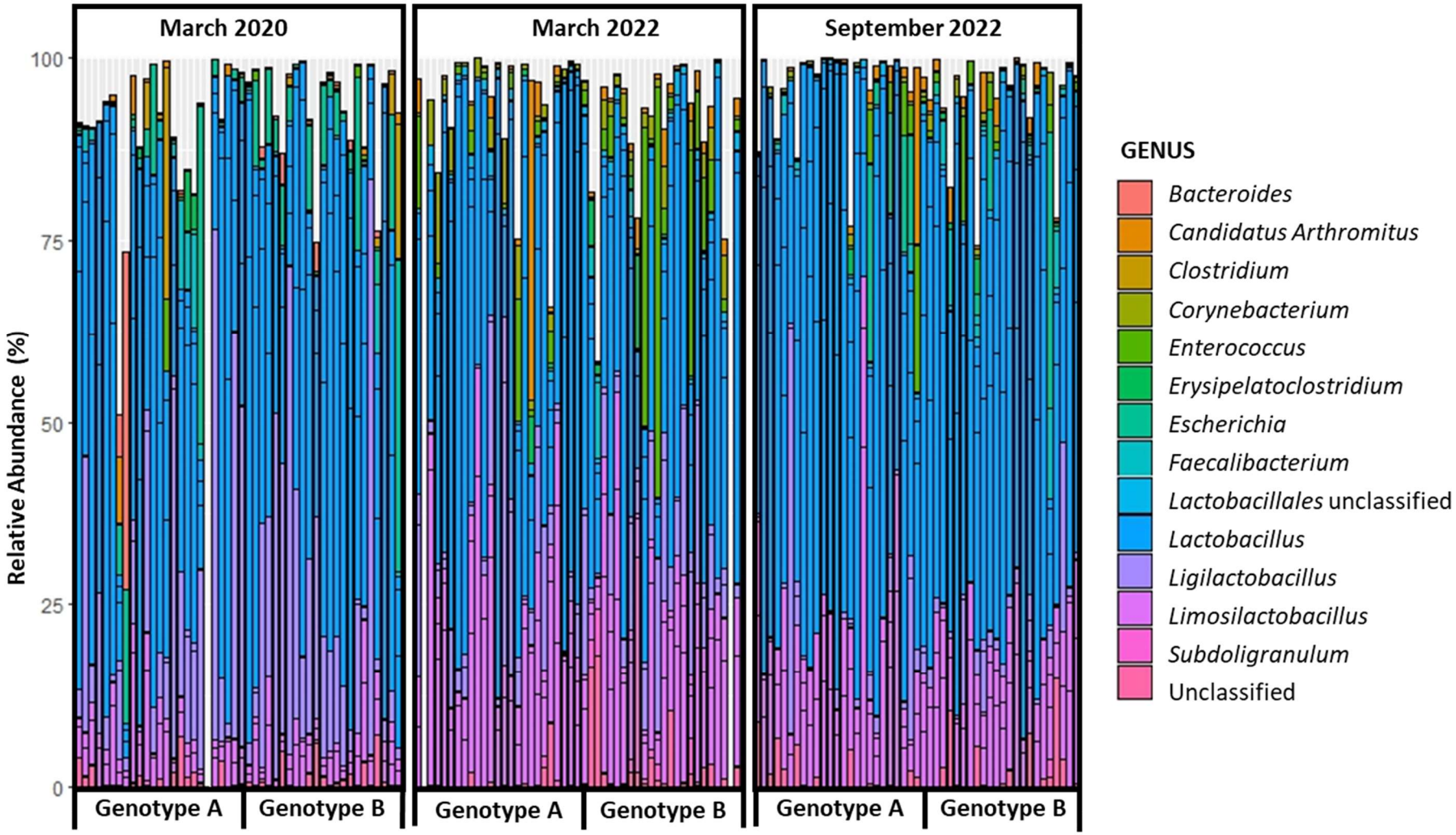


Figure 4.5 Relative abundance of top 20 genera in broiler faeces at 7 dph, across three collection point.

The faecal microbiomes were very similar across the collection points (Figs. 4.4, 4.5). Small but consistent shifts in microbial composition or function may indicate key taxa or metabolic pathways that contribute to performance differences, even if overall microbial diversity remains stable. By analysing these subtle differences, we can better assess whether specific microbial compositions are associated with improved or reduced growth. In this study, significance was determined at $P < 0.05$, meaning only statistically robust associations were considered biologically relevant. *Candidatus Arthromitus spp. SFB turkey*, *Clostridium spiroforme*, *Enterococcus hirae*, and *Lachnospiraceae* bacterium were among the top 20 taxa at all collection points (Fig. 4.6). Within the *Lactobacillaceae* family, *Lactobacillus crispatus, Lactobacillus gallinarum, Lactobacillus johnsonii, Limosilactobacillus vaginalis, Ligilactobacillus aviarius, Ligilactobacillus salivarius*, and *Limosilactobacillus reuteri* were consistently in the top 20 across all collection points.

In March 2020, the dominant species were *Lactobacillus gallinarum* (28%), *Ligilactobacillus aviarius* (19%), and *Lactobacillus crispatus* (19%). In March 2022, *Lactobacillus johnsonii* was the dominant species, accounting for an average of 36% of the faecal community, followed by *Limosilactobacillus vaginalis* (14%) and *Ligilactobacillus aviarius* (8%). In September 2022, like March 2022, *Lactobacillus johnsonii* was the dominant species, representing an average of 25% of the total chick faecal microbiome. In March 2020, *Lactobacillus crispatus* accounted for 21%, and *Lactobacillus gallinarum* for 17%. Unlike March 2020 and March 2022, *Ligilactobacillus aviarius* was only 1% of the faecal microbiome in September 2022.

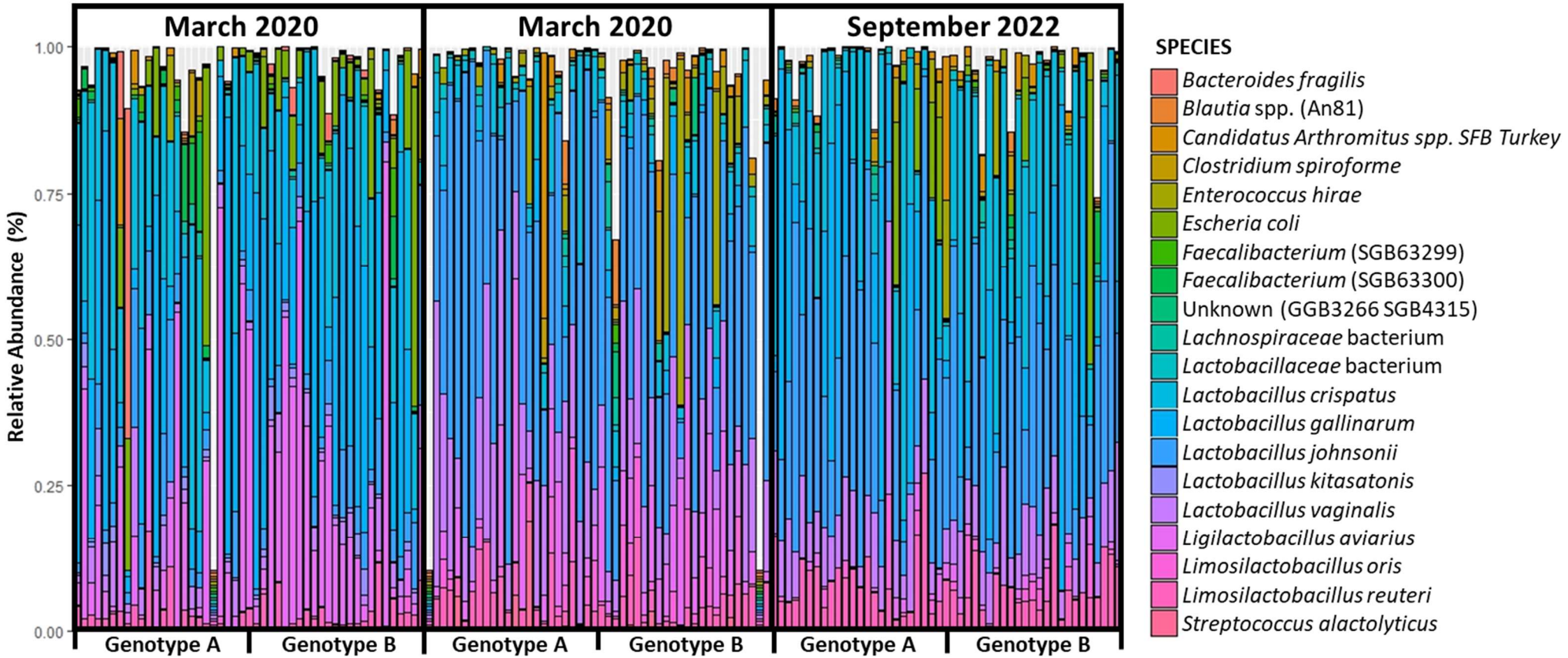


Figure 4.6 Relative abundance of top 20 species in broiler faeces at 7 dph, across three collection points.

Several significant differences emerged between genotypes, although there was considerable overlap between flocks. *Corynebacterium stationis* exhibited a significant difference in September 2022, with average abundance significantly higher in genotype B (Fig. 4.7A). *C. stationis* has been identified as persistent in poultry sheds following application of disinfectants (Luyckx et al., 2017) and sodium bisulphate (to treat accumulation of ammonia) (Joerger et al., 2020). However, its specific role and significance in poultry health and disease still need to be studied. *Lactobacillus crispatus* (Fig. 4.7B) showed significantly higher average abundance in genotype A in March 2020 and genotype B in September. The abundance of *L. aviarius* (Fig. 4.7C) was significantly higher in genotype B in March 2020, which had lower biological efficiency while *Candidatus Arthromitus* spp. SFB turkey (Fig. 4.7D) was in significantly greater average abundance in genotype B in September 2022. Although these differences do not exhibit a discernible genotypic pattern, they may be relevant to biological efficiency.

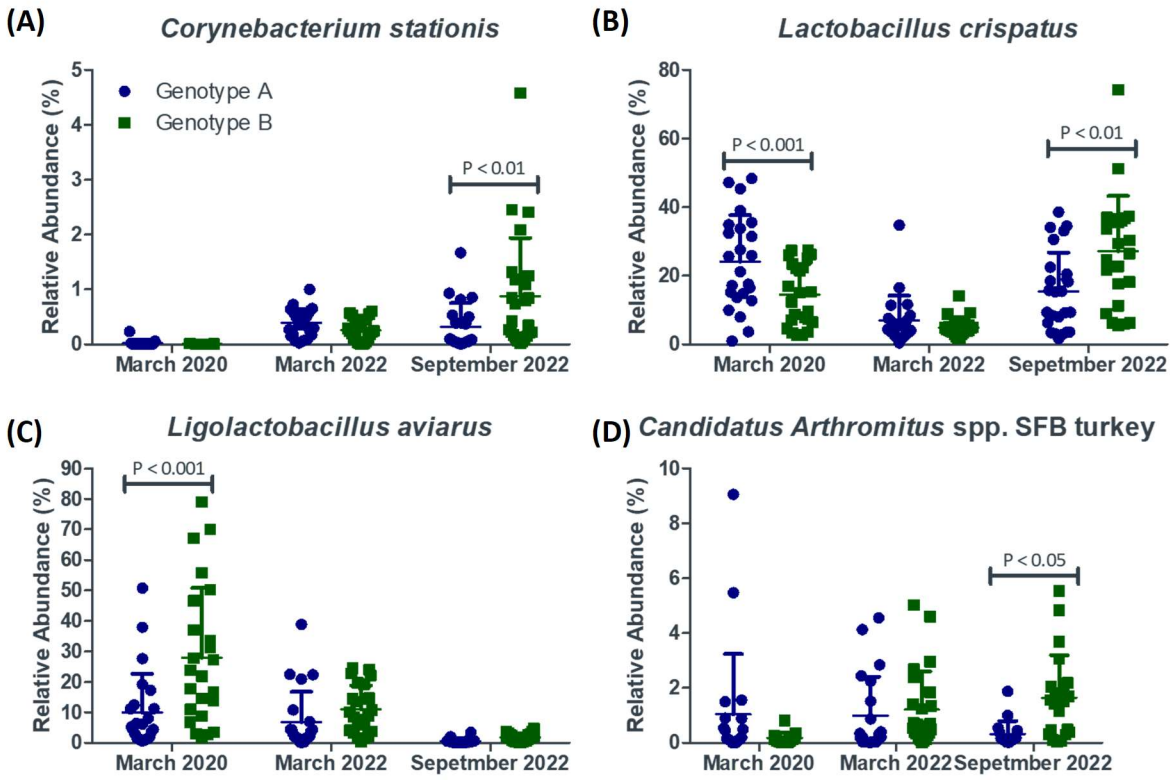


Figure 4.7 Species with a significant difference in abundance in broiler faeces at 7 dph, comparing genotypes within collection dates, determined with non-parametric t-test.

While *Lactobacilli* species were dominant, their relative abundances varied significantly between collection points. This variation could be due to differences in the specific strains and species present, as different Lactobacilli exhibit distinct metabolic functions and environmental adaptations. In March 2020, *L. johnsonii* (Fig. 4.8A) was less abundant than both collections in 2022 (t-test, $P < 0.0001$). Similarly, *L. vaginalis* (**Fig. 4.8B**) was more abundant in March 2022 (t-test, $P < 0.0001$) and September 2022 (t-test, $P < 0.05$) than March 2020. *Limosilactobacillus oris* (Fig. 4.7E) was in higher abundance in March and September 2022 (t-test, $P < 0.001$) whereas *L. aviarius* (Fig. 4.7C) was on average significantly more abundant in March 2020 (t-test, $P < 0.001$). Both *L. crispatus* (Fig. 4.7B) and *L. gallinarum* (Fig. 4.8F) had significantly higher abundances in March 2020 (t-test, $P < 0.001$) and September 2022 (t-test, $P < 0.001$).

Enterococcus hirae (Fig. 4.8C) exhibited an average higher abundance in March and September 2022 (t-test, $P < 0.01$) as this species was detected in a low number of birds from March 2020. In contrast, *E. coli* (Fig. 4.8D) was most abundant in March 2020 (t-test, $P < 0.01$), yet it was undetectable in March 2022 and found in only a few birds in September 2022. The higher *E. coli* populations could have contributed to the differences in biological efficiency of broilers in March 2020.

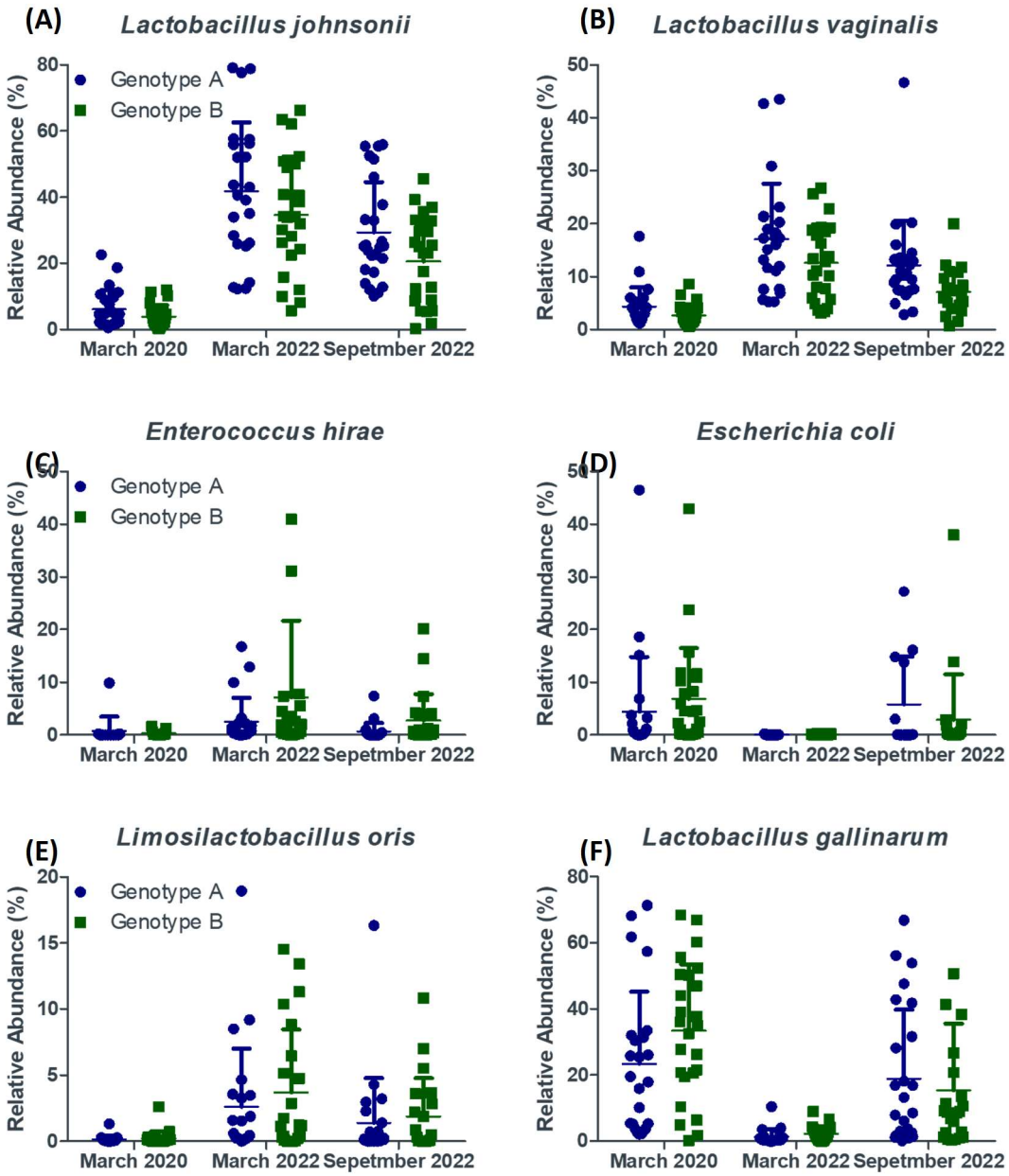


Figure 4.8 Species with a significant difference in abundance in broiler faeces at 7 dph in two genotypes, comparing collection dates, determined with non-parametric t-test.

No significant difference was observed in either α- (Fig. 4.9A) or β-diversity (Fig. 4.9B) between September 2022 and the March collection dates. However, a significant difference in β-diversity was detected between March 2020 and March 2022 (ANOVA, $P < 0.0001$).

The Bray-Curtis PCoA analysis revealed that samples from different collection dates tended to cluster together, indicating similarity. However, an overlap was observed

between the samples from September 2022, March 2020, and March 2022. This suggests that the microbial diversity in September 2022 was similar to that of both March collections. In contrast, the samples from the two March collections did not overlap, indicating distinct diversity differences between these cohorts.

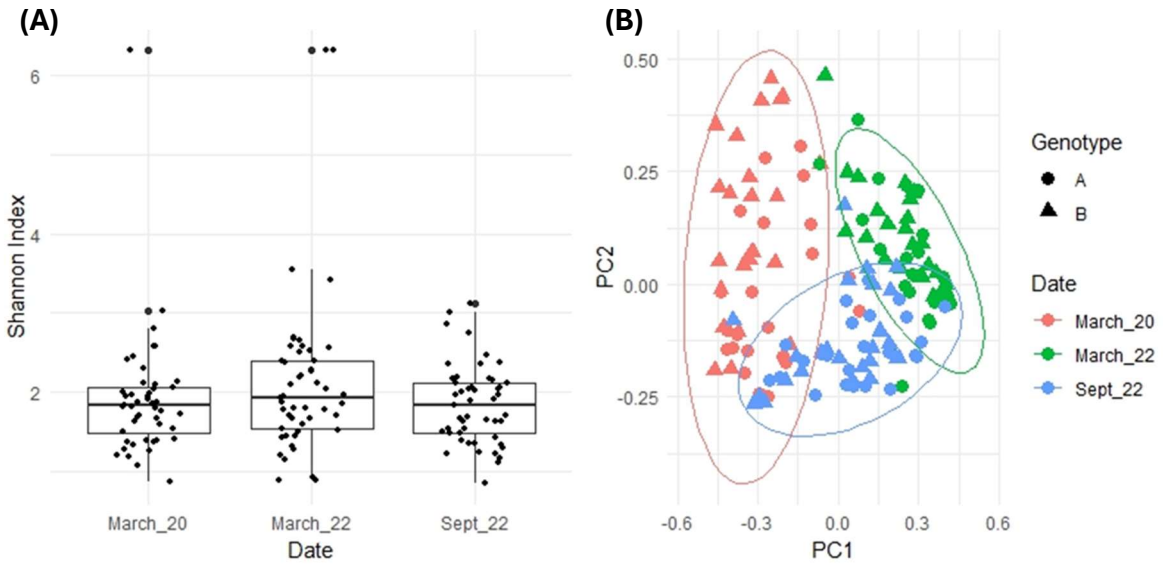


Figure 4.9 Broiler microbiome α-diversity measured by Shannon Index **(A)** and β-diversity measured by Bray-Curtis dissimilarities **(B)** at 7 dph, across three collections.

No significant difference was observed in α- (Fig. 4.10A) or β-diversity (Fig. 4.10B) between genotypes A and B, indicating similar microbiomes at this early stage. This finding is consistent with the similarity observed in faecal microbiomes across three different collection dates. Additionally, it suggests that significant differences in relative abundance between genotypes are not driving the overall diversity or composition of the microbiome.

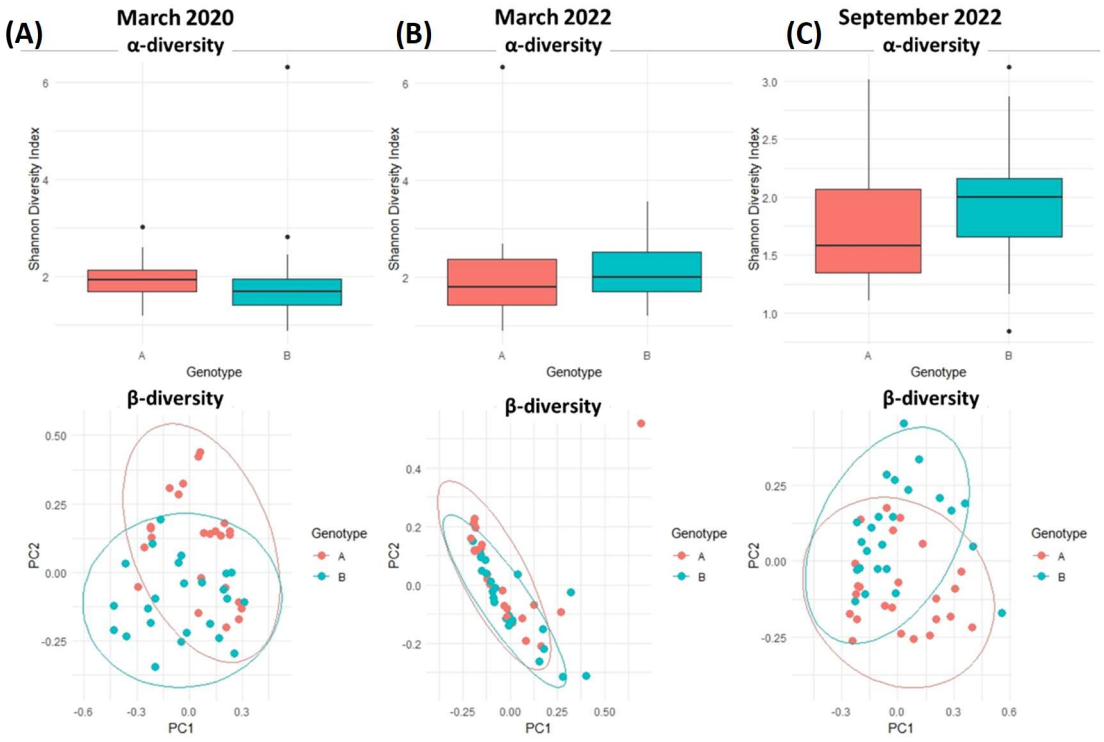


Figure 4.10 Broiler microbiome α-diversity measured by Shannon Index and β-diversity measured by Bray-Curtis dissimilarities comparing two genotypes in March 2020 **(A)**, March 2022 **(B)** and September 2022 **(C)**.

4.3.3.3 *Differences Between Performance*

Of the 147 broiler faecal samples collected at 7 dph, 39 were classified as low performers, while 73 were categorised as high performers based on IQR of BWG. The remaining 35 samples were classified as neutral performers. Once again, *Lactobacillaceae* emerged as the dominant bacterial family, consistent with the observations across all cohorts. Analysis of the top 20 most abundant species in both high and low performers revealed striking similarities, albeit with varying abundance levels in *Lactobacilli* species (Fig. 4.11).

Lactobacillus johnsonii emerged as the the most abundant species in low-performing broilers, comprising an average of 24% of the faecal microbiome. In contrast, it ranked as the second most abundant species in high-performing broilers, accounting for 19%. Conversely, *L. gallinarum* emerged as the dominant species in high-performing

broilers, representing 20% of the faecal microbiome. In contrast, it ranked as the fifth most abundant species in low-performing broilers, comprising only 9% of the microbiome.

Alistipes has been suggested as a marker of a mature caecal microbiome, but its presence does not necessarily indicate a favourable microbiome composition in early microbiome communities (Richards et al., 2019). Interestingly, *Alistipes* were present at similar levels in the faeces of high-performing (0.3%) and low-performing (0.2%) broilers at 7 dph, with no significant difference observed between the two groups. Therefore, *Alistipes* do not appear to be a reliable performance marker in faecal samples at 7 dph, at least at the genus level.

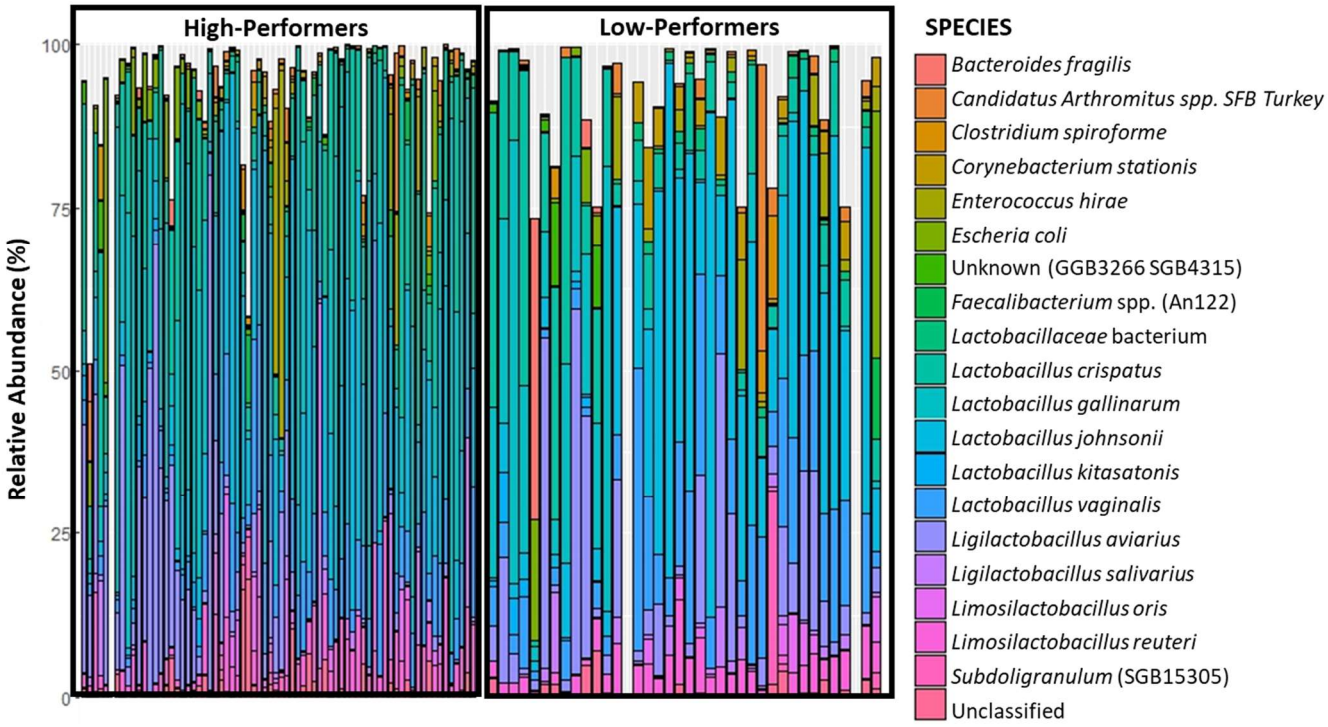


Figure 4.11 Relative abundance of top 20 species in faeces of higher and lower performing broilers at 7 dph.

High-performing broilers exhibited a significantly higher relative abundance of certain *Lactobacilli* species, including *L. gallinarum* (t-test, $P < 0.001$), *L. crispatus* (t-test, $P < 0.01$), and *L. reuteri* (t-test, $P < 0.01$), which may contribute to their biological efficiency. Conversely, low-performing broilers had a significantly higher relative abundance of other *Lactobacilli* species, such as *L. johnsonii* (t-test, $P < 0.01$) and *L. vaginalis* (t-test, $P < 0.01$). These findings suggest a potential link between *Lactobacilli* composition and broiler

performance (Fig. 4.12). Given that *Lactobacilli* are commonly associated with probiotic effects (Adhikari and Kwon, 2017, Pokorná et al., 2019), these results underscore the importance of *Lactobacilli* in microbiome development, performance, and gut health in broilers.

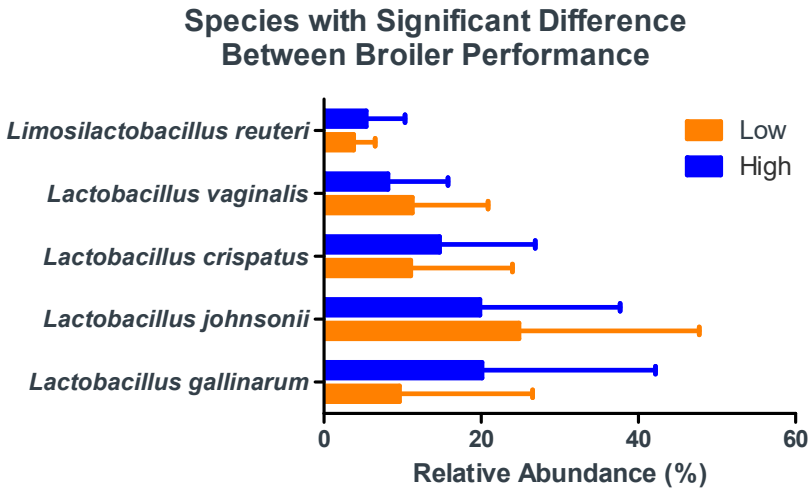


Figure 4.12 Average relative abundance and SD for species with significantly different abundances in the faeces of lower and high-performing broilers at 7 dph.

There was no difference in diversity between high and lower performers (Fig. 4.13). This suggests that the overall richness and diversity of bacterial species in the faecal microbiome at 7 dph do not underpin variations in performance. However, subtle discrepancies among specific and low abundant taxa could potentially play a role.

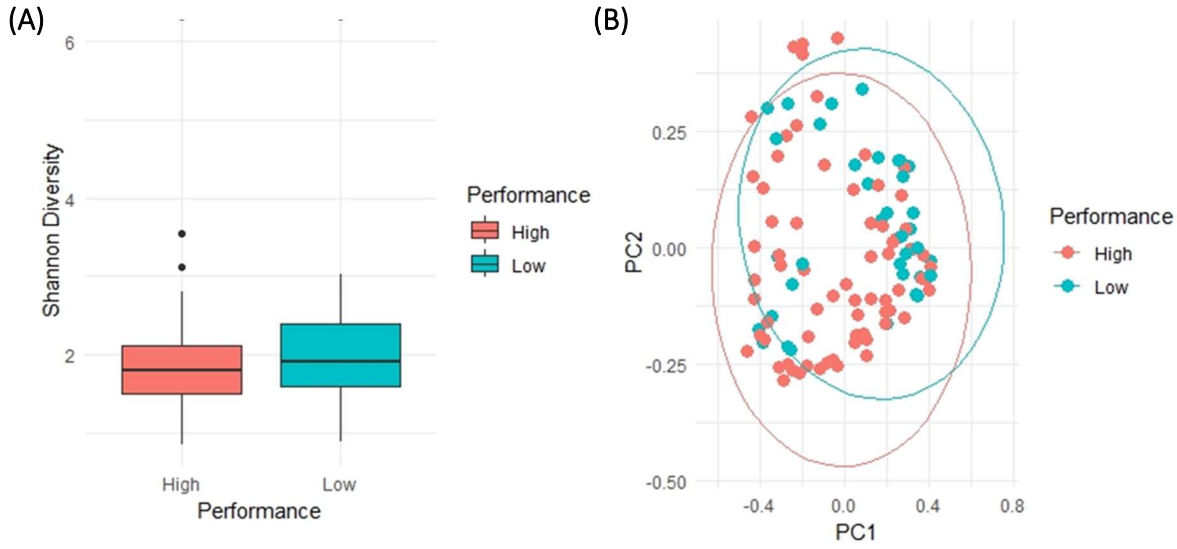


Figure 4.13 α-diversity measured by Shannon index **(A)** and β-diversity measured by Bray-Curtis dissimilarities **(B)** comparing faecal microbiome composition of low- and high-performing broilers. Data was generated with MetaPhlAn 4, phyloseq and ggplots2 in R.

4.3.3.2 *Antimicrobial Resistance and Virulence Factors*

The most prevalent resistance gene determinants observed conferred resistance to tetracycline, aminoglycoside, lincosamide, and streptogramin (Fig. 4.14A). These resistance genes were most abundant in samples collected in March 2022. Despite no antibiotics ever being used on this farm, a total of 75 AMR genes were identified through shotgun metagenomic sequencing across all collection dates (Fig. 4.14B). Samples collected in March 2022 exhibited the highest number of total AMR genes, totalling 1197, with an average of 41 genes per broiler; genotype A harboured 344 genes, while genotype B contained 546. In comparison, samples collected in March 2020, 651 AMR genes were recorded, averaging 22 per broiler, with 241 in genotype A and 221 in genotype B. The lowest count was observed in September 2022, with a total of 434 AMR genes detected, averaging 15 per bird, comprising 108 in genotype A and 186 in genotype B.

A total of 355 virulence factors were identified through shotgun metagenomic sequencing across all collection dates (Fig. 4.14C). In March 2020, 178 virulence factors were detected, with 84 in genotype A and 94 in genotype B, averaging 4 factors per broiler. March 2022 exhibited 85 virulence factors, with genotype A birds showing less than 1 factor

per broiler, while genotype B had an average of 3 factors per bird. This discrepancy between genotypes was notable. The contrast became even more pronounced in September 2022, when no virulence factors were found in the faeces of any broilers from genotype A. In contrast, all 92 virulence factors detected at this collection point belonged to genotype B, averaging 4 factors per broiler. While these are the average figures, one broiler had up to 19 virulence factors present, while 51% of broilers had none.

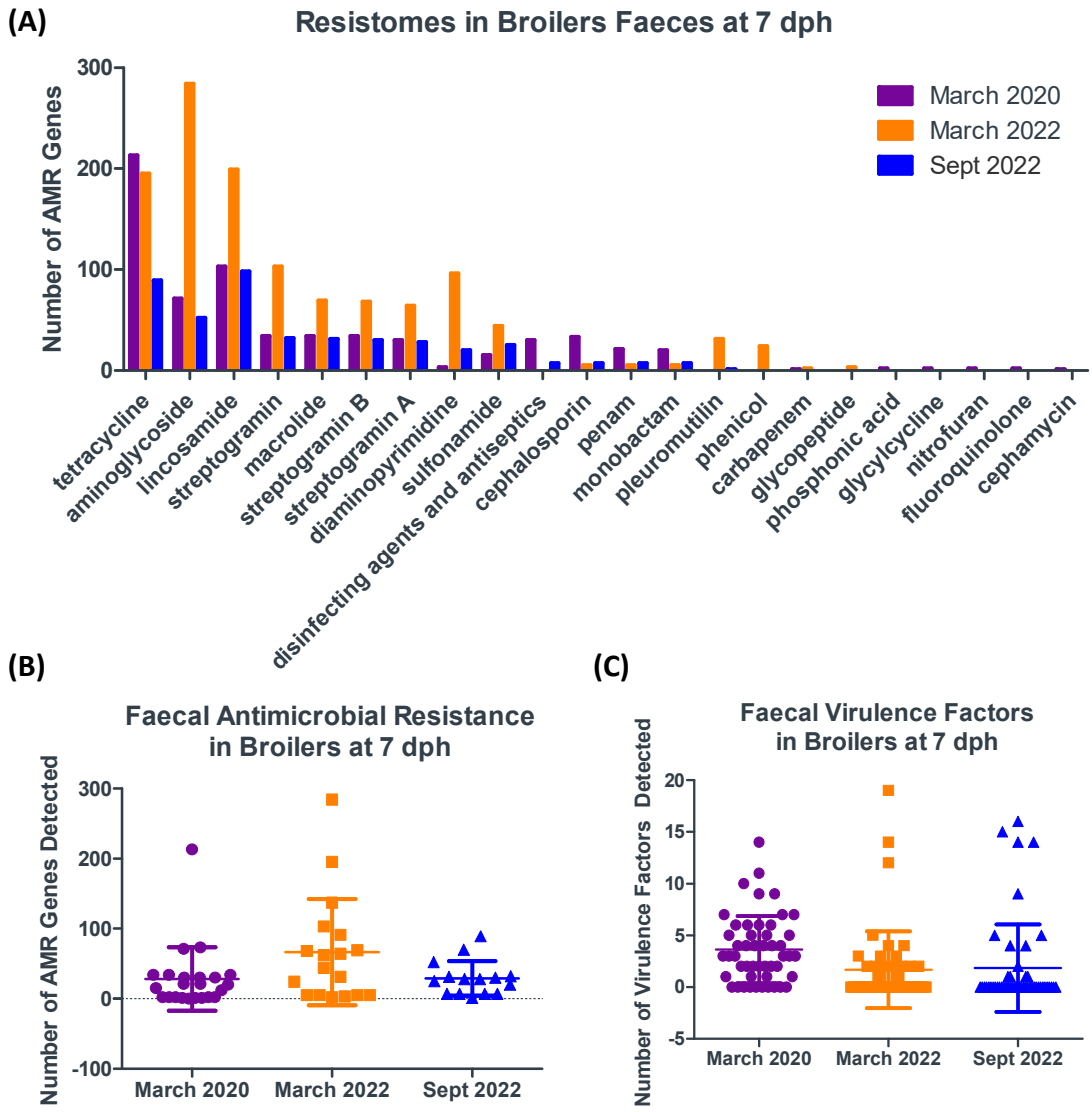


Figure 4.14 Carriage of AMR genes and virulence factors within broiler faeces at 7 dph across three collection dates.

4.3.4 Faecal Mycobiome of Broiler Chicks

In March 2020, 837,495 quality-filtered reads were obtained from ITS metabarcoding sequencing, whereas March 2022 yielded 67,808 reads, and September 2022 produced 463,450 reads. The observed disparity in read quantities, particularly in the March 2022 cohort (Fig. 4.15), likely reflects differences in sequencing quality. Despite this, 3,686 ASVs were identified across all samples, with taxonomic assignments available at the order level. It is important to note that this taxonomic resolution was limited due to sequencing variability, which could affect the accuracy of these findings.

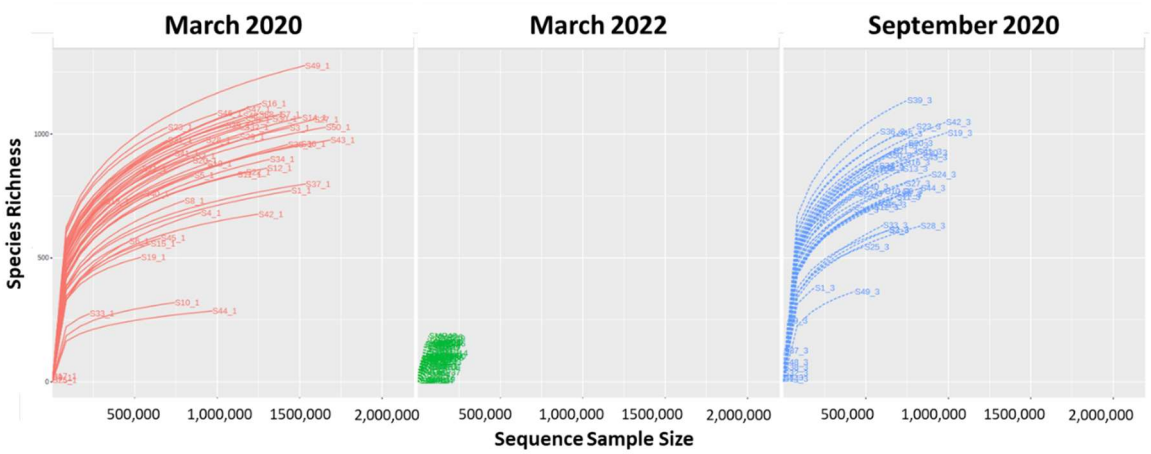


Figure 4.15 Rarefaction curves of ITS1 rRNA amplicon sequencing reads in broiler faeces at 7 dph, comparing three collection dates.

Despite the comparatively lower read count, the reads obtained from March 2022 exhibited higher quality, facilitating more accurate taxonomic assignments. This enhancement in sequencing quality likely contributed to the improved taxonomic resolution observed in March 2022 compared to the March 2020 and September 2022 cohorts. A significantly greater number of taxa remained unassigned in the March 2020 and September 2022 datasets (ANOVA, $P < 0.0001$) (Fig. 4.16).

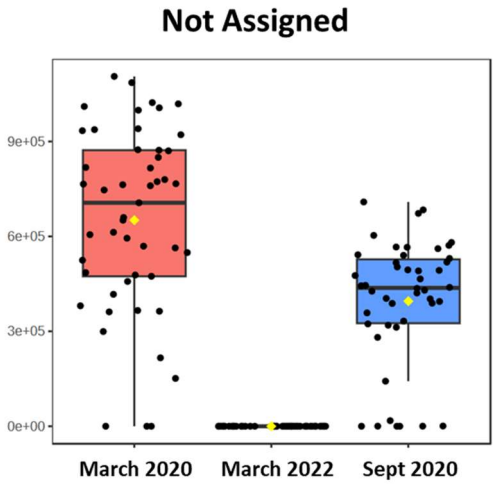


Figure 4.16 Filtered read count for Not Assigned taxa at each collection date.

In March 2020, the order *Eurotiales* predominated, while in March 2022, *Hypocreales* and *Pleosporales* were dominant, and in September 2022, *Saccharomycetales* dominated the fungal community (Fig. 4.17).

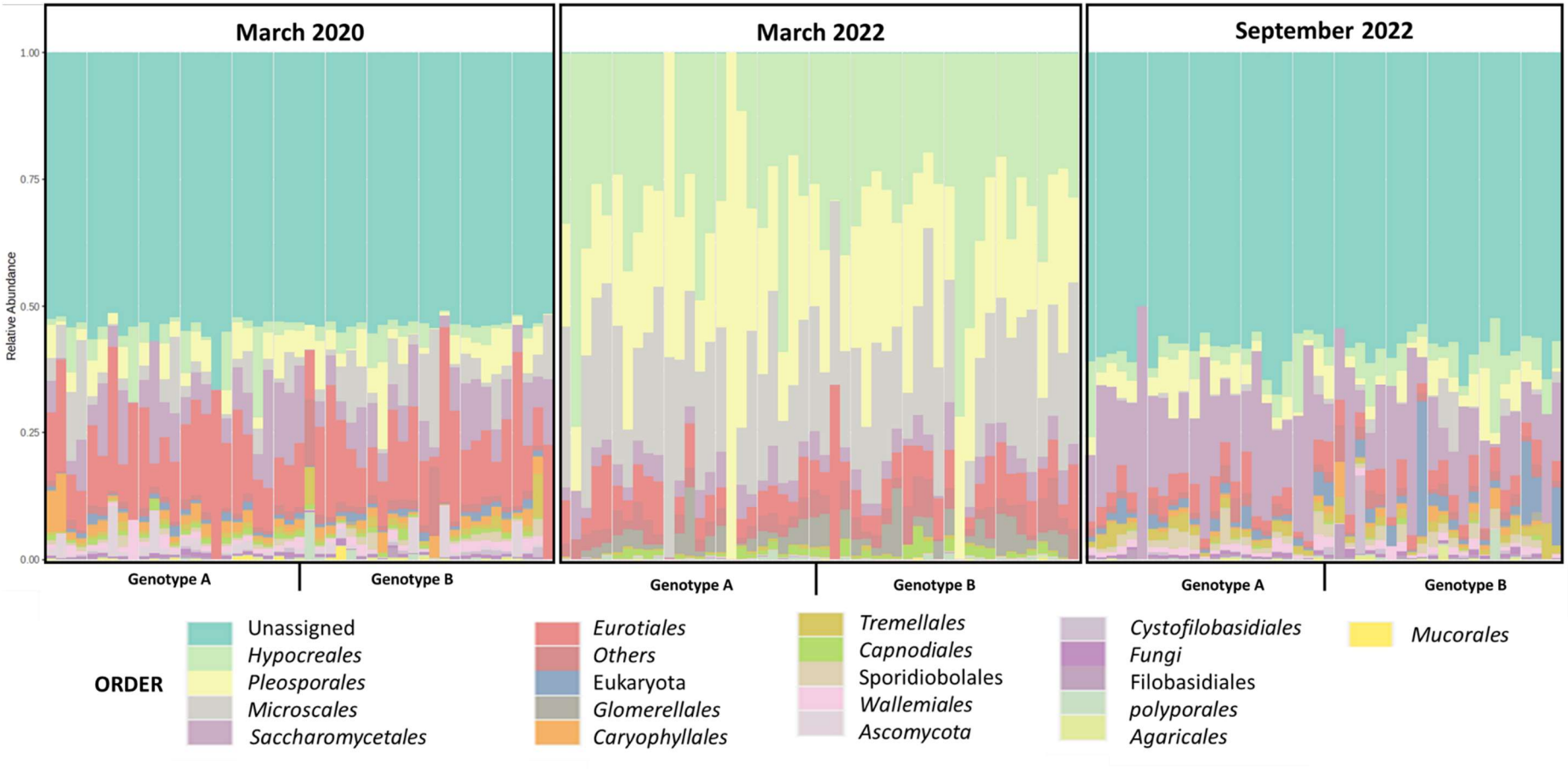


Figure 4.17 Relative abundance of orders in broiler faecal mycobiome at 7 dph.

In March 2020, the family *Aspergillaceae* was the most abundant, comprising 10% of the mycobiome (Fig. 4.18); unassigned taxa accounted for 66%, and other less abundant taxa contributed 7% of the total mycobiome. In March 2022, *Pleosporaceae* dominated, representing 27% of the mycobiome, *Hypocreales* at 26%, and *Microascaceae* at 24%. Similarly, in September 2022, unassigned taxa comprised 70% of the mycobiome, other smaller taxa at 9%, and *Saccharomycetales* at 4%. *Aspergillaceae* constituted 3% of the mycobiome in March 2022 and September 2022, indicating consistent abundance across these dates. The presence of numerous smaller taxa in March and September 2022 underscores the richness of the broiler faecal mycobiome at 7 dph whilst emphasising the extent of fungal taxa yet to be identified. *Caryophyllaceae* and *Dianthus* were identified, although they are flowers. This could be due to a couple of reasons. One possibility is misidentification, as we have established that while ITS metabarcoding is better for mycobiome analysis than shotgun sequencing, it still results in many unassigned ASVs. Another reason could be the high conservation of the ITS gene in eukaryotic cells

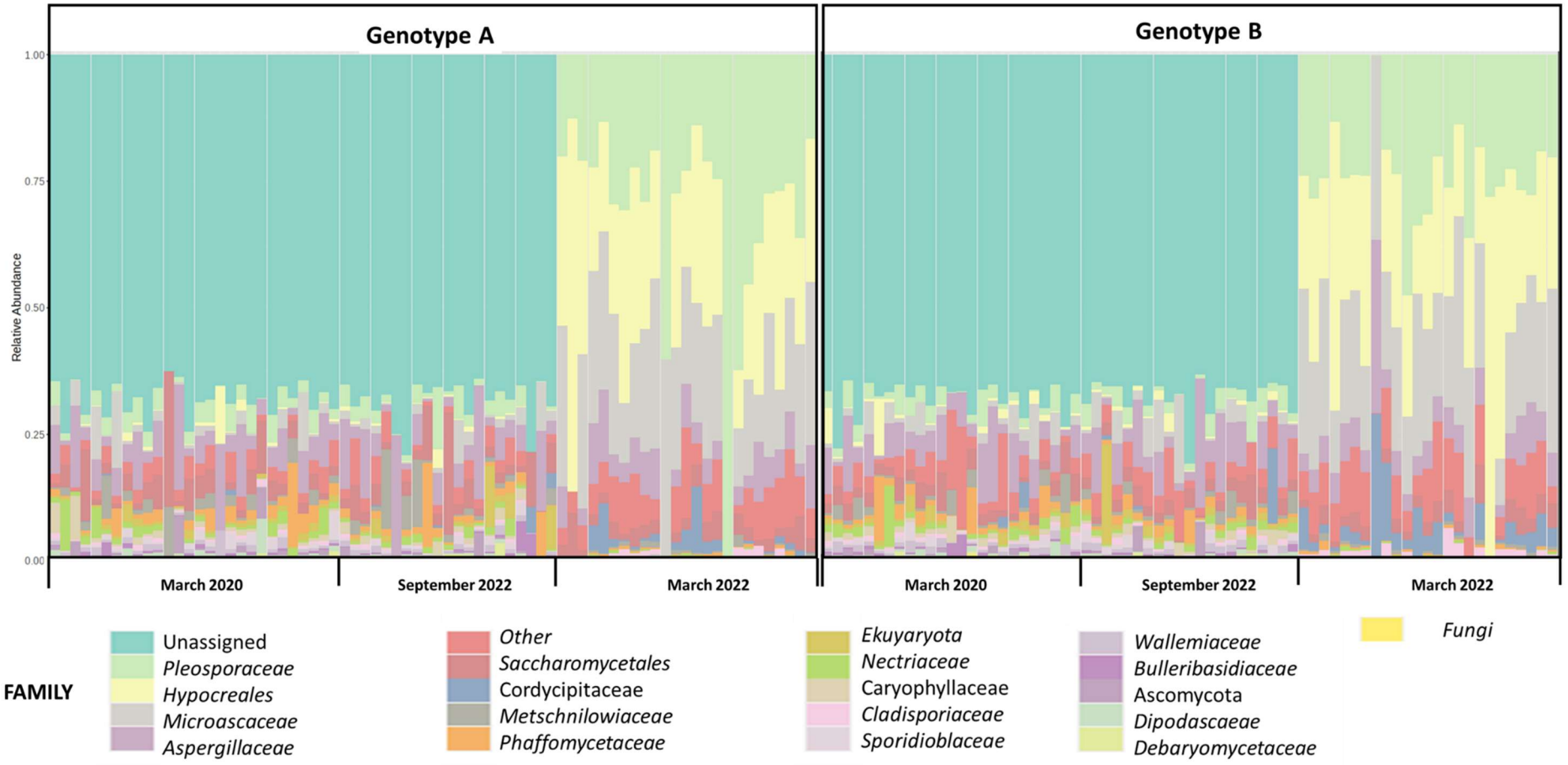


Figure 4.18 Relative abundance of families in broiler faecal mycobiome at 7 dph.

No significant differences in diversity were observed between the faecal mycobiome compositions of March 2020 and September 2022 (Fig. 4.19). However, α-diversity was on average lower in March 2022 compared to March 2020 (ANOVA, $P < 0.05$). Regarding β-diversity measured by Bray-Curtis dissimilarities, March 2022 exhibited significant differences from the other collection dates (ANOVA, $P < 0.0001$), with higher values indicating greater similarity between broiler faecal mycobiomes in March 2020 and September 2022. These findings align with the microbiome analysis, where September 2022 demonstrated greater similarity to March 2020.

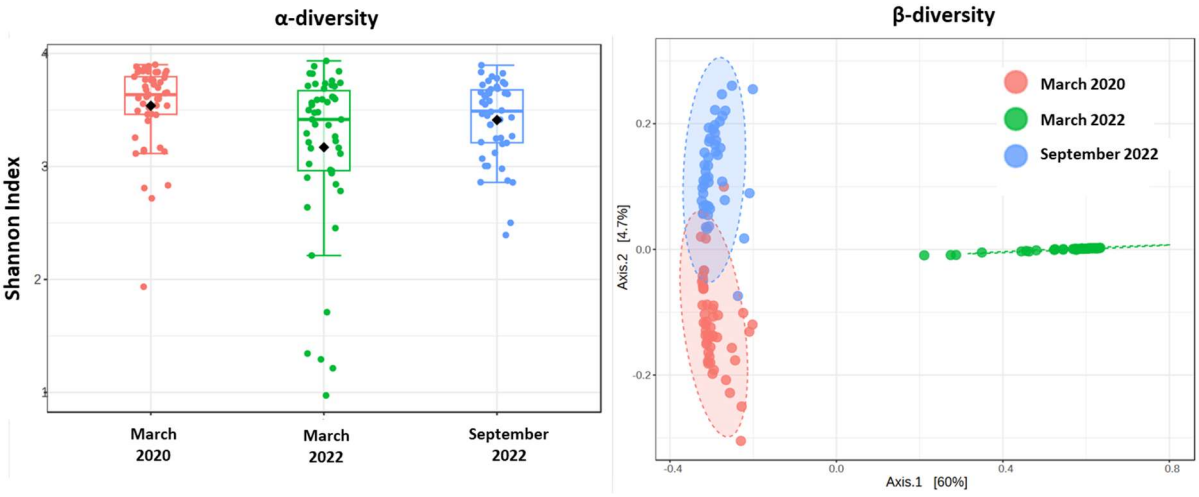


Figure 4.19 Broiler faecal mycobiome α-diversity (A) and β-diversity (B) across the three collection dates.

Across all collection dates, genotype B exhibited on average a higher α-diversity (Fig. 4.20A) compared to genotype A (t-test, $P = 0.04$). However, there was no significant difference in β-diversity (Fig. 4.20B), indicating that while genotype B demonstrated greater species richness than genotype A, there were no significant differences in community evenness between the two genotypes.

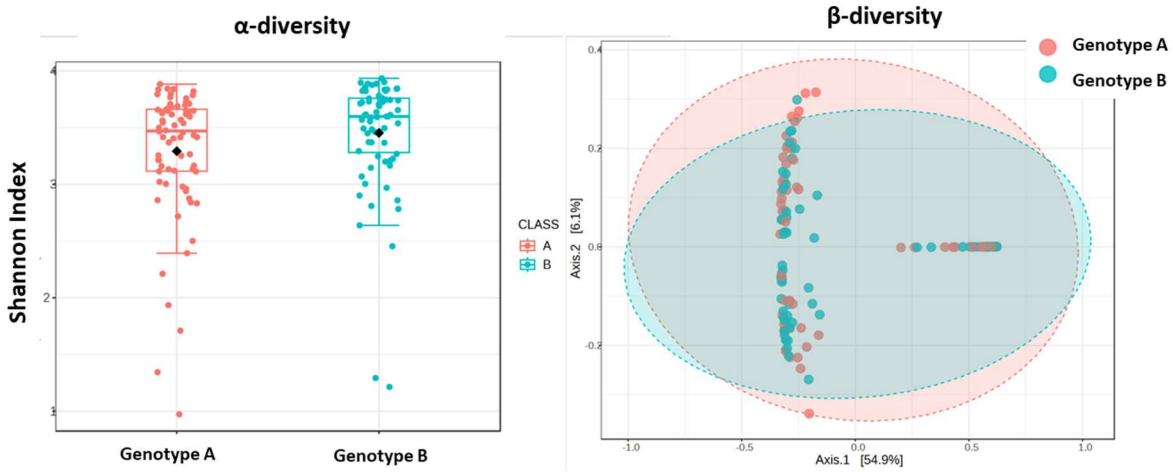


Figure 4.20 Broiler faecal mycobiome α-diversity **(A)** and β-diversity **(B)** between two genotypes.

There were 65 significant differences observed in mycobiome taxa between collection points (ANOVA, $P < 0.0001$). Among these differences, *Microascaceae* exhibited the most pronounced variation (Fig. 4.21A), with higher abundance detected in March 2020 and March 2022 and scarcely present in September 2022. Despite *Aspergillaceae* being detected across all samples, its abundance was notably higher in March 2020 (Fig. 4.21B), contrasting with its lowest abundance in March 2022. This discrepancy may underscore database bias, as *Aspergillus* was identified in shotgun metagenomic sequencing. Despite *Pleosporaceae*'s dominance in March 2022, its abundance was significantly higher in March 2020 (Fig. 4.21C). *Hypocreales*, another dominant taxon in March 2022, exhibited significantly higher abundance at this time than at other collection points (Fig. 4.21D). *Metschnikowiaceae* was absent in March 2022 samples but displayed low abundance in both March 2020 and September 2022 (Fig. 4.21E). This demonstrates the presence of low-abundance taxa contributing to the observed similarity in diversity between March 2020 and September 2022.

No statistically significant differences were observed in mycobiome diversity or taxa composition between low and high-performing broilers, nor between sexes. However, when collection date was considered a covariate, six significant differences were detected between genotypes (ANOVA, $P < 0.01$). Specifically, *Corynesporascaceae* exhibited significantly higher abundance in genotype B (Fig. 4.21F).

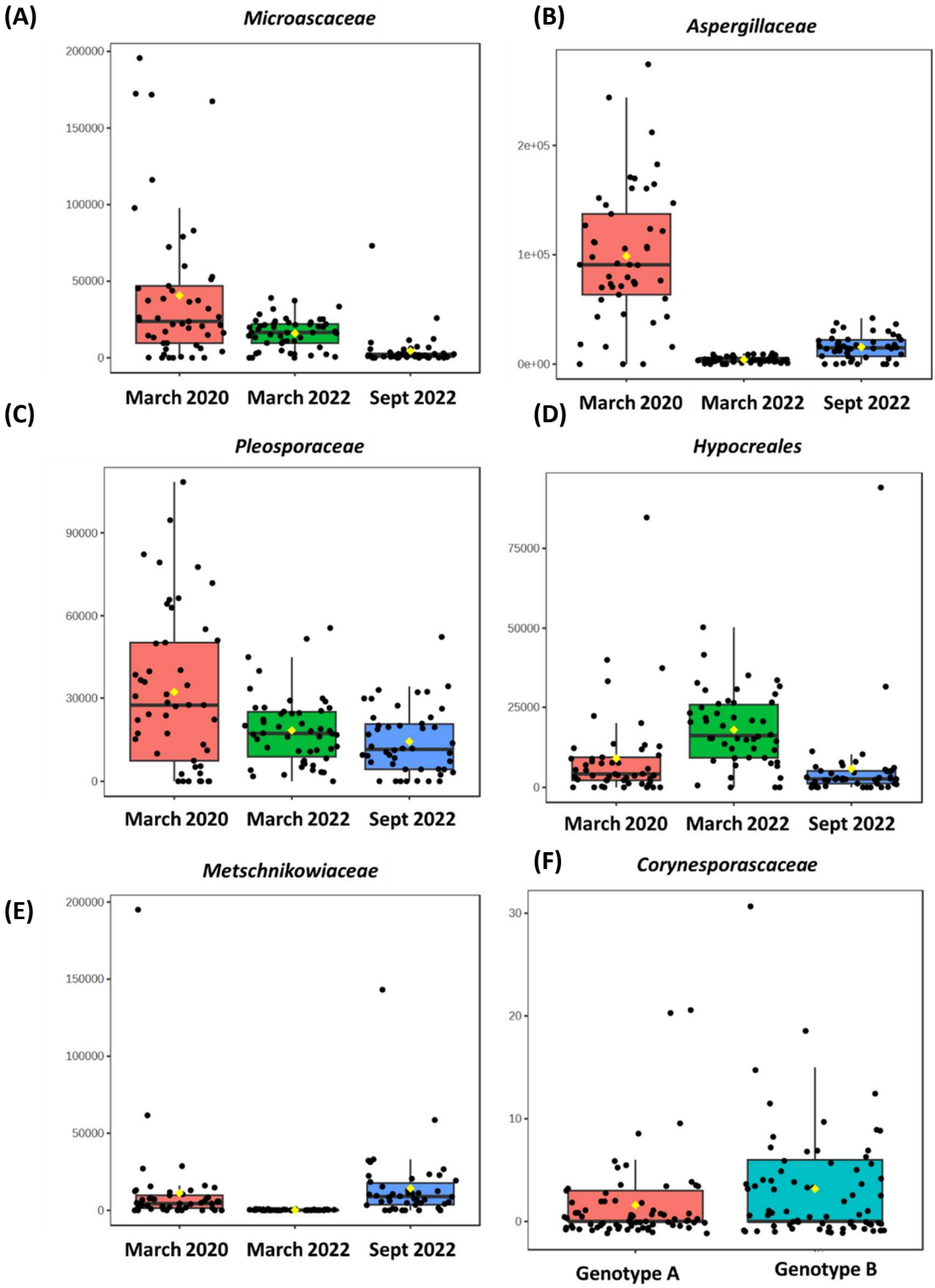


Figure 4.21 Filtered counts from taxa showed significant differences (ANOVA, P < 0.0001) between collection points **(A-E) and** between two genotypes **(F).**

The core mycobiome comprises fungal families prevalent in all samples at a relative abundance exceeding 0.01% (Fig. 4.22A). At this threshold, *Pleosporaceae*, *Aspergillaceae*,

Microascaceae, and the order *Saccharomycetales* exhibited prevalence rates of 90%, 100%, 70%, and 90%, respectively, across all broilers faecal samples at 7 dph collected over three distinct time points.

In March 2020, *Aspergillaceae* was present at an abundance of 0.07% in 90% of the samples (Fig. 4.22B). In contrast, in March 2022 and September 2022, its abundance decreased to 0.03% and 0.02%, respectively, with an occurrence rate of 80% in both cases (Fig. 4.22C-D). These findings reveal that the faecal mycobiome at 7 dph is inconsistent, particularly when compared to the consistent dominance of *Lactobacillaceae* in the faecal microbiome across various samples and cohorts. While the same fungal families are present across different samples and cohorts, suggesting the existence of a core mycobiome, the compositions still exhibit variability. Taxa *Aspergillaceae*, *Microascaceae*, *Pleosporaceae*, *Saccharomycetales* and *Hypocreales* were present in all sampling times, while March 2022 had the highest number of prevalent taxa which were absent from the other timepoints, including abundant members *Plectosphaerellaceae* and *Cordycipitaceae* (**Fig 4.21**)

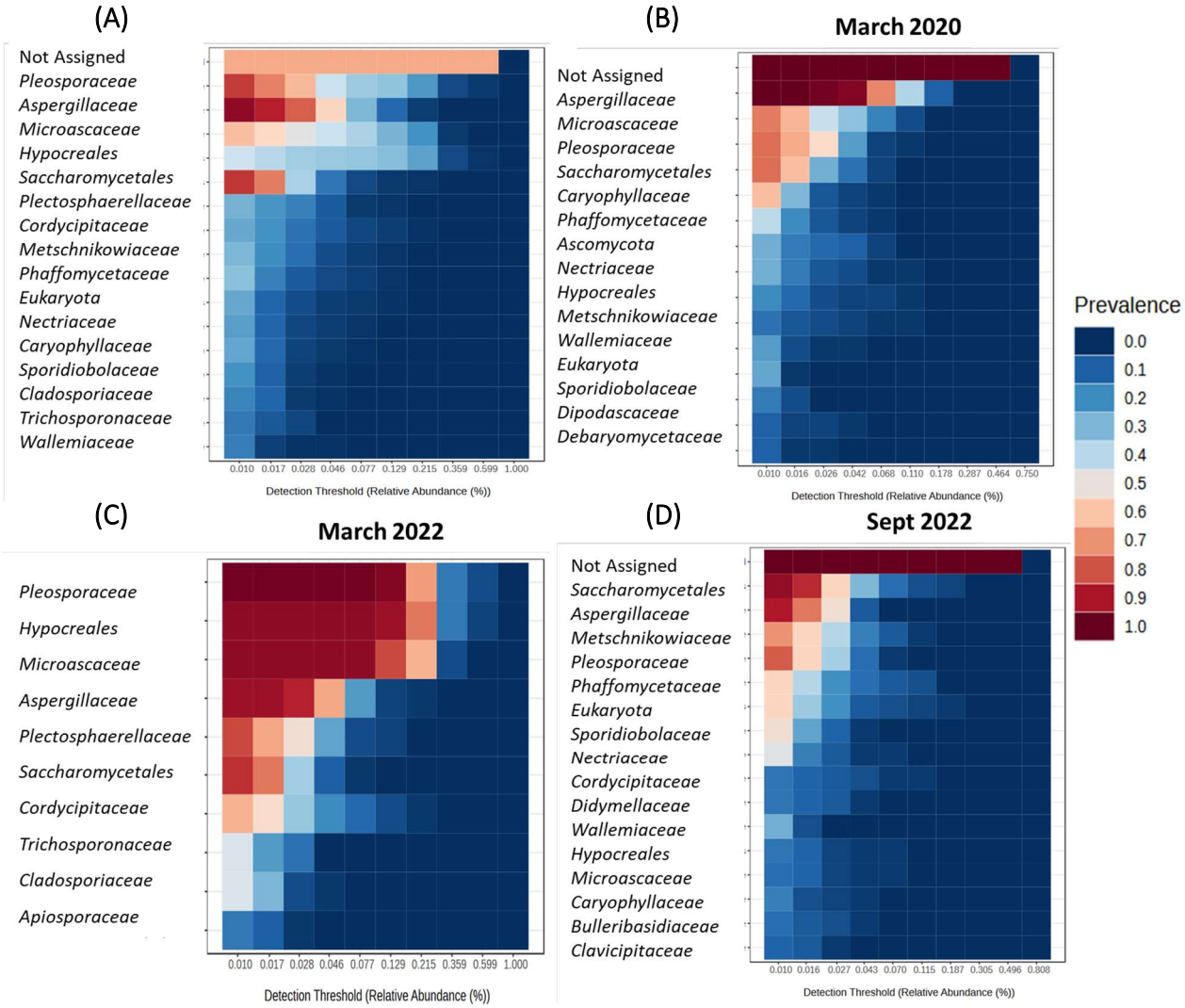


Figure 4.22 Heatmaps generated with MicrobiomeAnalyst 2.0 to see relative abundance and prevalence in cohorts of the core mycobiome families across the collection points **(A)** and between them **(B- D)**.

4.3.5 Correlating the Microbiome and Mycobiome

Faecal microbiome and mycobiome α-diversity showed no significant differences (Fig. 4.23A), suggesting that bacterial and fungal species do not affect each other's species richness in broiler chicks. However, there was a significantly positive relationship between the β diversities of the microbiome and microbiome (Fig. 4.23B), meaning that samples with more diverse bacterial populations also tend to have more diverse fungal populations.

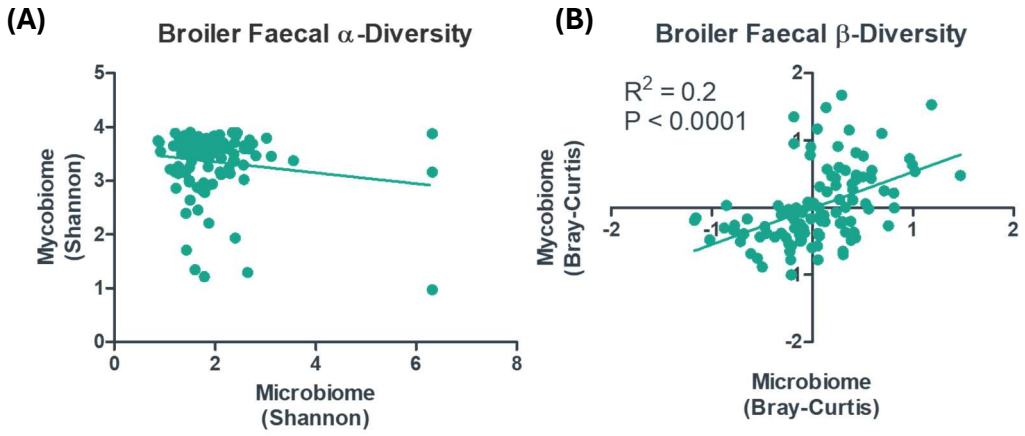


Figure 4.23 Linear regression models correlating microbiome and mycobiome faecal α- (A) and β- (B) diversity in broiler faeces at 7 dph.

Both microbiome and mycobiome faecal β-diversity exhibit a positive relationship with body weight (Fig. 4.24), supporting the concept that diversity benefits performance. It is important to note that these observations are specific to the early stage of 7 dph and the correlation is 0.8% for microbiome and 23% for the mycobiome indicating that other variables are influencing the body weight.

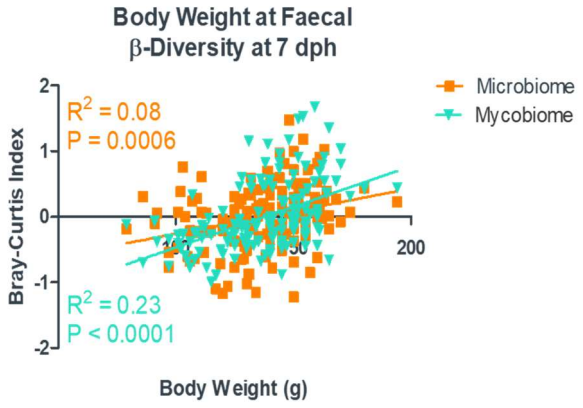


Figure 4.24 Linear regression model of β-diversity and body weight at 7 dph.

4.3.6 Faecal Metabolome of Broiler Chicks

This study identified 176 metabolites in the broiler faeces at 7 dph across the three collection dates. The ANOVA results identified 142 significant differences in metabolite concentrations between the three collection points (P = <0.0001) (Fig. 4.24A). Interestingly, March 2020 and September 2022 had 138 faecal metabolites shared between the two collections, with only 39 metabolites present in the faeces of all broilers at 7 dph despite similarities in the microbiome profile. This suggests a temporal variation in the faecal metabolome compositions, as demonstrated by the distinct clustering without any overlap in the PCoA plot (Fig. 4.25B). Unlike the microbiome, these findings suggest a unique relationship between faecal metabolomes and collection date.

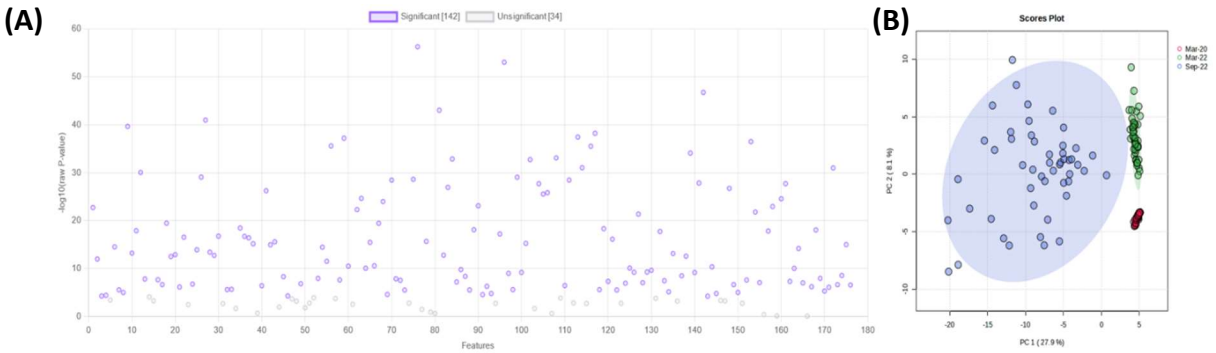


Figure 4.25 ANOVA results (P < 0.0001) comparing three collection dates (A) and PCoA plot demonstrating faecal metabolome compositions across three collection dates (B).

The analysis revealed 15 metabolites with significant genotype differences across all three collection dates (Fig. 4.26). These metabolites play crucial roles in various physiological processes. For instance, amino acids such as arginine, asparagine, aspartate, glycine, histidine, leucine, methionine, phenylalanine, tyrosine, and valine are involved in protein synthesis. Sugars like glucose, lactose, and sucrose, which are fermented by the GI microbiome to produce SCFAs, also showed differences. The lack of differences in SCFAs could indicate microbiome immaturity or low fermentation levels at this early stage. Succinate is rapidly converted as an intermediate in the production of SCFA propionate and was the only faecal metabolite to have a significantly higher concentration in genotype B

compared to genotype A. Lastly, trimethylamine N-oxide (TMAO), converted from trimethylamine (TMA) by the GI microbiome, was among the metabolites with significant differences.

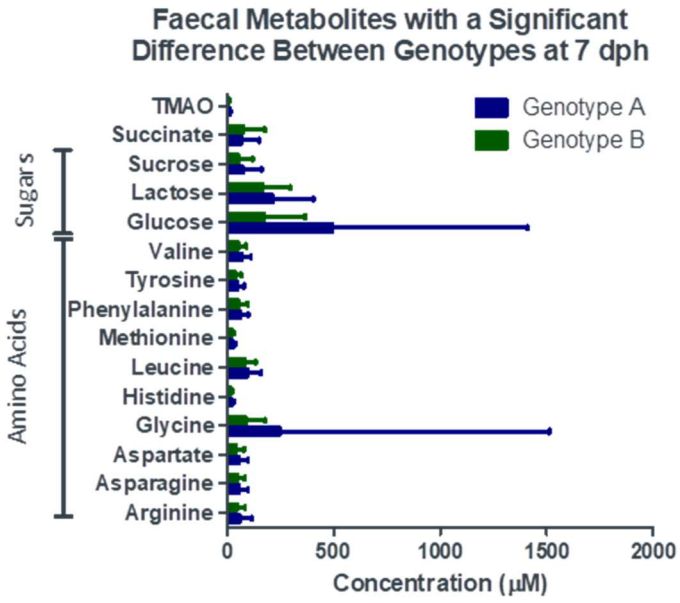


Figure 4.26 Metabolites that had a significant difference in concentrations between genotypes, showing the average concentration and SD for each genotype across three collection points.

Table 4.1 Average concentrations and SD of metabolites with a significant difference between genotypes, visualised in **Fig. 4.26**.

	GENOTYPE A			GENOTYPE B		
METABOLITE	Average Concentration	SD	# birds	Average Concentration	SD	# birds
Arginine	57.5	57.3	56	43.0	40.0	57
Asparagine	54.7	42.2	70	44.8	36.4	75
Aspartate	53.4	44.1	54	41.2	37.0	56
Glucose	493.4	915.1	52	167.9	197.7	59
Glycine	244.2	1267.5	70	84.0	93.0	75
Histidine	18.8	11.8	58	14.0	9.5	66
Lactose	212.9	190.9	70	164.1	131.9	75
Leucine	95.3	63.4	70	78.1	54.3	75
Methionine	25.3	11.3	56	19.6	8.9	56
Phenylalanine	60.1	38.8	70	52.4	41.1	75
Succinate	64.3	86.9	70	72.8	102.3	75
Sucrose	70.1	91.7	70	50.9	67.8	75

TMAO	8.7	5.5	70	5.4	4.0	73
Tyrosine	47.6	29.7	70	36.3	29.2	74
Valine	64.4	45.2	70	51.9	37.2	75

TMAO exhibited average higher concentrations in genotype A in March 2020 with a significant difference in March 2022 (ANOVA, $P < 0.01$) and no difference in September 2022 (Fig. 4.27A). Additionally, a positive trend can be observed between TMAO levels and β-diversity for both the microbiome ($R^2 = 0.16$, $P < 0.0001$) and mycobiome ($R^2 = 0.05$, $P < 0.0001$) (Fig. 4.27B). This suggests that higher TMAO levels in faeces are associated with a more diverse GI microbiome.

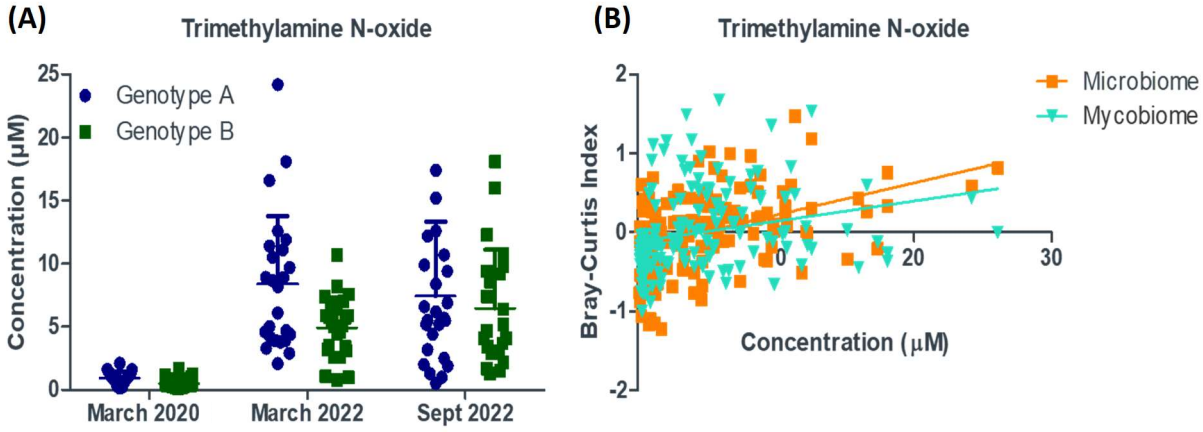


Figure 4.27 TMAO concentrations in broiler faeces across genotypes at different collection dates (A) and TMAO positive correlation to β-diversity (B).

This study found a negative trend between faecal TMAO and body weight at both 7 dph ($R^2 = 0.16$, $P < 0.0001$) and 35 dph ($R^2 = 0.19$, $P < 0.0001$) (Fig. 4.28). This may indicate that higher levels of TMAO are associated with lower body weight at this age. However, no relationship was observed between TMAO levels and BWG over the growth period. Furthermore, there was no significant difference in TMAO concentration between high- and low-performers, suggesting that TMAO levels do not directly influence growth rate or overall performance in terms of weight gain or that there are other factors involved with growth rate which reduce the influence of TMAO over time. This could imply that TMAO may be involved in other physiological processes affecting body weight rather than directly

impacting growth dynamics. These findings have practical implications for broiler management, highlighting the potential role of TMAO in understanding and optimising broiler performance.

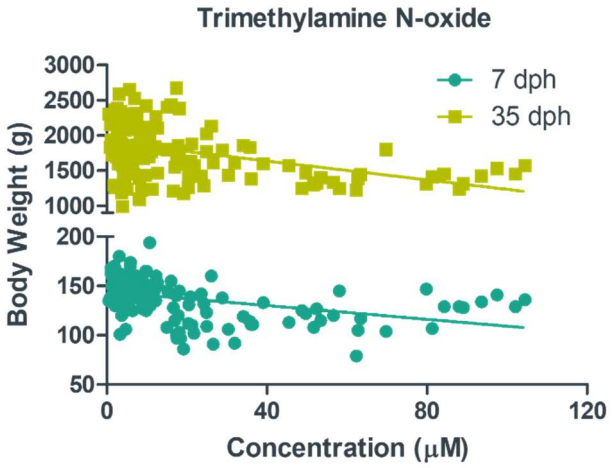


Figure 4.28 Linear regression model demonstrates a negative relationship between faecal TMAO at 7 dph and body weight at 7 and 35 dph.

Lactate and glucose were the most abundant metabolites in all faecal samples across all collection points. The high lactate levels are likely due to the dominance of *Lactobacilli* in the microbiome, as lactate is a standard metabolic product of these bacteria. Glucose (Fig. 4.29B), essential for cellular processes, and high glucose in the faeces could indicate poor absorption in the SI (Marcos et al., 2023). Despite the stability and detectability of these metabolites in faeces, there were no significant differences in lactate or glucose concentrations (Fig. 4.29A) and no observed relationships between these metabolites and body weight.

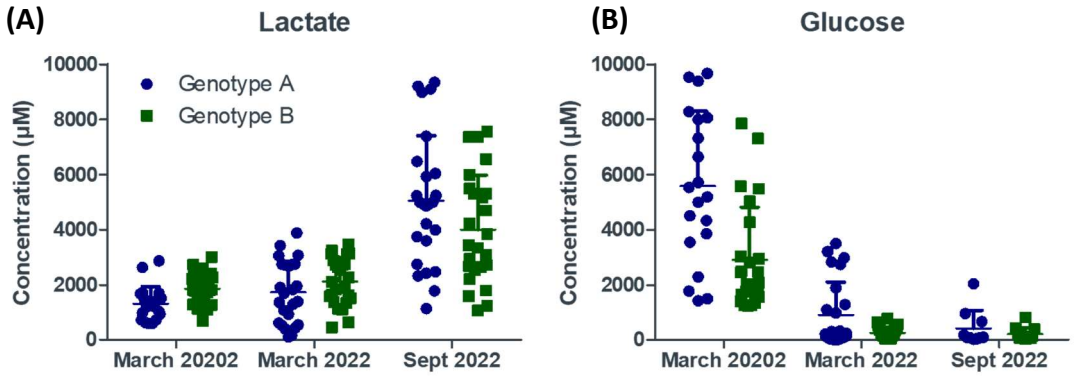


Figure 4.29 Broiler faecal concentrations of lactate (A) and glucose (B) at 7 dph between two genotypes across and three collection dates.

Lactate concentrations were significantly higher in high-performing broilers (Fig. 4.30A), potentially linked to differences in *Lactobacilli* composition. Additionally, high-performing broilers had significantly more acetate, though there were no differences in the levels of other SCFAs or taurine (Fig. 4.30B). Taurine, which is proposed as a potential GI microbiome marker of dysbacteriosis due to its role as the most common bile acid conjugate in chickens (Bailey, 2010), did not show a significant difference between performance groups. This suggests that while lactate and acetate may be associated with higher BWG, taurine is not.

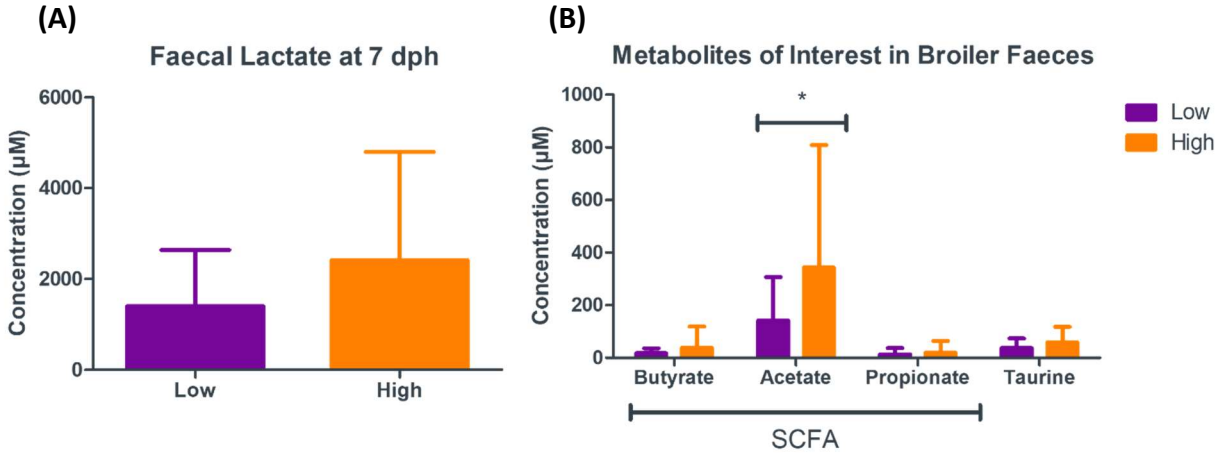


Figure 4.30 Average faecal lactate (A), SCFA and taurine (B) concentrations in higher and lower performing broilers at 7 dph.

4.3.7 Investigating the Broiler Microbiome to Aid in Phosphorus Nutrition

In the March 2020 cohort, an untargeted ^{1}H-NMR metabolomics analysis of broiler chick faeces uncovered noteworthy disparities, particularly in the presence of faecal myo-inositol, despite the absence of phytase in the diet. Genotype A exhibited significantly higher levels of myo-inositol than genotype B (Fig. 4.31), suggesting a potential variance in the GI microbiome's capacity to produce phytase. This discrepancy may affect overall biological efficiency, considering genotype A's higher average body weight at 7 dph in March 2020. Species with significant differences in relative abundance were also observed in the composition of the microbiome (Figs. 4.4-4.6) and the mycobiome (Fig. 4.21) between genotypes. However, no differences in diversity were observed, suggesting low abundant taxa may be responsible for or contribute to the activity.

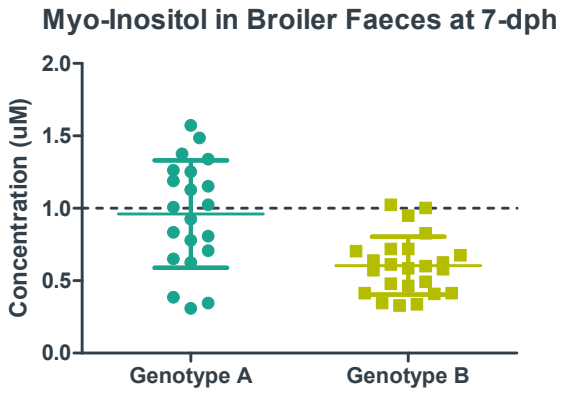


Figure 4.31 Two-way ANOVA on metabolomics data demonstrated significantly more myo-inositol in birds from genotype A than genotype B (P = 0.0014).

Applying the Human3 tool to shotgun metagenomic sequences of broiler chick faeces at 7 dph revealed the presence of pathway 'PWY-4702: phytate degradation I'. Furthermore, in the taxonomic assignment of these sequences, phytase-producing organisms such as *E. coli*, *Cladosporium* spp., and *Rhizomucor pusillus* were identified (Jatuwong et al., 2020, Selle and Ravindran, 2007).

4.3.7.1 *Gene Mining*

3-Phytase and 6-Phytase are enzymes that hydrolyse phytate (myo-inositol hexakisphosphate) at different positions: the third and sixth carbon atoms of the inositol ring, respectively (Liu et al., 1998). While 3-Phytase is typically derived from fungi and bacteria and often works best in acidic conditions (Kerovuo et al., 1998), 6-Phytase is commonly found in plants and some bacteria and may have different optimal conditions (Viveros et al., 2000). Both enzymes are used to improve phosphorus bioavailability in animal feed, with their selection depending on specific digestive and feed composition needs.

Genome mining (performed by QIB Bioinformatician Dr Sumeet Tiwari) revealed a greater presence of genes encoding for 3-phytase (EC 3.1.3.8) than 6-phytase (EC 3.1.3.26) in the broiler chick faecal microbiome from March 2020. Although genotype A had

significantly greater myo-inositol concentrations in the faeces, there was no significant difference in the number of phytase genes in genotypes A and B (Fig. 4.32A). There was a significant correlation between the number of 3-phytase genes and the myo-inositol concentration (Fig. 4.32B), which gave further evidence of the phytase degradation process occurring. To further understand the sources of microbial phytase, microorganisms were isolated from the faeces and screened for evidence of enzymatic activity.

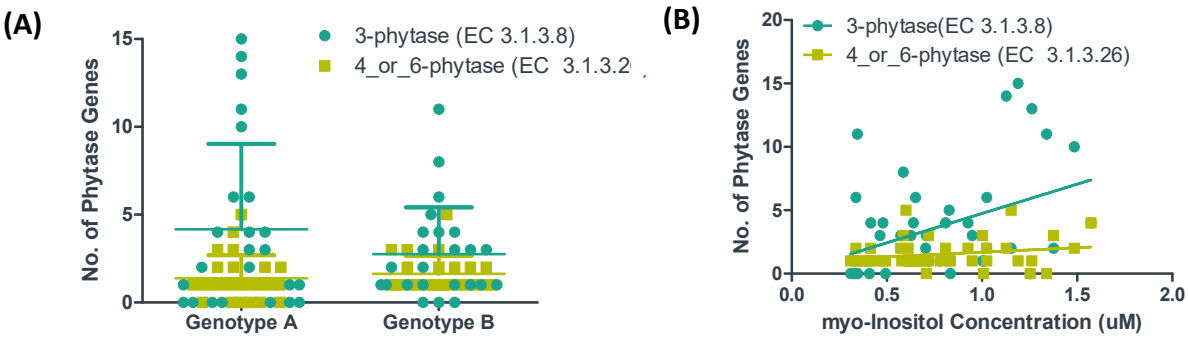


Figure 4.32 No significant difference was found in the number of phytase genes between genotype A and genotype B. (B) Linear regression model demonstrated a significant correlation (P=0.0072) between the presence of genes encoding for 3-phytase (EC 3.1.3.8) in samples with high Myo-inositol concentration. However, there was no significant effect (R^2=0.1564).

Seventeen taxa that had phytase genes were identified from the shotgun metagenomic sequences of broiler chick faeces. These comprised three phylum-level classifications: *Proteobacteria, Firmicutes*, and *Candidatus*. Among them were eight strict anaerobes: *Subdoligranulum, Pseudoflavonifractor, Rubneribacter, Rhodotorula, Oscillibacter, Clostridium, Anaerotignum,* and *Anaeromassilibacillus*. Additionally, two obligately anaerobic taxa were identified: *Eubacterium* and *Erysipelatoclostridium*. Two taxa were facultatively anaerobic: *Shigella* and *Escherichia*. Lastly, two taxa were aerobic: *Massilimicrobiota*, commonly found in the chicken caecum (Gilroy et al., 2021), and *Janthinobacterium*, typically found in soil and water (Gillis and Logan, 2015), although it was also detected in low abundance in the faecal samples from March 2020. Understanding

the aerobic, anaerobic and pathogenic characteristics of potential phytase-producing bacteria is vital for isolating and characterising such strains. *Escherichia coli* emerged as a significant contributor, encoding both 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26) genes (Fig. 4.33).

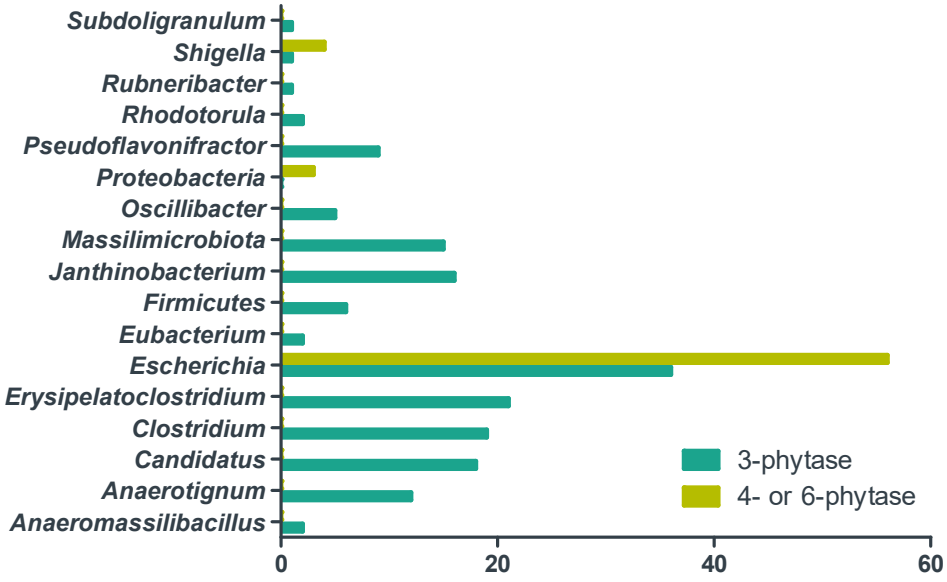


Figure 4.33 Phytase gene class and abundance in each taxon.

4.3.7.2 *Screening for Possible Phytase Activity*

Three strains from the QIB culture collection, each annotated with phytase production genes, were chosen to validate the screening process. No noticeable liquid or colony growth enhancement was observed with *E. cloacae* (data not shown). Both *B. subtilis* and *C. bovis* exhibited improved colony growth and liquid growth in the presence of Pa (Fig. 4.34). Employing these positive controls demonstrates that the gene alone (as seen with the *E. cloacae* strain) does not necessarily indicate active phytase production.

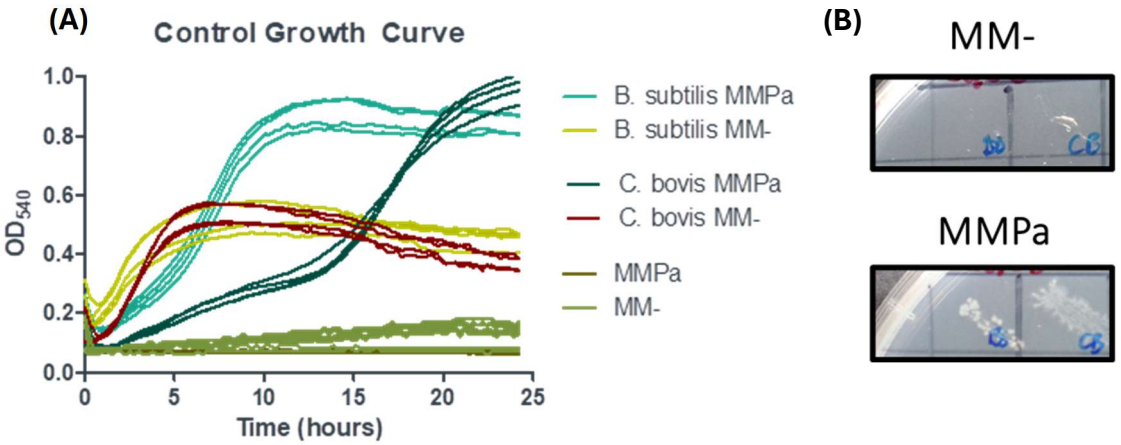


Figure 4.34 Growth curves (A) and solid growth (B) of two positive controls for developing the screening assays alongside media blanks with Pa (MMPa) and without (MM-). BS, *B. subtilis*, CB, *C. bovis.*

Of 13 broiler chick faecal samples exhibiting myo-inositol concentrations >1.0 µM, 84 isolates were obtained across four selective media types (Fig. 4.35A). 74% (64) of these isolates demonstrated enhanced growth in minimal media when Pa was added (Fig. 4.35B). During the screening process, *C. bovis* consistently outperformed *B. subtilis* in the liquid assay, regardless of whether the isolates were obtained from sub-culturing or glycerol stocks. These isolates showing improved growth in the presence of phytase will be subject to further investigation to quantify enzyme activity. No isolates were successfully obtained on the RB medium, and no yeasts were identified. This outcome is likely attributed to the dominance of bacteria over fungi in the faecal microbiome.

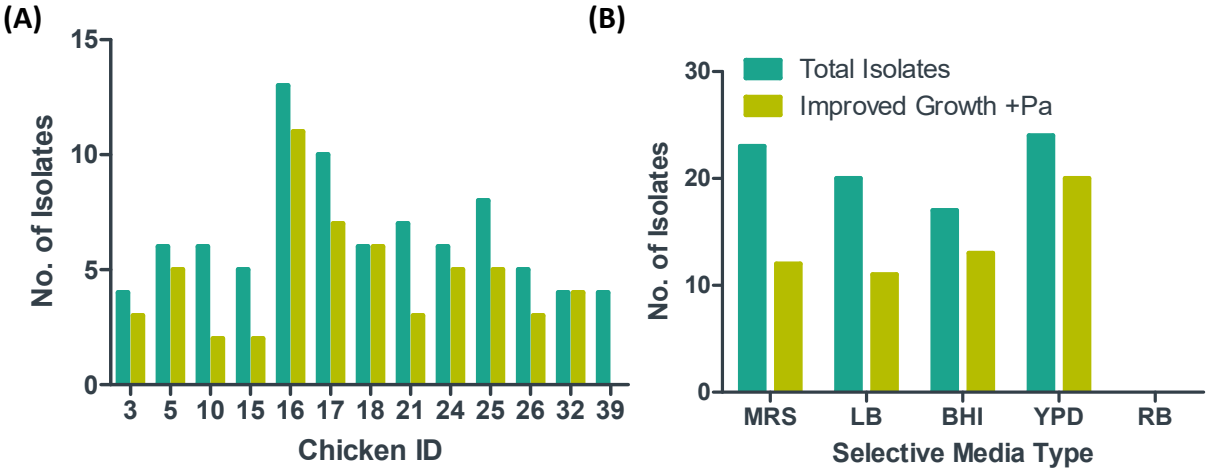


Figure 4.35 The number of total isolates and those with improved growth in the presence of Pa in the individual chickens (A) and across the different selective media types (B).

Improved colony growth was noted in 44% (37) of isolates when cultivated in minimal media with Pa, resembling the growth pattern seen in isolate 2 (Fig. 4.36B). Furthermore, 32% (27) of isolates displayed enhanced growth in the liquid screening assay, akin to isolates 3 and 4 (Figs. 4.36C-D). Among the 84 isolates analysed, 19% (16) exhibited improved growth in both the colony-forming and liquid phytase screening assays, mirroring the characteristics of isolate 1 (Fig. 4.36A). Conversely, 5% of the isolated strains showed no growth in either assay.

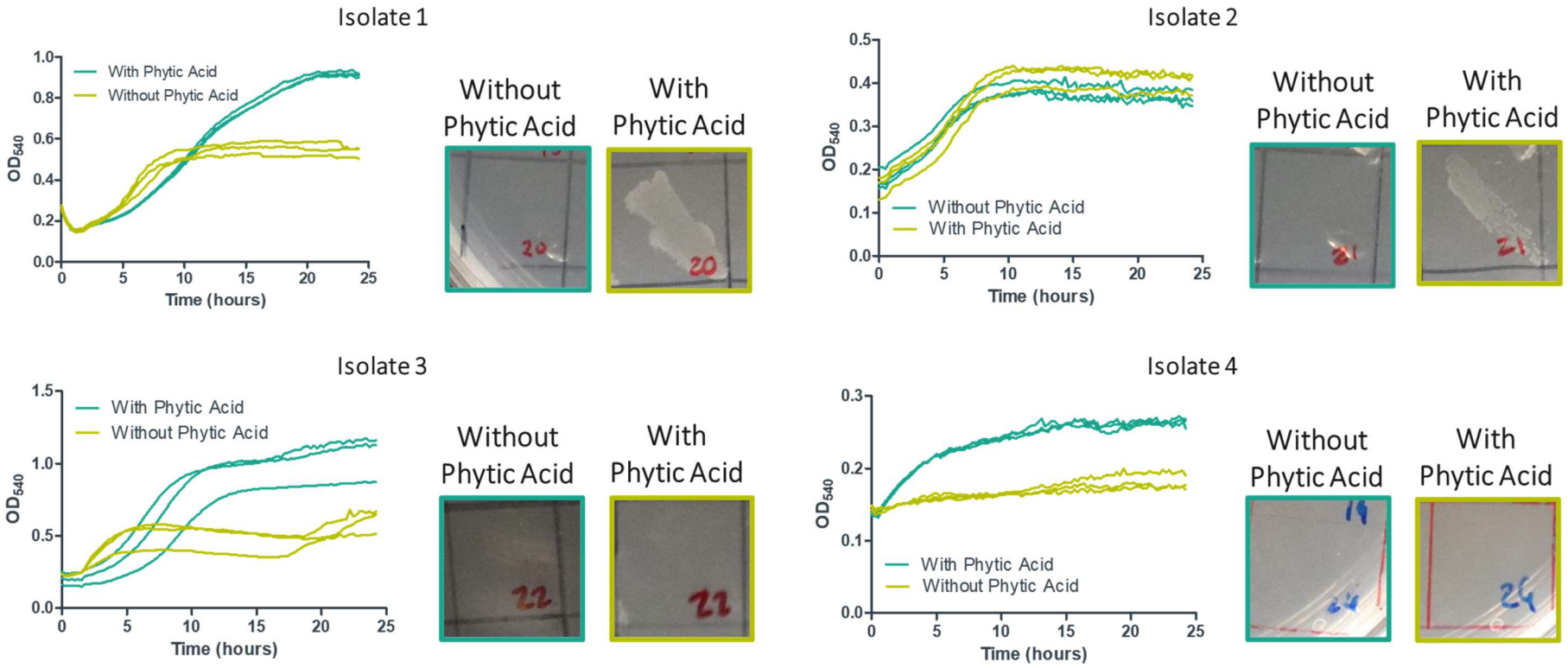


Figure 4.36 Example isolates of growth in liquid and solid assays.

4.3.7.3 *Whole Genome Sequencing and Annotation of Isolates with Phytase Activity*

Out of the 16 strains exhibiting enhanced growth in solid and liquid screening assays, whole-genome sequencing was conducted, and the data were submitted to the QIB culture collection. Surprisingly, *E. coli* was the sole species identified in both the bioinformatics analysis and screening experiments. Upon annotation of these 16 strains' whole genomes, none harbour previously identified phytase genes. Instead, acid phosphatase was detected, a pathway with broad specificity that shared homology to 4-phytase (Fig. 4.37). Additionally, two strains of *Proteus mirabilis* were isolated, representing novel findings regarding phytase activity. However, given its potential pathogenicity, the suitability of *Proteus mirabilis* as a probiotic candidate is questionable.

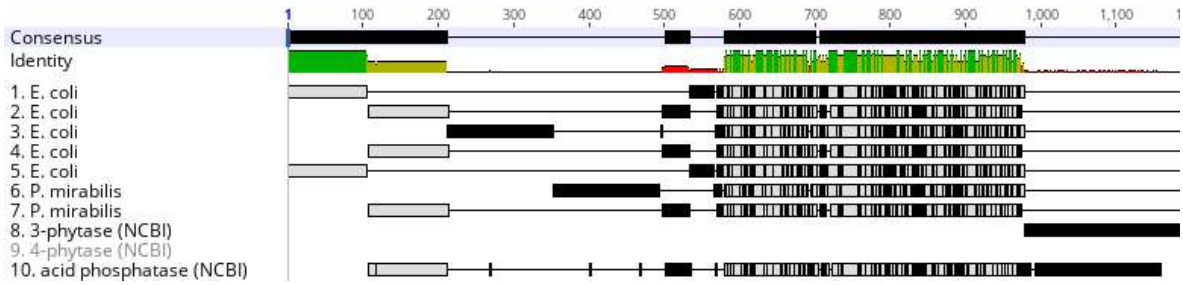


Figure 4.37 Predicted phytase nucleic acid alignment.

Acid phosphatase plays a crucial but nonspecific role in phytase production within the lower GI tract. While phytase production and Myo-inositol absorption predominantly occur in the SI, acid phosphatase facilitates phytase production in the distal gut (Viveros et al., 2000). By targeting the inositol phosphate intermediate, acid phosphatase accelerates phytate hydrolysis (Żyla et al., 1989, Zyla et al., 1995a, Zyla et al., 1995b). However, research indicates that the synergistic interplay between microbial phytase and acid phosphatase activity levels is somewhat limited in the degradation of phytates found in barley, maise, and soybean meal — all crucial plant-based components of animal feed (Näsi et al., 1999).

4.3.7.4 *Quantification of Phytase Activity*

Phytase activity is typically measured in phytase units (FTU), with one FTU defined as the enzyme quantity that releases 1 micromole of inorganic phosphorus per minute from 0.0051 mol/l sodium phytate at 37°C and pH 5.50 under standardised test conditions (Liu et al., 1998). All 16 strains exhibiting enhanced growth in solid and liquid assays were evaluated for phytase activity.

Among these strains, an *E. coli* isolate (culture collection # QIB120) exhibited the highest phytase activity, albeit lower than the positive control, registered at 1 FTU/mL (Fig. 4.38A). Notably, lactic acid-producing bacteria, such as *Lactobacilli* and *Enterococcus* spp. which had no phytase genes, yielded false positive results in the assay, a phenomenon observed in analogous studies (Singh et al., 2013). In comparison to the commercially available phytase sourced from *Aspergillus niger*, acid phosphatase alone proved insufficient for notable phytase activity (Fig. 4.38B).

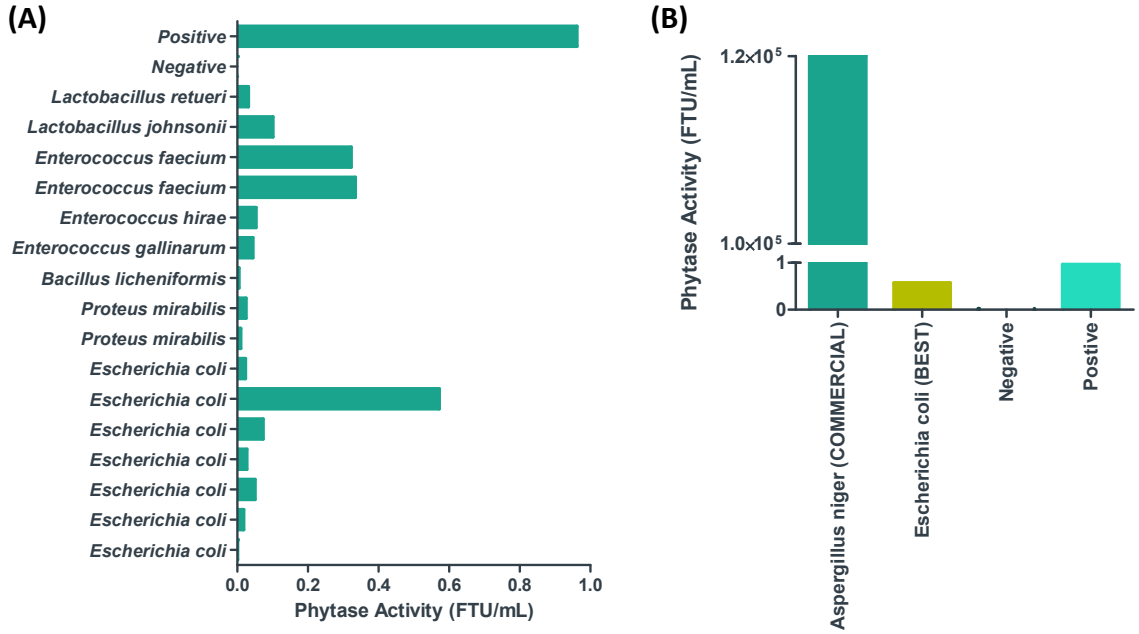


Figure 4.38 Phytase activity from 16 isolates showed improved growth in solid and liquid assays (A) compared to the strain with the most phytase activity commercially available (B).

4.3.7.4 *Faecal Myo-Inositol at Other Collection Dates*

This investigation conducted on March 2020 faecal samples prompted an exploration of myo-inositol levels in samples from March 2022 and September 2022. Myo-inositol was detectable in the faecal metabolome of all samples despite the continued absence of supplemented phytase in the diet, an occurrence not previously documented in the literature. No significant difference was observed between genotypes at any collection points (Fig. 4.39).

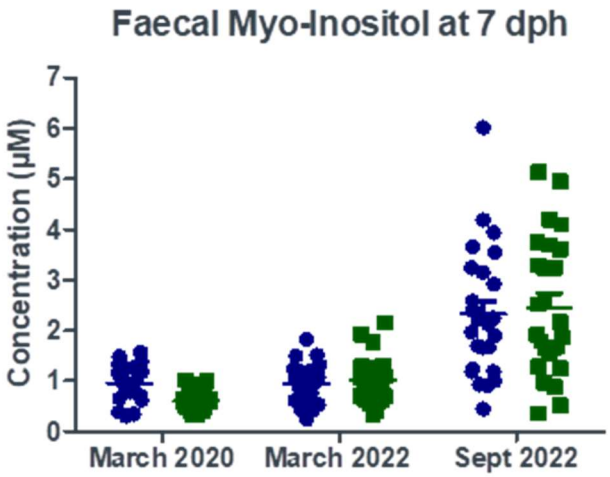


Figure 4.39 Faecal myo-inositol concentration in broilers at 7 dph in two genotypes across three collection points.

4.4 DISCUSSION

The faecal microbiome composition shared similarities in composition and showed no differences in species richness or diversity across all collection dates. Bacterial communities were as expected based on reports in the literature (Yeoman et al., 2012, Jurburg et al., 2019, Schokker et al., 2021). There are major similarities and overlaps in the microbiome, with no significant differences between bacterial families. However, there are some differences between species, and potential differences between strains, as different strains can elicit varying effects.

In contrast, the faecal mycobiome exhibited significant differences, with two collection dates showing high similarity and the third being markedly distinct. Unlike the microbiome, research has described the mycobiome as transient without much consistency (Davies et al., 2022, Robinson et al., 2020b, Robinson et al., 2022b). On the other hand, the faecal metabolome showed considerable variation in metabolite composition across all collection points, despite the chick faecal metabolome being described as consistent (Beauclercq et al., 2019b). Despite the variability in metabolite composition, a selection of specific faecal compounds were consistently detected, albeit in differing concentrations, indicating that valuable metabolic information can be obtained even at this early developmental stage.

Despite significant differences in body weight between genotypes within collection dates, no significant difference between genotypes was found at either 7 or 35 dph when combining all three cohorts. This suggests that there is no consistent overall genotypic effect on biological efficiency, highlighting the variability of birds across batches. These findings underscore the necessity of including cohort as a significant factor in analyses, as they demonstrate inherent variation even under controlled conditions.

Genotype B demonstrated slightly improved biological efficiency in terms of weight gain in March 2020 and March 2022. However, in September 2022, in contrast to the previous cohorts, genotype A and B had similar levels of productivity. With the genotypes, environment, feed specifications, and microbial composition are consistent across collection dates but the variation in performance implies that another factor is influencing biological efficiency.

The species that showed significant differences in relative abundance varied across collection dates and genotypes. One notable result from this study is the importance of *Lactobacilli*. Significant differences in the relative abundance of *Lactobacilli* species were observed between low and high-performing broilers. This underscores the importance of using sequencing techniques that allow species-level taxonomic resolution. It is not sufficient to merely assess the presence of *Lactobacilli* (Angelakis and Raoult, 2010); the specific species of *Lactobacilli* present are central in for the gut health and performance of the broilers (Adhikari and Kwon, 2017). *Lactobacillus* has been suggested to elicit a polarising effect on the caecal microbiome with a metanalysis determining relatively equal numbers of positive and negative interactions with other taxa (Zou et al., 2018). A possible

explanation is excess lactate production by *Lactobacillus* and reduced conversion to butyrate (Ducatelle et al., 2018a).

A diverse microbiome is likely to be accompanied by a diverse mycobiome in chicks, possibly because both benefit from good gut health or favourable environmental factors. This suggests the importance of considering the effects on the mycobiome when managing the microbiome. For instance, probiotics, prebiotics, and feed additives have been shown to impact the mycobiome (Ward et al., 2019), and since this area is less researched, the effects on gut health and performance still need to be better understood.

The faecal microbiome's and mycobiome's β-diversity show a significant relationship with body weight at 7 dph, although the exact mechanisms remain unclear. It may be beneficial to use fungal diversity as an indicator for microbiome maturity and performance instead of bacteria, as fungal communities generally have lower species richness (Davies et al., 2022, Robinson et al., 2022a, Robinson, 2019), making it easier to detect a general loss of diversity. Since the mycobiome diversity demonstrated a positive trend with the microbiome diversity in this study, monitoring fungal diversity could potentially provide insights into the bacterial microbiome changes. This approach could facilitate the identification of changes in diversity more effectively than focusing on bacterial diversity alone. However, whether this correlation will persist as bacterial communities undergo successional changes, and the GI microbiome matures is unknown.

The mycobiome in March 2022 was notably distinct, with significant differences in diversity, species richness, and dominant species compared to March 2020 and September 2022. However, the low read count in March 2022 likely contributed to these observed differences. These two other collection dates exhibited similar characteristics when comparing collection dates and individual broiler faecal samples within each cohort, suggesting consistency. Furthermore, March 2022 samples exhibited a higher number of virulence factors and AMR genes per sample. While speculative, one theory is that the distinct mycobiome in March 2022 may be linked to the increase in AMR genes. This could be due to the production of different fungal metabolites, such as antibiotics (Karwehl and Stadler, 2016), which exert different pressures on the gut bacteria.

Global political changes led to supplier shifts for some dietary components. Even with identical diet specifications, variations in feed preparation can occur due to differences in raw material quality and nutrient content. For example, if one batch uses soybean meal

with 38% crude protein and another uses soybean meal with 42% protein, the feed composition will differ. Similarly, variations in wheat protein and carbohydrate levels can alter the feed (Geier et al., 2009). These differences may explain the observed variations between hatches. Additionally, seasonal differences, with two collections in spring and one in autumn, could affect external temperatures and humidity, impacting conditions inside the shed. These factors highlight the importance of understanding the microbiome and the influence of industry factors on gut health and performance.

It is possible that TMAO and TMA-producing bacteria play a crucial role in early microbiome development but may not be beneficial for performance later in life. TMAO levels demonstrated a negative trend with body weight at 7 and 35 dph, suggesting a potentially detrimental impact on growth or metabolic processes as the birds mature. Early microbiome diversity is critical for creating a healthy and resilient gut environment (Rodrigues et al., 2020), potentially aiding in nutrient absorption, immune function (Broom and Kogut, 2018), and pathogen resistance (Kogut, 2019). However, high TMAO levels could reflect a microbiome composition that, while beneficial for initial development, might shift towards less efficient nutrient processing or other metabolic activities that hinder optimal growth later on. Further research is needed to elucidate the specific roles of TMAO and TMA-producing bacteria at different developmental stages and their long-term impacts on broiler performance. Understanding these dynamics could help optimise microbiome management strategies to enhance growth and health outcomes throughout the broilers' lifecycle. This could involve adjusting dietary components, probiotics, or other interventions to balance early microbiome benefits with long-term performance goals.

Exploring the broiler chick faecal metabolome and microbiome provided an opportunity to investigate a nutritional aspect crucial for performance. Poultry diets typically contain phytate and phytate-bound phosphorus, but this form of phosphorus is only partially available to the birds (Selle and Ravindran, 2007). Complete degradation of phytate releases both phosphorus and myo-inositol available for absorption, which is known to improve metabolic performance (Sommerfeld et al., 2018) and suggested to benefit feed conversion rates (Walk et al., 2018). Increased phosphorous bioavailability through the degradation of Pa by phytase not only aids in bone mineralisation but also reduces phosphorus pollution in the environment (Walters et al., 2019). However, poultry have inadequate endogenous phytase activity and thus require phytase derived from *A.*

niger or *E.* coli as a feed additive (Menezes-Blackburn et al., 2015). Including inorganic phosphate in their diet represents a significant expense, as phosphorus ranks as the third most costly ingredient, following energy and protein (Lim et al., 2024). Given the slim profit margins in poultry farming and the fact that feed is their greatest expense, this is a significant consideration.

Phytase is produced by both bacteria (Singh et al., 2013) and fungi (Olstorpe et al., 2009). Recent studies in laying hens have demonstrated that between 62% and 74% of dietary phytate is enzymatically degraded by the microbiome (Sommerfeld et al., 2020). However, this aspect has yet to be extensively studied in broilers, although myo-inositol supplementation has demonstrated improved feed efficiency in broilers (Sommerfeld et al., 2018). The establishment of poultry phytase diets predates the removal of AGPs from farms (Selle and Ravindran, 2007) and the emergence of a deeper understanding of the microbiome's role in performance and gut health. With advancements in management practices and a growing appreciation for the microbiome's influence, there is potential for re-evaluation. Despite the persistent exclusion of phytase from the diet, the discovery of endogenous phytase activity in broiler chicks suggests a possible pathway forward. By leveraging better management strategies and modulating the broiler chick microbiome, there may be opportunities to reduce reliance on phytase feed additives. This could lead to more sustainable and efficient poultry production practices while maintaining optimal performance and gut health.

These findings suggest that broiler chicks possess significant endogenous phytase activity as early as 7 dph. This discovery challenges the long-standing reliance on external phytase supplements and emphasises the importance of considering genotype-specific microbiome interactions and the potential influence of environmental and industry factors on productivity. Moreover, the correlations between mycobiome and microbiome diversity and performance metrics underscore a potential link between bacteria and fungi at this early development stage.

4.5 ADDITIONAL ACKNOWLEDGEMENTS

The generous support from the BBSRC and the QIB Impact Acceleration Award of £10,000, as well as the Society of Feed Technologists' Edgar Pye Research Scholarship of £1,250, enabled the investigation of the broiler microbiome in the context of phosphorus nutrition. I am deeply appreciative of the assistance provided by the dedicated team at QIB, including Dr Sumeet Tiwari, who contributed to gene mining and offered valuable technical support, and Rebecca Sutcliffe, James Choi, and Dr Jacob Scadden, who aided with purification and growth curve of isolates. *Chryseobacterium bovis* FI11139 and *Enterobacter cloacae* FI11317 were isolated from fermented cereal food kunu zaki by Dr Nwanneka Akinyemi.

CHAPTER 5

SPATIAL VARIATION OF THE BROILER GASTROINTESTINAL TRACT AT 13-DAYS POST HATCHING

5.1 INTRODUCTION

Significant variations in the relative microbiome composition across different GI tract segments in chickens have been documented (Oakley and Kogut, 2016, Wielen et al., 2002, Zhou et al., 2021, Oakley et al., 2014, Gong et al., 2007). Consequently, no single description can encapsulate the entirety of the intestinal microbiome. Studies utilising 16S rRNA sequencing in poultry have further highlighted noteworthy differences in composition corresponding to various ages and spatial locations within the GI tract (Glendinning et al., 2019).

The caecum plays a pivotal role in the GI tract, serving as a site for fermentation and producing short-chain fatty acids (SCFAs) (Bauer et al., 2019). It harbours the highest diversity and density of bacteria, making it a commonly studied area for assessing the intestinal microbiome. Despite its high microbial concentration and vigorous fermentation activity, the composition of the caecal microbiome differs from that of other GI tract regions (Glendinning et al., 2019). However, analysing caecal droppings or content does provide insight into digestive function but not necessarily the microbial composition of the upper GI tract or colon (Andreani et al., 2020). Additionally, birds typically empty their caecum every 24-48 hours (McNab, 1973), usually in the morning, potentially influencing the composition observed depending on the time of caecal collection.

The faecal microbiome is recognised as variable (Oakley and Kogut, 2016). Yet, it remains the least invasive and most efficient sampling method, allowing for data collection without sacrificing birds. Key factors shaping the composition of the faecal microbiome include age, diet, and environmental influences (Schokker et al., 2021). Notably, faecal samples include representatives of microbial populations from the upper gut (ileum) and caecum (Schokker et al., 2021).

At 13 dph, commercial broilers enter the second stage of their three-staged feeding programme, transitioning to a grower diet with elevated nutrient levels, including calcium, to foster swift skeletal development (Acres, 2018). Drawing from established knowledge on the successional changes of the chicken GI microbiome, the presence of initial colonisers such as *Lactobacillus* and *Enterococcus* can be observed from 0 dph, followed by rapid colonisers like *Escherichia* and *Shigella* emerging around 7 dph (Glendinning et al., 2019, Jurburg et al., 2019). By 10 dph, a greater presence of *Ruminococcus*-like fast-growing taxa

is noted (Glendinning et al., 2019, Jurburg et al., 2019, Mohd Shaufi et al., 2015). The GI microbiome and metabolome attain a relatively stable state from 20 dph onwards (Mohd Shaufi et al., 2015), thus rendering the assessment of gut health and performance at 13 dph pivotal for exploring potential gut-based interventions before broilers transition to the final stage of their life cycle on the finisher diet, typically around day 21 dph. Notably, existing literature predominantly examines the bacterial aspect of the microbiome. In contrast, the mycobiome, or fungal component, appears primarily influenced by environmental factors such as diet (Robinson et al., 2022a). The present research suggests that broilers at 13 dph do not exhibit discernible successional changes or expected abundances in the GI mycobiome, with the greatest diversity observed in the upper GI tract (Robinson et al., 2020a).

As the cost of shotgun metagenomic sequencing declines, there's a growing recognition of the non-bacterial elements within the GI microbiome in poultry research (Mulholland et al., 2021, Gilroy et al., 2021). This advanced sequencing technique allows for exploring functional genes and pathways in GI tract content samples, transcending the limitations of traditional 16S rRNA gene sequencing (Durazzi et al., 2021). Notably, poultry GI tracts harbour a remarkable diversity and abundance of bacteriophages, although research on Archaea sequences remains limited (Gilroy et al., 2021). Similarly, investigations into the faecal mycobiome of broilers are sparse, with prevailing research focusing more on the chicken shed environment (Sugiharto, 2019), external features (such as beak and comb), or the respiratory tract (Mulholland et al., 2021).

5.1.2 Aims & Objectives

The primary aims and objectives of this thesis chapter are as follows:

1. Investigate the suitability of faecal sampling as a method for characterising performance of broilers by analysing the variability of the microbiome, mycobiome, and metabolome across three GI tract locations in 10 broilers at 13 dph.
2. Explore the interplay between the broiler microbiome's fungal and bacterial components to understand their relationship and potential impact on performance.

3. Characterise differences in broiler performance based on body weight and serum metabolome profiles at 13 dph, shedding light on potential health and performance indicators in broiler production systems.

Hypothesis

Faecal sampling can be a reliable proxy for assessing broilers' performance at 13 dph. I predict that the variability observed in the compositions of the microbiome, mycobiome, and metabolome across three GI tract locations — jejunum, caecum, and faeces — at 13 dph will be indicative of overall performance. Furthermore, I hypothesise that differences in broiler performance based on body weight and serum metabolome at 13 dph can be identified. This hypothesis will be tested through shotgun metagenomic sequencing to characterise the bacterial microbiome, ITS1 rRNA metabarcoding to characterise the fungal microbiome, and ^{1}H-NMR spectroscopy to analyse the metabolome of the gut locations and serum.

5.2 METHODS

5.2.1 Sample Collection and Storage

As described in Chapter 2: Methods, Aviagen collected serum and gastrointestinal (GI) content from the jejunum, caecum, and colon (containing faeces) of 10 broilers at 13 dph. In brief, ten seemingly healthy juvenile broilers of mixed sex and body weight were randomly selected and euthanised by cervical dislocation at Aviagen, Edinburgh, by trained personnel as part of routine disease monitoring in February 2021. GI content samples were flash-frozen and transported to QIB on dry ice, where GI content was stored at -80°C and serum at -20°C.

Microbial and metabolomic analyses followed the methodologies detailed in Chapter 2. DNA was extracted using Maxwell RSC PureFood GMO and Authentication kit (Promega) (Methods 2.3.1.1), with sequencing conducted using shotgun metagenomics (Methods 2.3.2.3). Bioinformatic processing and taxonomic classification were performed as described in Methods 2.4.2, and statistical analyses were conducted as described in Methods 2.5.

5.3 RESULTS

5.3.1 Sex and Weight Distribution of Ten Broilers at 13 days post-hatching

The mean broiler weight at 13 dph was 385.2 g (SD ± 83.4) (Fig. 5.1). Five broilers had a bodyweight greater than the IQR 2, which was 380.5 g, categorising them as ‘over’ performers. These broilers included all four males and a single female. The remaining five female broilers were less than IQR2, classing them as ‘under’ performers.

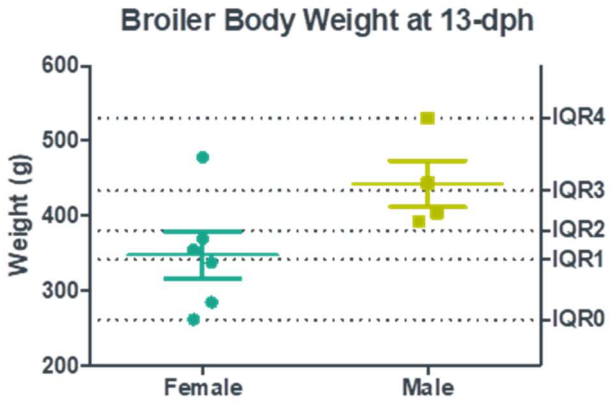


Figure 5.1 Vertical scatter graph demonstrating the weight and sex distribution of 10 broilers at 13 dph, with IQR on the right Y-axis.

5.3.2 Microbiome of the Small Intestine (Jejunum), Caecum and Colon (Faeces) at 13 Days Post-Hatching

5.3.2.1 *Determining Sample Quality*

The DNA yield reflects the expected genomic material for each sample type. The caecum is known to have the greatest density and diversity of bacteria. It, therefore, has the highest DNA yield with an average of 225.4 ng/µl (SD ± 133.2), followed by faeces, which had an average of 22.2 ng/µl (SD ± 18.6) and then jejunum with an average of 6.7 ng/µl (SD ± 7.1) (Fig. 5.2).

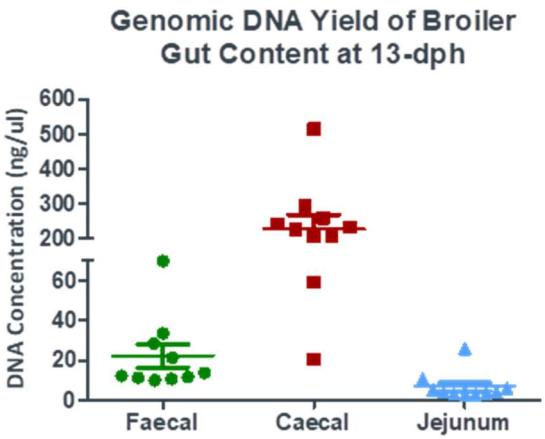


Figure 5.2 Genomic DNA yield from three GI locations using the Promega Maxwell RSC PureFood GMO Authentication kit, quantified with the dsDNA broad-range Qubit Assay.

Despite having the highest genomic DNA yield of all GI tract locations, the caeca had the lowest number of reads passing quality filtering (Fig 5.3). The shotgun metagenomic sequencing determined an average of 41,200,451 reads that passed quality filtering in the caeca. The majority (99.97%) were bacterial reads, and 0.03% were viruses, which include VLPs, such as bacteriophages. No eukaryote or archaeal reads were found in the caecum. In faeces, a total of 349,311,180 reads passed quality filtering. Like the caecum, 99.98% of these reads belonged to bacteria. In addition to the bacteria, 0.02% of reads were attributed to viruses and 62 eukaryote reads, equating to less than 0.00002% of the total faecal microbiome. No archaeal reads were found in the faeces. The jejunum samples had 223,445,696 reads pass quality filtering. Bacteria was again the most abundant domain, accounting for 99.993% of all jejunal reads. A lower number of viruses were detected in the jejunum, only accounting for 0.007%. Still, this GI tract location had the highest number of eukaryotes (accounting for 0.003% or 6,703 reads) and was the only sample type that had an archaeal read identified found in a single broiler. The identified archaea were an unclassified species from the *Methanocaldococcus* genus. Recognising the limitations imposed by a low number of reads is crucial, as it may impact the confidence with which results from mycobiome analysis can be interpreted compared to microbiome analysis.

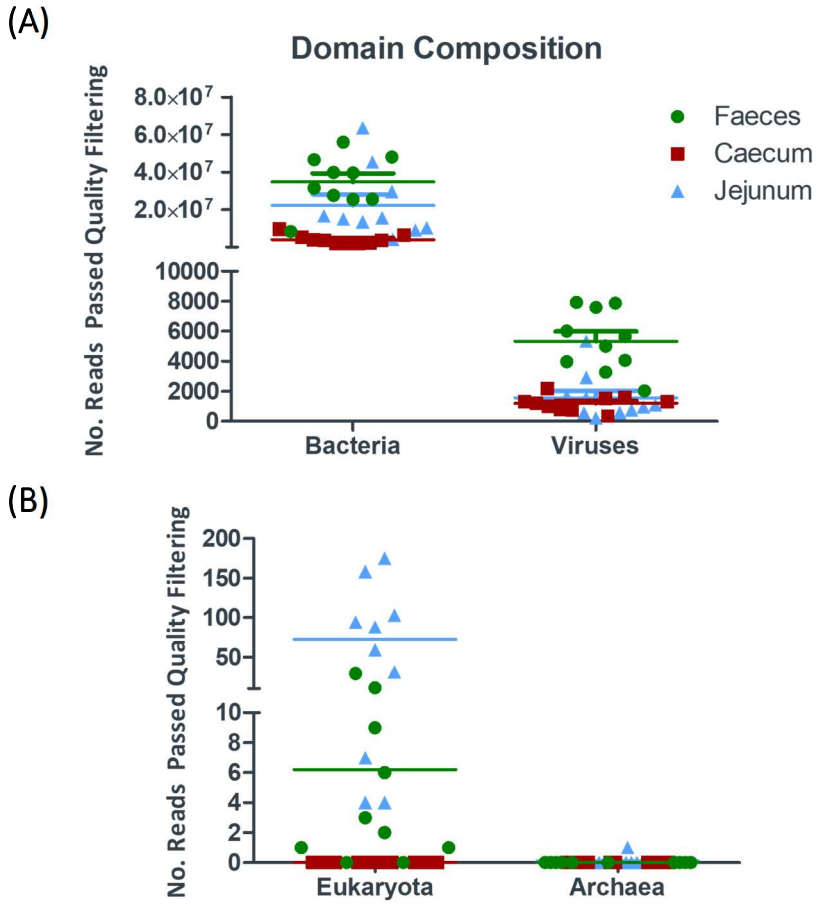


Figure 5.3 Vertical scatter graph depicting the number of shotgun metagenomic reads that passed quality filtering (using FastQC) for each domain in three GI tract locations.

Only three eukaryotes were assigned species from the shotgun metagenomic sequencing. These were protozoan parasite *Eimeria tenella* and yeasts *Debaryomyces hansenii* and *Candida glabrata*. Currently, shotgun metagenomic databases are inadequate at assigning eukaryote taxonomy. Therefore, I used the same genomic DNA for simultaneous ITS1 rRNA metabarcoding sequencing. There was no significant difference in the number of ASVs that passed quality filtering between these GI locations (Fig. 5.4).

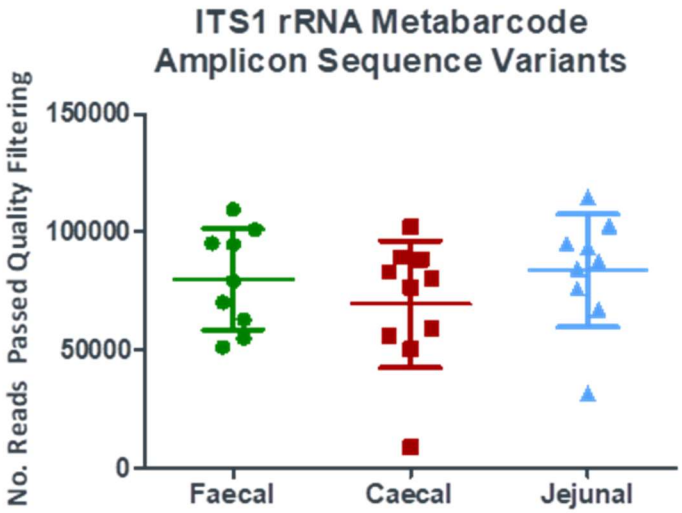


Figure 5.4 Vertical scatter graph depicting the number of ITS1 rRNA metabarcoding reads that passed DADAist2 quality filtering and were assigned individual ASVs.

5.2.2.2 *Spatial Variation of Bacterial Composition, Abundance and Diversity*

There were 38 genera across all GI tract locations from shotgun metagenomic analysis. Eight were viral genera; one was fungal, and the rest belonged to bacteria. Seven of the 38 identified belonged to taxonomic groups higher than genus. The composition of the faecal and jejunal microbiome was very similar and distinct from that of the caecum, although they shared several genera. The most dominant genera were *Blautia*, *Escherichia*, *Lactobacillus*, *Oscillibacter* and *Subdoligranulum*, which only accounted for 50-75% of bacteria in the caecum, whereas they represented 80-99% of faecal and jejunal samples (Fig. 5.5).

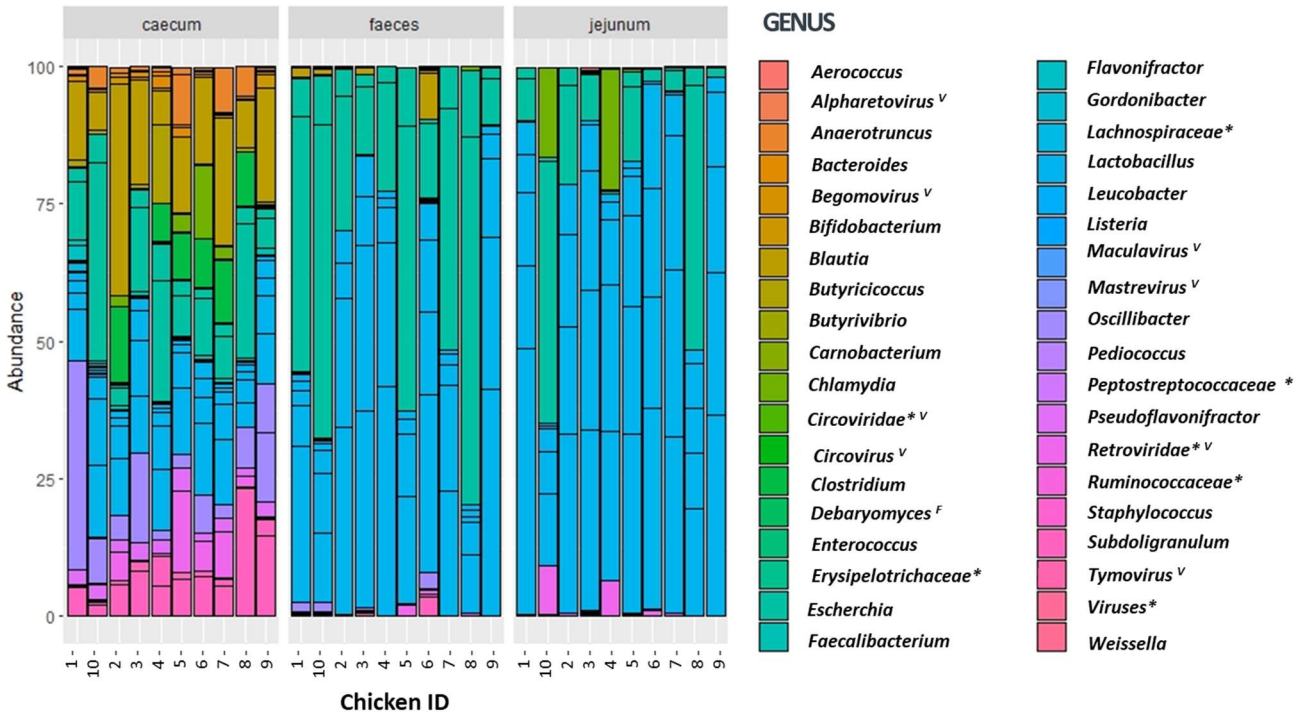


Figure 5.5 Stacked bar graph to visualise microbiome composition of genera in three GI tract locations of broilers at 13 dph, with superscripts of the legends demonstrating * = taxonomic levels higher than genus, ^{V} = viral domain, ^{F} = fungal domain, generated using the phyloseq R package.

Of the ten most abundant species across the three GI tract locations, five of these are *Lactobacillus* species (Fig. 5.6): *Lactobacillus crispatus*, *Lactobacillus johnsonii*, *Lactobacillus reuteri*, *Lactobacillus salivarius* and *Limosilactobacillus vaginalis* were found in all three GI tract locations. *Escherichia coli* is the most dominant species in the faeces in half the broilers and is found in high abundance in both the jejunum and caecum, alongside unclassified *Escherichia*. Three of the top 10 species, *Ruminococcus torques*, unclassified *Oscillibacter* and *Subdoligranulum* were only found in the faeces and caecum, not the jejunum. Furthermore, these three species were not found in every broiler faecal sample at 13 dph but were found in all caecal samples.

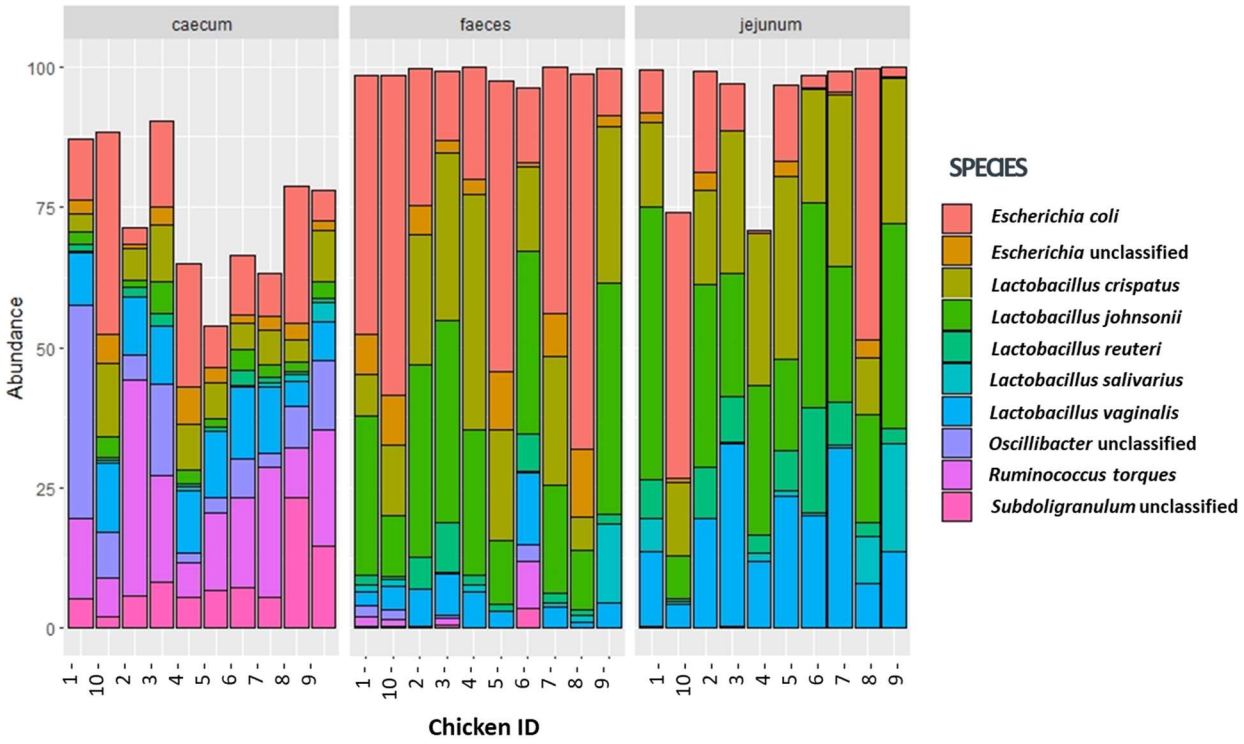


Figure 5.6 Stacked bar graph generated in the phyloseq R package to visualise the relative abundance of the top 10 species across three GI tract locations in broilers at 13 dph.

Twelve taxa had significant differences in abundance between the three GI tract locations (Fig. 5.7). Of the 12, nine had significantly higher relative abundance in caeca, which is known to have the highest density of bacteria (Oakley and Kogut, 2016). In the caecum *Anaerotruncus colihominis*, *Clostridium saccharolyticum*, *Faecalibacterium prausnitzii*, *Pseudoflavonifractor capillosus*, unclassified *Subdogranulum*, *Bacteroides xylanisolvens*, unclassified *Oscillibacter*, *Ruminoccocus torques* and, *Subdoligranulum variable* relative abundances were significantly higher than in both faeces and jejunum. *Limosilactobacillus vaginalis* was the only species to be considerably higher in abundance in the jejunum when compared to the caecum or faeces. Unclassified *Escherichia* had a significantly higher abundance in faeces than jejunum and caecum compositions. Finally, *Lactobacillus johnsonii* had a significantly higher abundance in the jejunum and faeces than the caecum.

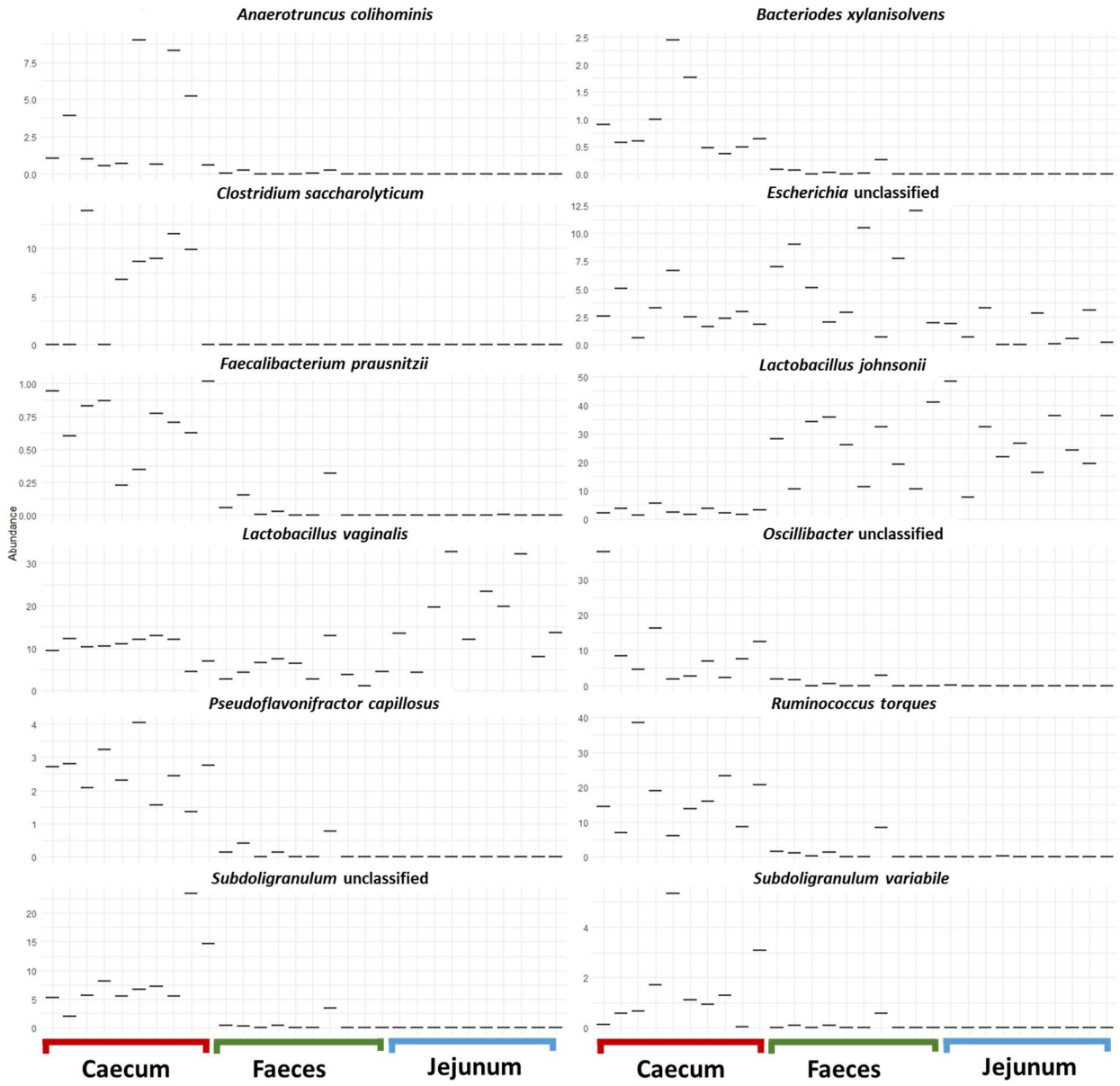


Figure 5.7 The 12 taxa with significant differences between GI tract locations using the Wilcoxon test (P = <0.005) with relative abundance on the Y-axis to display the difference between broilers.

The Shannon Index incorporates species richness and evenness when measuring the α-diversity of the microbiome. The caecum had the highest α-diversity of all three GI tract locations (Fig. 5.8). The caecum Shannon Index was significantly greater than both the faeces and jejunum (Kruskal-Wallis test, P < 0.0001). This demonstrates that the caecum

has a rich and even microbiome, which can be visualised in the stacked bar graphs of relative abundance (Fig. 5.6).

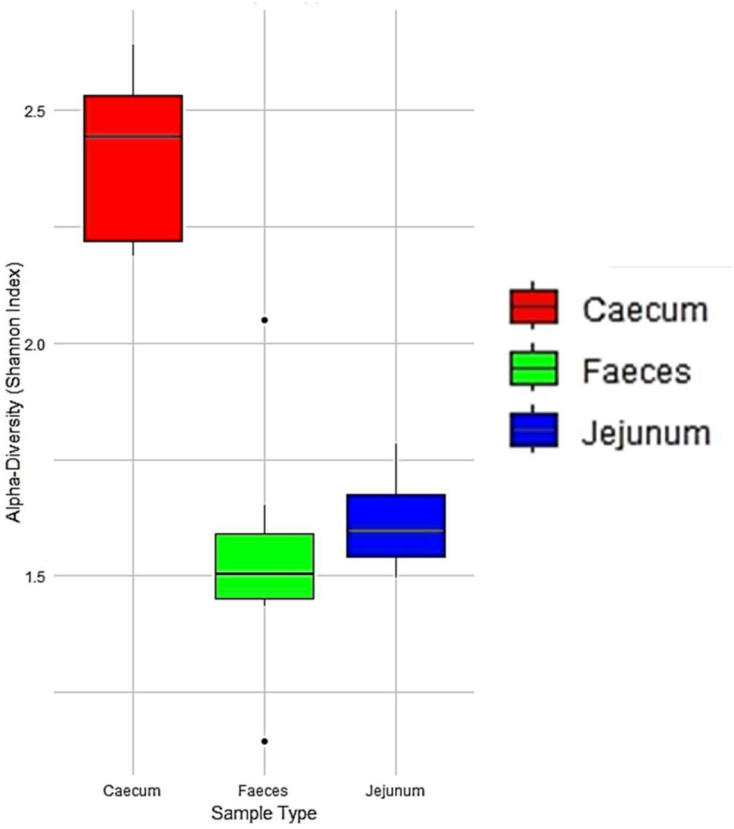


Figure 5.8 Whisker plot depicting the α-diversity of the microbiome, using Shannon Index, of three GI tract locations in broilers at 13 dph, generated in phyloseq R package.

With PCoA, the closer data points are plotted, the more similar the microbiomes are regarding β-diversity. PCoA determined Axis 1 (X-axis) to have a variance of 39.1%, whilst Axis 2 (Y-axis) has 21.7%, representing moderate variability. The fact all jejunal (blue) and faecal (green) samples cluster on the right of Axis 1, whereas the caecal (red) samples cluster on the left, demonstrated the dissimilarity to the caecum (Fig. 5.9). Five faecal microbiomes and two jejunal microbiomes have a similar β-diversity, they can be seen to cluster in the top right quadrant, with the remaining eight jejunal and five faecal microbiomes clustering in the bottom right. Complimentary to the α-diversity, the β-diversity also demonstrated greater similarity between the faeces and jejunum, with the caecum having a distinctly different composition. No significant differences were found

between sex at 13 dph, which has been reported to cause differences in chicken microbiome from as early as 3 dph (Lumpkins et al., 2008).

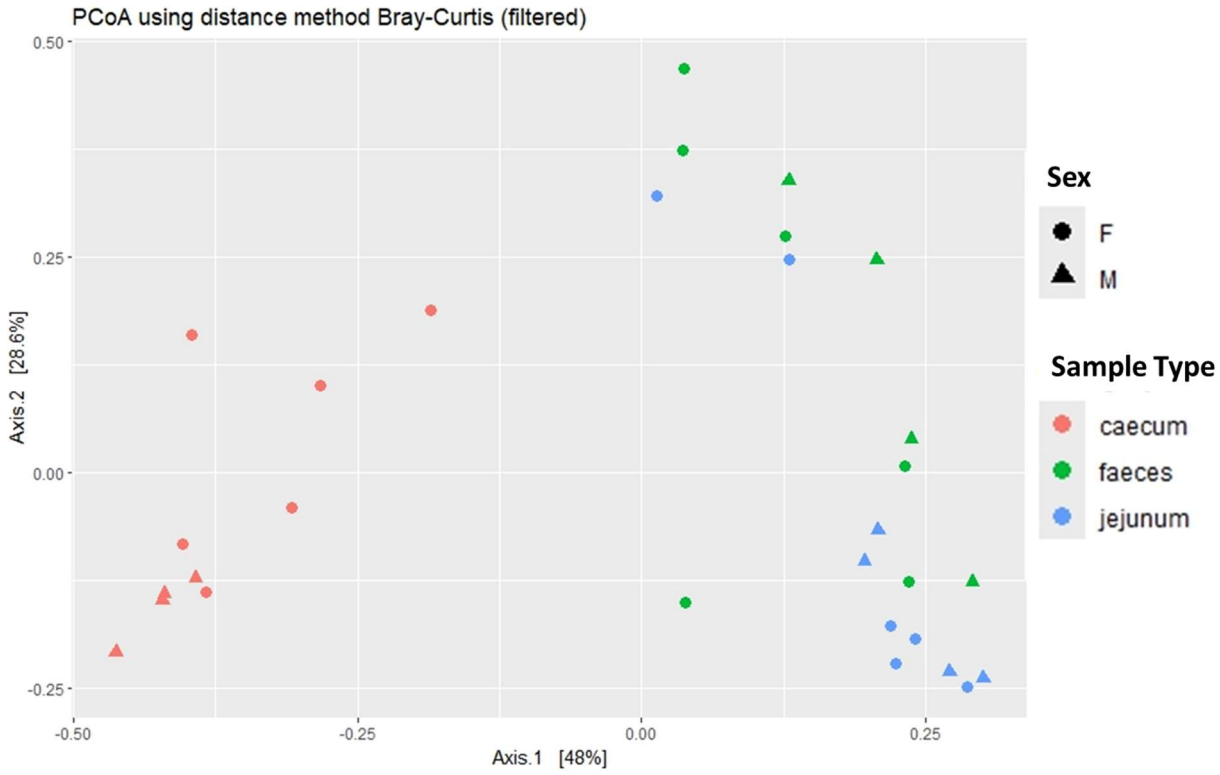


Figure 5.9 PCoA of β-diversity, measured with Bray-Curtis.

Shotgun metagenomic sequencing identified AMR genes and virulence factors in the broiler gut microbiome. The caecum contained the highest number of AMR genes (**Fig. 5.10A**), consistent with its greater microbial density, followed by faeces, which showed higher inter-individual variation. The jejunum had the lowest AMR gene abundance. Identified resistance genes spanned multiple antimicrobial classes, including tetracyclines, with both intrinsic and acquired resistance mechanisms present.

Virulence factors were most abundant in faeces (**Fig. 5.10B**) and included genes related to adhesion, immune evasion, and toxin production. However, there was no significant correlation between virulence factors and broiler body weight at 13 dph, suggesting minimal impact on early growth performance.

While AMR genes are common in gut microbiota, their potential for horizontal gene transfer raises food safety concerns, particularly regarding resistance to medically

important antibiotics. Similarly, although the detected virulence factors are not directly linked to disease, their presence underscores the complexity of microbial interactions in the gut. Further studies are needed to assess their persistence and significance throughout the broiler production cycle.

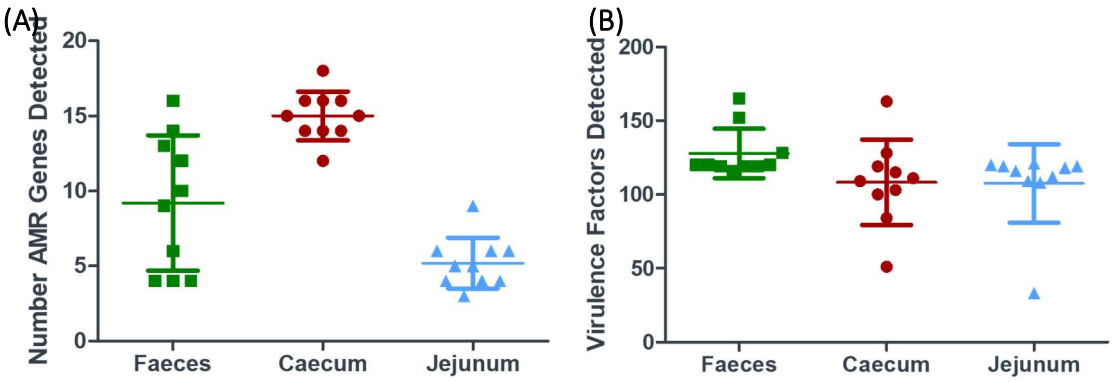


Figure 5.10 Vertical scatter graph depicting the number and standard deviation of (A) AMR genes and (B) virulence factors in three GI tract locations, identified with the ARIBA bioinformatics tool in Galaxy.

In summary, the shotgun metagenomic results indicate that the faecal and jejunal microbiomes exhibit significant similarity according to bacterial composition. At the same time, the caecum shows a distinct structure, higher density, and high diversity, as anticipated from genomic DNA extractions and existing literature. Faecal sampling demonstrated the least variability between individual broilers at 13 dph. With a clearer understanding of bacterial microbiome composition in three GI tract locations, it is essential to further characterise the fungi.

5.2.2.3 *Spatial Variation of Fungal Composition, Abundance and Diversity*

The composition of fungi in the gut microbiome, the mycobiome, was identified using a metabarcoding sequencing strategy. Taxonomy is assigned based on the ITS rRNA gene, which is highly conserved in fungi. Compared to shotgun metagenomic sequencing,

which is highly specific and uses deep sequencing for strain-level identification of microorganisms, sequencing the ITS rRNA gene does not permit the same level of specificity. Fungal species, such as those in the *Aspergillus* genus, which have similar ITS rRNA genes, cannot be distinguished; therefore, the genus is the lowest taxonomic level for abundance analyses.

ITS rRNA sequencing revealed 1407 ASVs across the three sample types in broilers at 13 dph, which were assigned to 501 taxonomic features. The caecum exhibited the least number of ASVs, totalling 146, trailed by the jejunum at 353, and the faeces at 480. This finding contradicts existing literature, which typically asserts the SI's supremacy in fungal density (Robinson et al., 2020a). The *Saccharomycetales* order (ascomycete yeasts) was the most abundant in the jejunum and caecum, whereas *Eurotiales* (green and blue moulds) were dominant in the faeces (Fig. 5.11).

Figure 5.11: Stacked bar graph comparing the composition of fungal orders in broiler caecum, colon (faeces) and SI (jejunum) at 13 dph, identified using ITS1 metabarcoding sequences.

When comparing the faecal and jejunal orders, the only significant difference was between the most abundant *Saccharomycetales* and *Eurotiales*. There was a significantly higher abundance of *Saccharomycetales* in the jejunum compared to the faeces (P = <0.001) and in the caecum compared to faeces (P = <0.0001) (Fig. 5.12A). There was a significantly higher abundance of *Eurotiales* order in the faeces than in the jejunum (P = <0.001) and caecum (P = <0.0001) (Fig. 5.12B). When comparing the caecum to faeces, 15 significant differences at the order taxonomic level were discovered and 12 significant differences in abundance between the jejunum and caecum (data in Appendix 1). This suggests that the faeces and jejunum have a more similar composition with only two significant differences between orders.

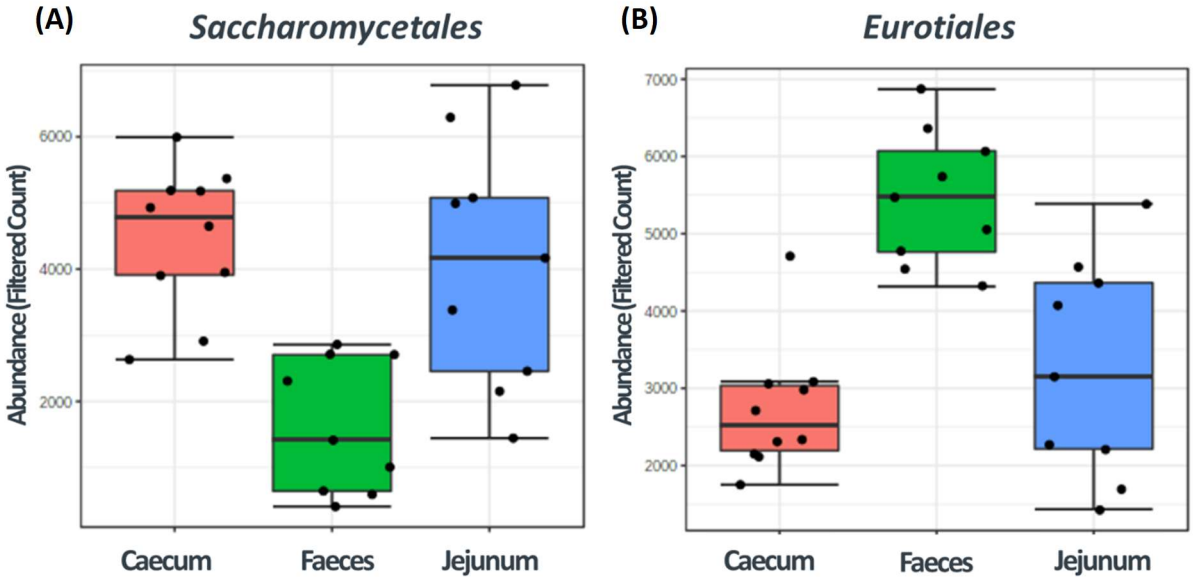


Figure 5.12 Box plot of dominant fungal orders with significant differences in abundance: (A) *Saccharomycetales* and (B) *Eurotiales* in caecum, faeces, and jejunum of broilers at 13 dph.

The top 10 most abundant genera accounted for 68% - 96% of all caecum samples, 86% - 95% of all faecal samples and 80% - 97% of all jejunum samples (Fig. 5.13). This demonstrates that the most abundant fungi are responsible for most of the composition. In faecal samples, *Aspergillus* accounted for up to 60% of the total abundance, whereas yeast *Diutina* accounted for 66% and 78% of the total abundance in the caecum and jejunum, respectively. In the caecum, all other taxonomy accounted for an average of 7%

abundance in each sample. An average of 6% of the ASVs in the caecum samples were unassigned. All other taxonomies accounted for 9% of the faecal composition in the faecal samples. Only 1% of faecal ASVs were unassigned, suggesting that this is a well-characterised sample type, which is beneficial for identifying a marker of performance or gut health. The jejunum had the greatest number of unassigned ASVs, averaging 10%. Additionally, the jejunum relative abundance outside of the top 10 genera accounted for 10% of the relative abundance. This indicates that the jejunum hosts a richer fungal population, but diversity analysis will provide greater clarity.

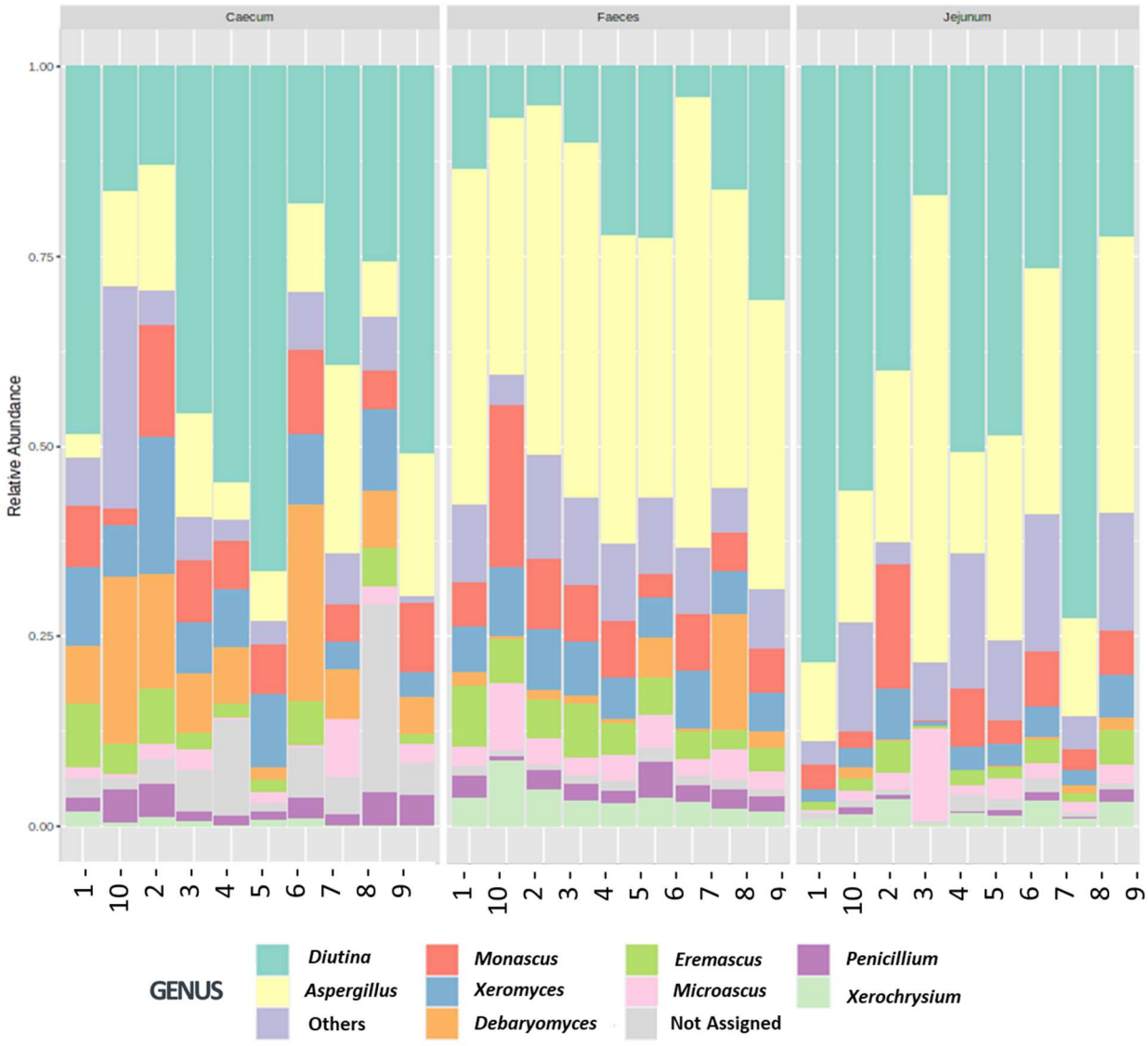


Figure 5.13 Stacked bar graph depicting the relative abundance of the top 10 fungal genera in broilers' caecum, faeces, and jejunum at 13 dph.

From the top 10 most abundant genera (Fig 5.13), there was a significant difference between eight genera when comparing GI locations. These were *Debaryomyces*, *Xerochrysium*, *Aspergillus*, *Penicillium*, *Xeromyces*, *Diutina*, *Microascus* and *Eremascus*. As mentioned, across all GI tract locations, *Diutina* and *Aspergillus* were the most abundant genera, with *Diutina* having significantly greater abundance in the caecum and jejunum compared to the faeces (Fig. 5.14A) and *Aspergillus* having significantly greater abundance in the faeces (Fig. 5.14B).

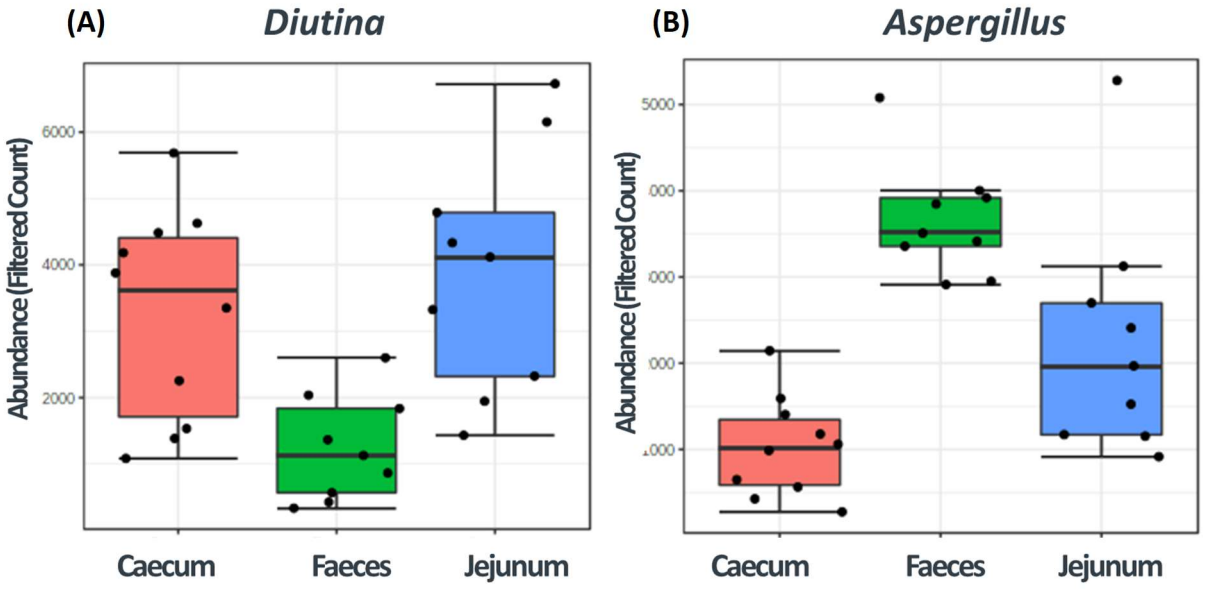


Figure 5.14 Box plot of the two most abundant genera, (A) *Diutina* and (B) *Aspergillus*, with significant differences between sample types.

A total of 50 significant differences in fungal genera abundances were observed when comparing the three locations (Mann–Whitney U, P=<0.05, Table 5.1). Specifically, there were 17 genera with a significant difference between faeces and jejunum, while 31 genera showed significant differences between faeces and caecum. The most pronounced disparities were found between jejunum and caecum, with 49 genera exhibiting significant differences in relative abundance across GI tract locations. Notably, the least variation was observed between faeces and jejunum, highlighting their closer compositional resemblance. These findings suggest that faecal sampling adequately captures the fungal composition of the jejunum but fails to represent the entirety of the distinct caecal composition. This observation is significant because previous research indicates that the SI

harbours a greater diversity and abundance of fungi in poultry (Robinson et al., 2022a, Davies et al., 2022), contrasting with the bacterial microbiome, which is most abundant and diverse in the caecum and least so in the jejunum. While the caecum may be a better representation of the most abundant bacterial microbiome, the faeces and jejunum appear to be more proficient in capturing the resident fungal microbiome.

Table 5.1 List of 50 genera with significant differences in abundance between sample types in ascending order of P value (most significant to least significant) (Mann–Whitney U, P=<0.05).

GENUS	P value	FDR	GENUS	P value	FDR
Epicoccum	<0.001	0.003	*Filobasidium*	0.006	0.021
Debaryomyces	<0.001	0.003	*Kodamaea*	0.009	0.028
Mycosphaerella	<0.001	0.003	*Vagicola*	0.009	0.028
Vishniacozyma	<0.001	0.003	*Zymoseptoria*	0.009	0.028
Xerochrysium	<0.001	0.003	*Metschnikowia*	0.011	0.031
Aspergillus	<0.001	0.003	*Trichosporon*	0.011	0.031
Microdochium	<0.001	0.003	*Thermoascus*	0.011	0.031
Gamsia	<0.001	0.003	*Acremonium*	0.011	0.032
Gibberella	<0.001	0.003	*Paecilomyces*	0.012	0.032
Neoascochyta	<0.001	0.003	*Sporobolomyces*	0.013	0.035
Kazachstania	0.001	0.005	*Apiotrichum*	0.022	0.056
Penicillium	0.001	0.005	*Trichomonascus*	0.023	0.056
Fusarium	0.001	0.006	*Issatchenkia*	0.027	0.065
Lecanicillium	0.001	0.007	*Botrytis*	0.027	0.065
Itersonilia	0.001	0.007	*Microascus*	0.029	0.065
Xeromyces	0.001	0.007	*Aureobasidium*	0.029	0.065
Alternaria	0.001	0.007	*Eremascus*	0.031	0.066
Leucosporidium	0.002	0.007	*Hannaella*	0.031	0.066
Papiliotrema	0.002	0.008	*Cyberlindnera*	0.033	0.066
Thermomyces	0.002	0.010	*Mollisia*	0.033	0.066
Monographella	0.002	0.010	*Didymella*	0.033	0.066
Diutina	0.003	0.012	*Diaporthe*	0.035	0.066
Corynespora	0.003	0.013	*Clavispora*	0.035	0.066
Dioszegia	0.004	0.015	*Hyphopichia*	0.035	0.066
Parastagonospora	0.005	0.018	*Wallemia*	0.044	0.081

The α-diversity determines composition differences within the sample. The Shannon Index incorporates both the richness and evenness of the community. There were

no significant differences in the mycobiome Shannon Index between the three GI tract locations (Fig. 5.15). The faecal samples exhibited the least variable α-diversity, indicating a relatively consistent community in terms of richness and evenness. However, it is important to consider the low number of reads, which complicates interpretation.

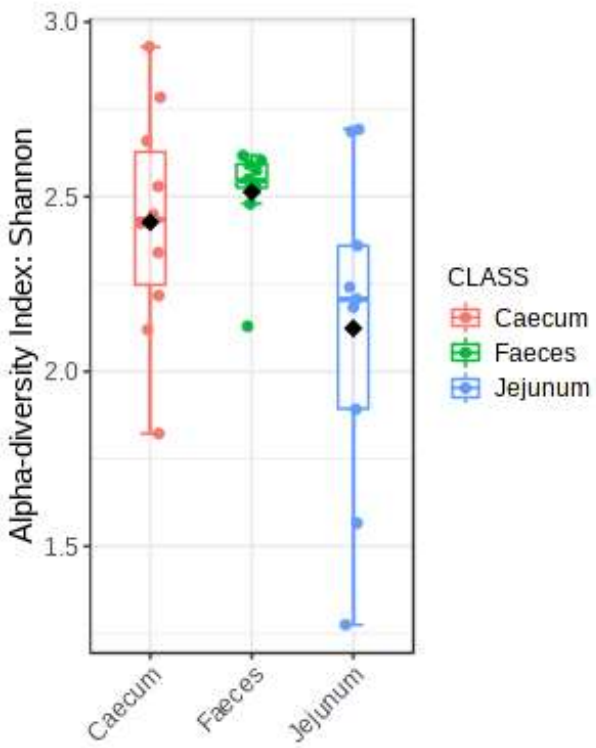


Figure 5.15 Vertical scatter box plot displaying the mycobiome α-diversity using the Shannon Index for ten broilers at 13 dph, comparing three GI tract locations.

Fungal β-diversity in the samples was assessed using Bray-Curtis in a PCoA (Fig. 5.16). Like the findings in α-diversity, the faecal samples cluster closely together, indicating high similarity and consistency in faecal fungal communities. Furthermore, the faecal samples completely overlap with the jejunum samples, while the jejunum samples, in turn, exhibit overlap with the caecal samples. This observation suggests that the jejunum shares similarities with the faecal and caecal samples, whereas the faecal and caecal communities exhibit distinct differences. Pairwise analysis (PERMANOVA, P=<0.05) revealed that there was a significant difference in the β-diversity of faeces compared to the caecum (P= 0.001) and jejunum (P=0.002) as well as the caecum compared to the jejunum (P=0.002).

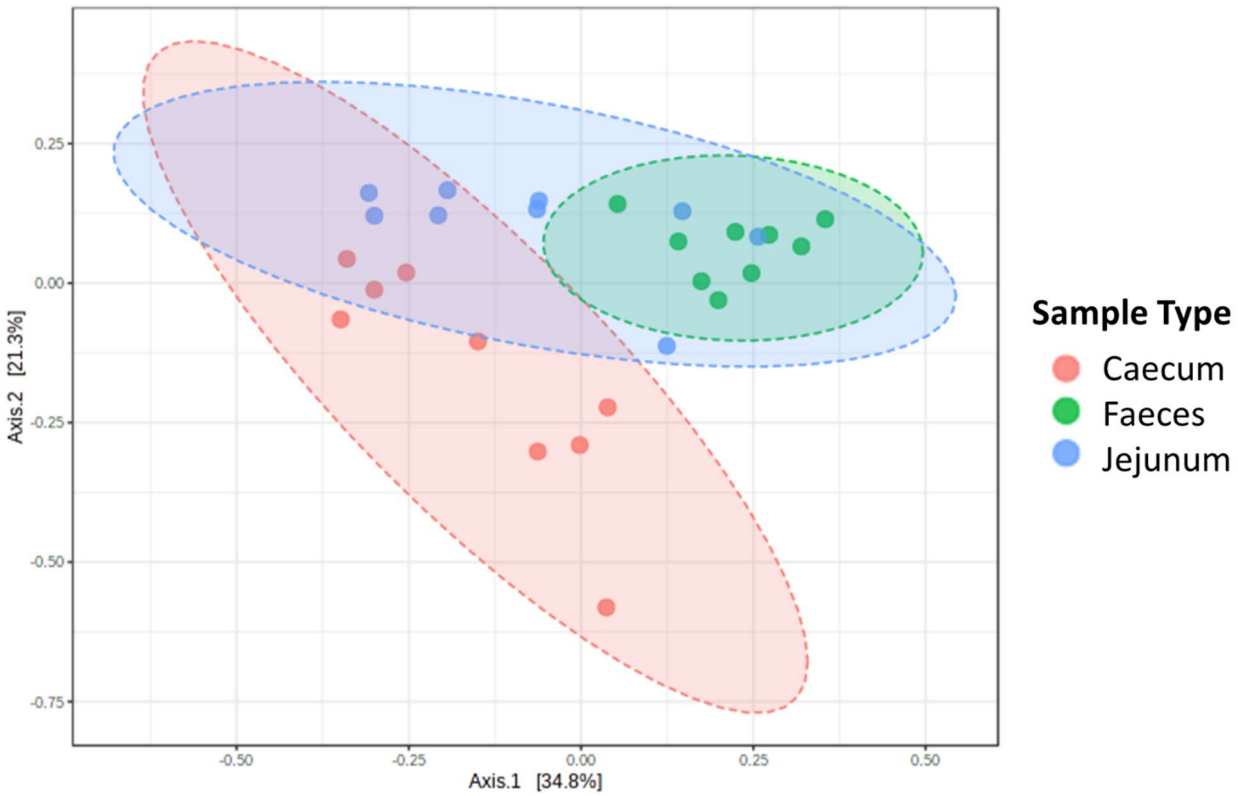


Figure 5.16 PCoA of β-diversity, measured with Bray-Curtis. Ellipses demonstrate average distribution for each sample type.

The mycobiome has the most consistent community in the faeces compared to the caecum and jejunum, as determined by α- and β-diversity analysis. Additionally, the faecal mycobiome was the best characterised in terms of taxonomic assignment, and the most abundant genera accounted for up to 97% of the total composition. The similarity observed between faecal and jejunal samples implies that faecal sampling adequately reflects the fungal community of the upper GI tract. Nonetheless, the jejunal sample exhibits greater richness and a higher presence of unknown species, as indicated by shotgun sequencing and PCoA analysis. It appears that the jejunal sample can encompass the entirety of the faecal community and some of the caecal communities. This aligns with existing literature indicating that the SI harbours a diverse fungal community. However, certain species present in the caeca are not captured by the jejunal or faecal samples. However, akin to bacterial communities, faecal fungal communities appear notably distinct from those found in the caecum. This disparity may partly stem from the contrasting intestinal environments of the caecum and colon, characterised by differences in pH, oxygenation, and physiological

structure (Grond et al., 2018, Ravindran et al., 2016). The increased density and diversity of bacteria in the caecum could influence the fungal composition variations. Additionally, the virome presents another potential factor impacting fungal and bacterial composition, warranting further exploration.

5.2.2.4 *Spatial Variation of Viral Composition and Abundance*

Describing the viral aspect of the gut microbiome has limitations due to suboptimal genomic DNA extraction methods tailored specifically for viruses. Consequently, distinguishing between free bacteriophages and those integrated into the genome was not possible. Additionally, the low counts and variability across samples necessitated pooling virus compositional data from all birds to comprehensively understand viral compositions across the three different GI tract locations in broilers at 13 dph.

Viruses constitute a small fraction of the microbiome across different regions of the GI tract, representing 0.02% of reads in faecal samples, 0.03% in the cecum, and 0.007% in the jejunum, indicating a low relative abundance in the upper GI tract (Fig. 5.17). In the cecum, the dominant virus is *Parvoviridae*, while *Caudovirales*, a major bacteriophage order, prevails in both faeces and jejunum. This suggests a higher incidence of bacteriophages in the small and large intestines than in the caecum. *Podoviridae* represented 3% of the faecal virome and had been identified in the gut of sentinel birds (Day et al., 2015) and healthy chicken faeces (Lima et al., 2017); *Retroviridae* was similarly identified in these studies and was identified in all broiler sample types of these broilers, representing 0.6-0.7% of the gut virome in broilers at 13 dph.

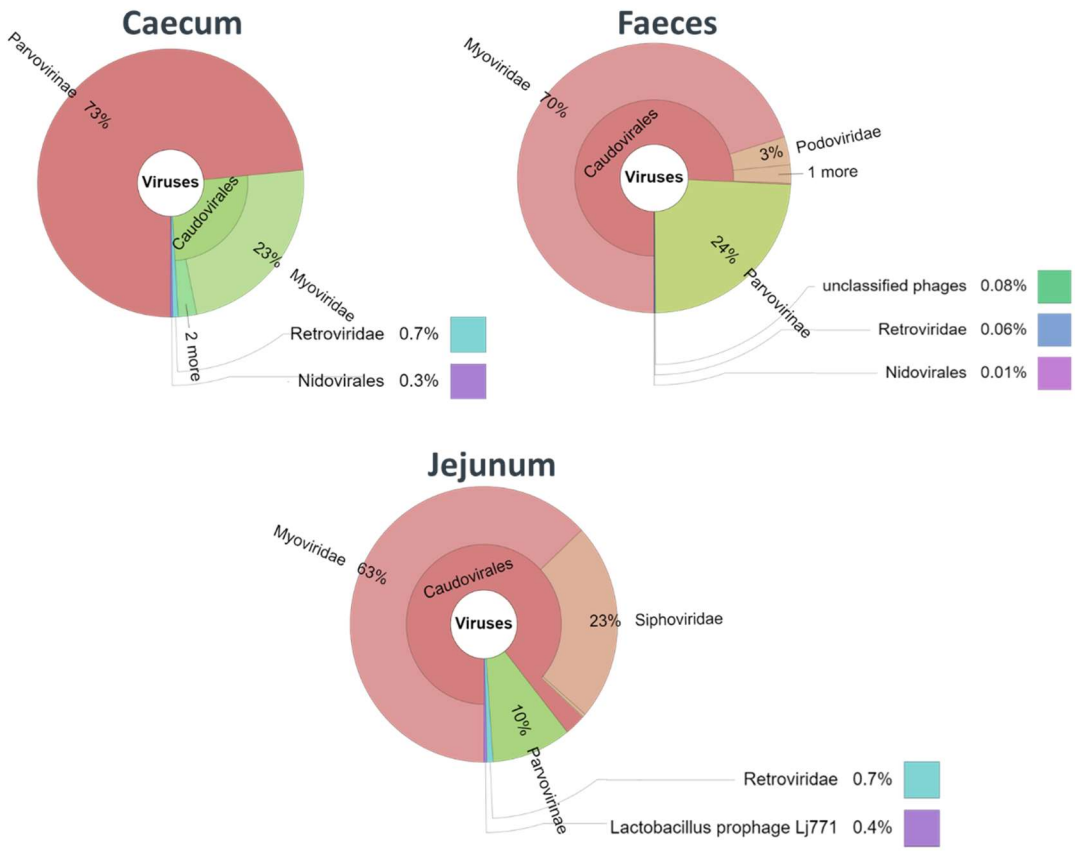


Figure 5.17 Pie-diagrams generated using Krona-Kraken2 display the pooled composition of total viral abundance in caecum, faeces and jejunum.

5.2.3 Comparing and Correlating Bacterial and Fungal Diversity

Despite the caecum's significantly higher bacterial α-diversity, there was no difference in the fungal α-diversity between the three GI tract locations (Fig. 5.18A). The β-diversity revealed all three GI microbiomes to be significantly different, with the caecum having the most diverse microbiome, followed by the faeces and then the jejunum in both bacterial and fungal microbiome communities (Fig. 5.18B).

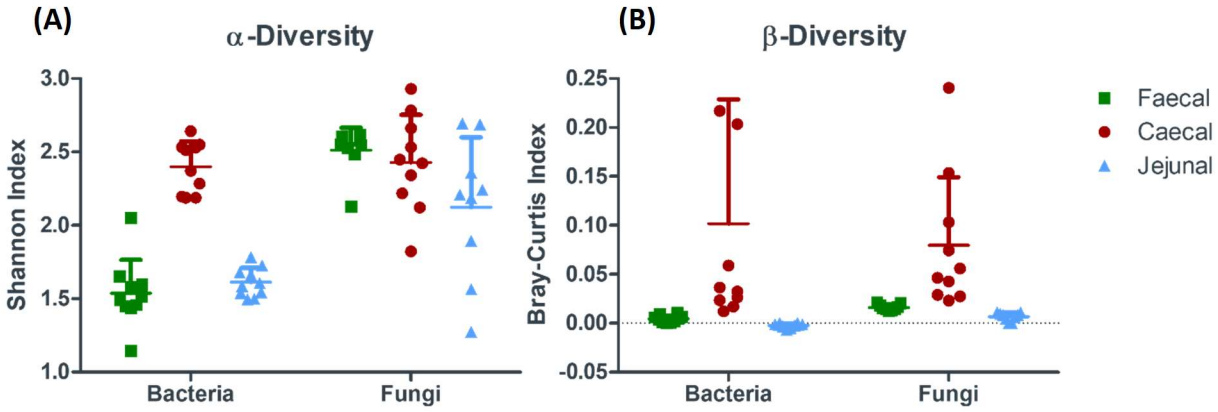


Figure 5.18 Vertical scatter graph comparing **(A)** α-diversity using Shannon's index and **(B)** β-diversity using Bray-Curtis index in three GI tract locations in broilers at 13 dph.

There were no significant correlations between microbiome and mycobiome α-diversity within the different GI locations (Fig. 5.19A). However, a positive, significant correlation and effect between the microbiome and mycobiome for all GI locations can be seen between the β-diversity measurements (Fig. 5.19B). This suggests that regardless of GI location when looking between samples, increased microbiome β-diversity is associated with increased mycobiome β-diversity.

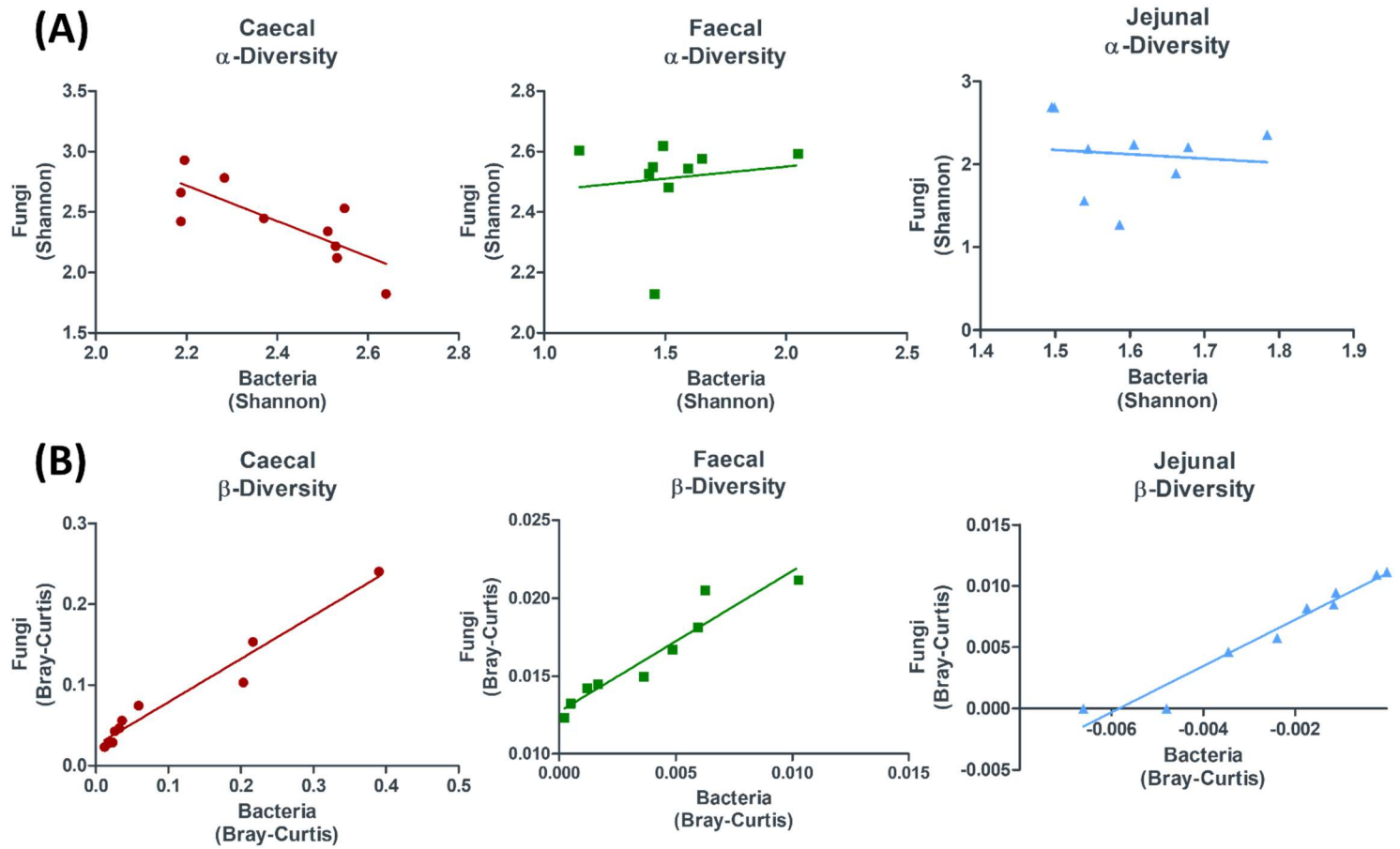


Figure 5.19: Linear regression of different GI locations in broilers at 13 dph, modelling microbiome and mycobiome α-diversity using Shannon's index **(A)** and β-diversity using Bray-Curtis index **(B)**. Caecum: P=<0.0001, R^2=0.96. Faeces: P=<0.0001, R^2=0.91. Jejunum: P=<0.0001, R^2=0.95.

5.2.4 Spatial Variation of the Metabolome in the Small Intestine (Jejunum), Caecum and Colon (Faeces) at 13 days post-hatching

Three hundred and thirty-six compounds were profiled across three GI tract locations using untargeted ^{1}H-NMR. There were 204 significant differences in metabolite concentration between the caecum, jejunum, and faeces (Kruskal-Wallis, P=<0.05) (Fig. 5.20). A full table of compounds and P values is available in Appendix 2. There were no significant differences when comparing 'over' and 'under' performing birds or when comparing sexes (data not shown). As with the microbiome, there is no consistent metabolome for the entire gut; each GI tract location has a distinct metabolome relevant to the microbial community and physiological structure.

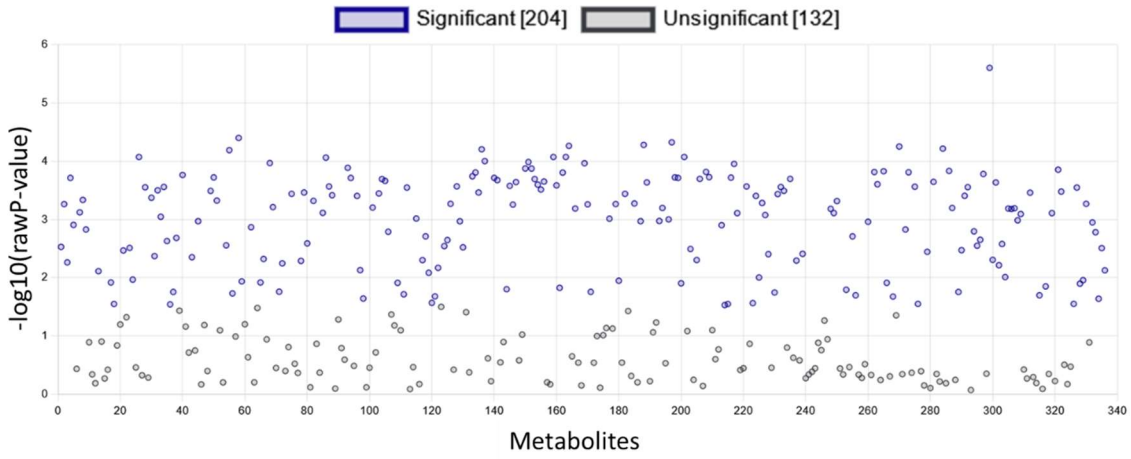


Figure 5.20 Scatter dot plot exhibiting 204 metabolites with a significant difference (Kruskal-Wallis, P=<0.005) in concentration in at least one GI location, acquired with untargeted ^{1}H-NMR and graph generated with MetaboAnalyst 5.0 R package.

The compound with the greatest difference in concentration between GI tract locations was trimethylamine (Fig. 5.21). There was a significantly greater concentration in faeces than in the caecum or jejunum (P = <0.0001) and significantly higher concentrations in the caecum than the jejunum (P = <0.0001). Trimethylamine is an amine which is converted from TMAO by the distal gut microbiome (Shehata, 2022), which is why there is a lower concentration in the SI site, the jejunum.

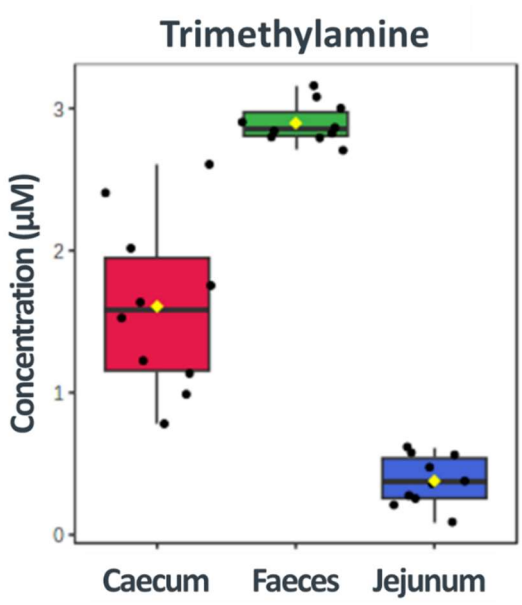


Figure 5.21 Box plot exhibiting the difference in concentration of trimethylamine.

Glucose and lactate were the most abundant compounds detected across all sample types. There was significantly greater glucose in the faeces and jejunum than in the caecum, with the jejunum having the highest concentration of all three GI locations (Fig. 5.22A). Lactate concentrations were significantly higher in faecal samples than in all other GI locations, and jejunum had a significantly higher concentration than caecum (Fig. 5.22B). Lactic acid-producing bacteria were the most abundant organism in all sample types and comprised more than 50% of the faecal and jejunal microbiomes (Fig. 5.6), which explains the high abundance of lactate across GI locations.

Butyrate is a short-chain fatty acid produced in the caecum (Bauer et al., 2019). There was significantly more butyrate in the caecum than in the faeces and jejunum, and significantly more butyrate in the jejunum compared to the faeces (Fig. 5.22C). Butyrate is essential for the modulation of the chicken's gut microbiome (Zou et al., 2019). Supplementation with butyric glycerides alters the microbiome in broilers (Yang et al., 2018), although effects on performance are unknown. As this metabolite is vital to gut health, it is disappointing that this compound is less detectable in the faeces as it could prove a valuable marker.

Taurine was previously suggested as a caecal marker for dysbacteriosis (Bailey, 2010) , as taurine is chickens' most common bile acid conjugate. If more taurine was detected, it would suggest an excessive breakdown of bile acid conjugates during dysbacteriosis.

However, at 13 dph, taurine was only significantly higher in the jejunum samples (Fig. 5.22D), where it is absorbed (Yuan and Wang, 2010). There was no significant difference between caecal and faecal taurine concentrations; therefore, either sample type would be suitable for detecting this compound.

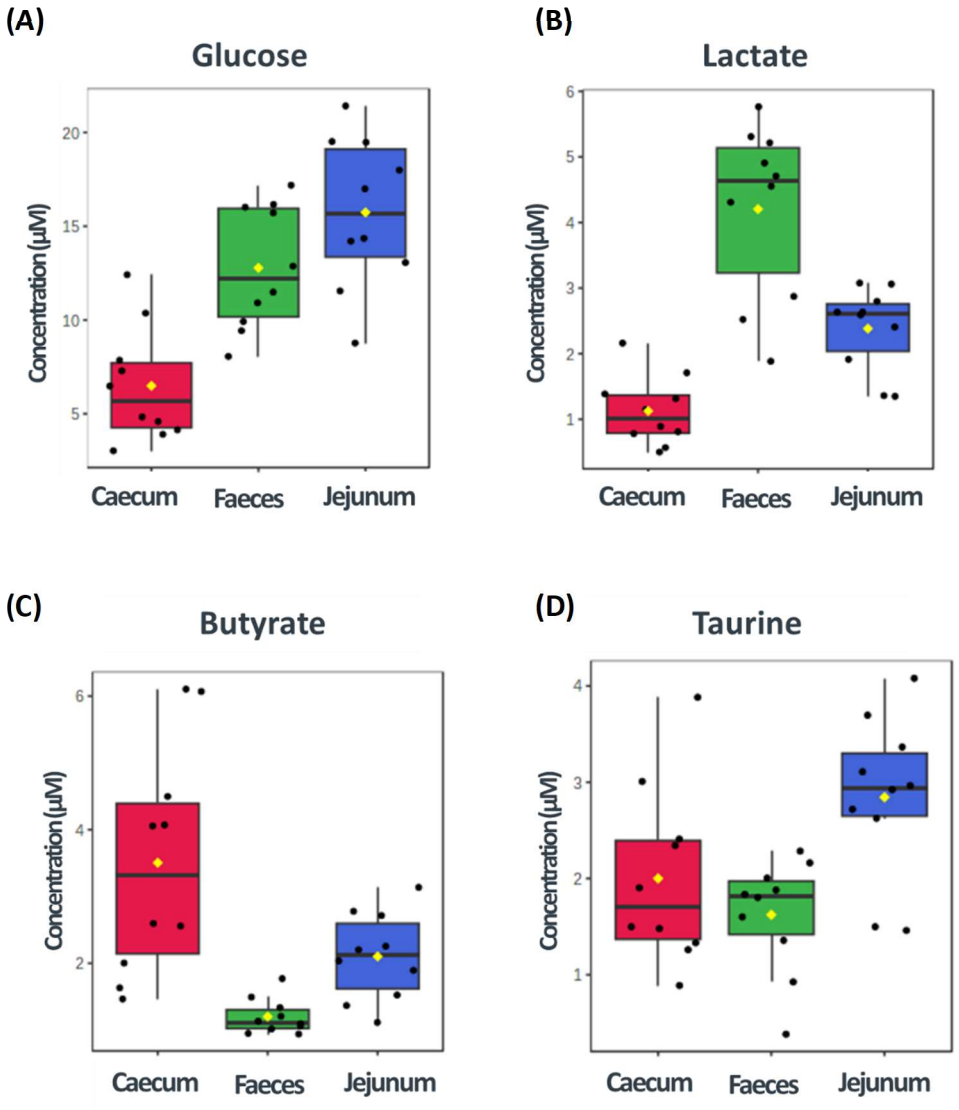


Figure 5.22 Examples of four metabolites showing significant differences between GI content locations: (A) glucose, (B) lactate, (C) butyrate and (D) taurine.

Functional analysis using Human3 revealed a total of 352 pathways. Surprisingly, faeces exhibited the highest count at 335 pathways, followed closely by the jejunum with 330, while the caecum presented the lowest count at 276 pathways. This finding is unexpected considering the caecum's previously noted high diversity and density of

bacteria. Interestingly, no pathways for taurine or butyrate were detected in the shotgun metagenomic data.

The most prevalent pathway across all GI locations was PWY-7219, responsible for adenosine ribonucleotides de novo biosynthesis (Fig. 5.23A). Its dominance underscores its essential role, although its relative abundance was only 0.0003%, indicating low detection across all pathways. Noteworthy is the similarity between the second most abundant pathways in the caecum and faeces, while the second most abundant pathway in the jejunum showed minimal detection in the distal GI locations. Regarding the most abundant compounds identified, five lactate pathways (Fig. 5.23B) and four glucose pathways (Fig. 5.23C) were observed. Despite glucose concentrations being highest in the jejunum, the faeces had the highest relative abundance of glucose pathways.

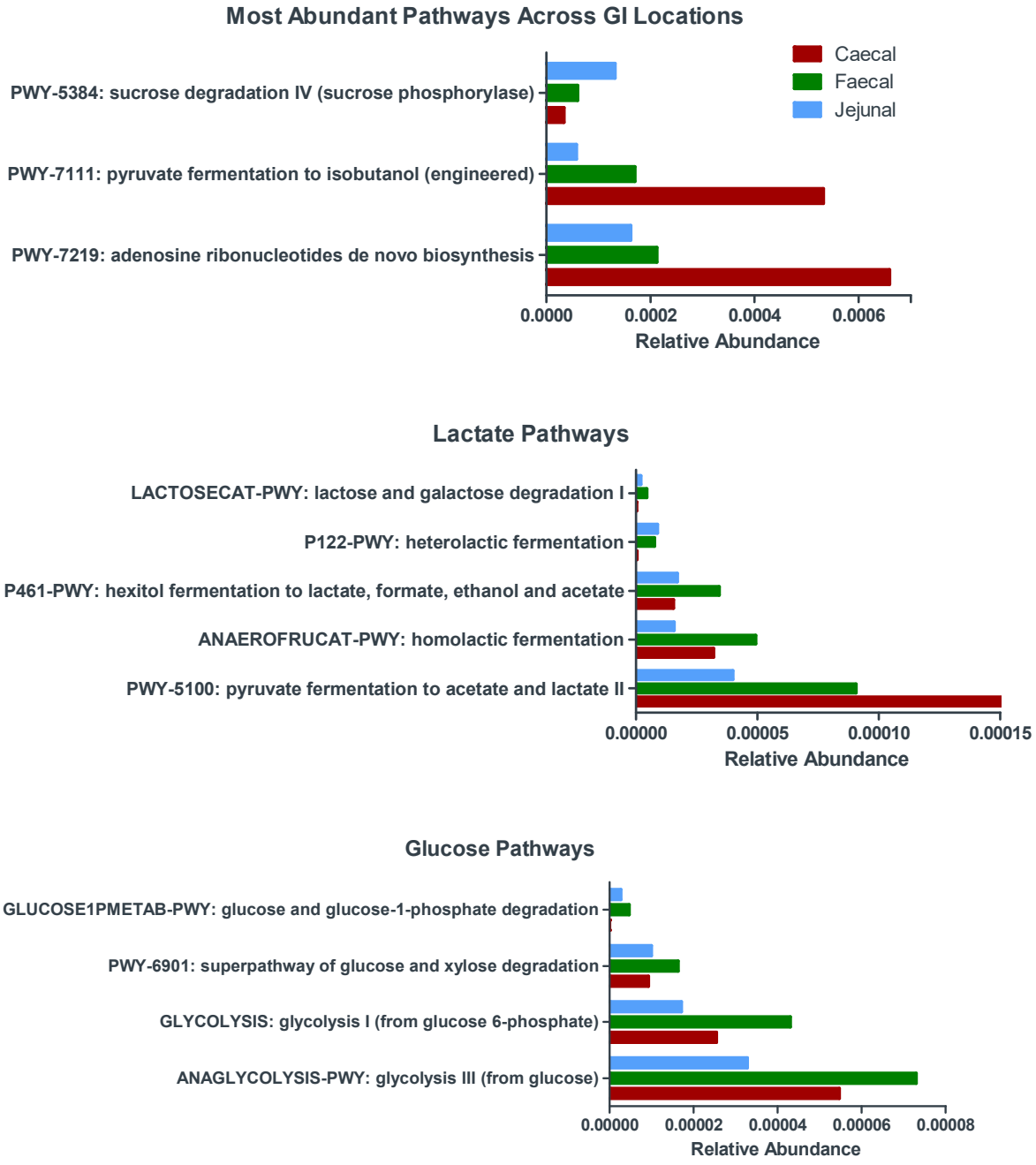


Figure 5.23 Horizontal bar graphs depicting the average relative abundance of (A) the most abundant metabolic pathways, (B) lactate metabolic pathways and, (C) glucose metabolic pathways from the Humann3 functional analysis.

In addition to the 204 significant differences in metabolite concentrations across the three GI locations, the caecum's metabolic profiles (i.e., the composition of compounds) were distinctly different from the other GI tract locations (Fig. 5.24). The faecal and jejunal

metabolic profiles can be seen clustering on top of each other, showing that the metabolite profiles of these two sample types are very similar, whereas caecum samples cluster entirely separately. This demonstrated that the faecal sample captured the metabolome of the SI but not the caecum.

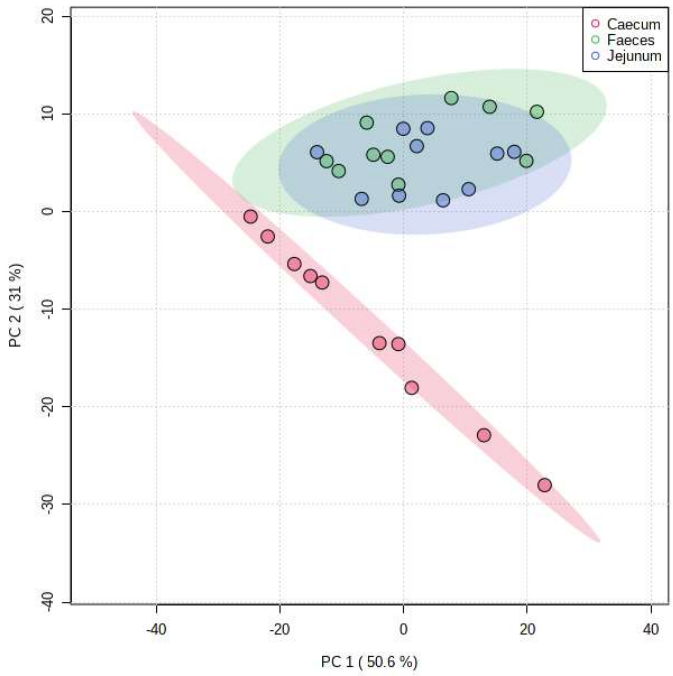


Figure 5.24 PCoA of metabolite profiles. Samples with similar profiles cluster closer together. Ellipses represent the average distribution. Generated using MetaboAnalyst 5.0 R package.

5.2.5 Metabolome of the Serum at 13 days post-hatching

Untargeted ^{1}H-NMR analysis of integrated peaks identified 140 metabolites across all broiler serum samples at 13 dph. After low abundance filtering, 95 metabolites remained (**Fig. 5.25A**). No significant difference between sexes (t-test, P=<0.05) (data not shown) was observed. However, protocatechuate was identified as having a significantly greater concentration in over-performing birds when birds were categorised as 'under' or 'over' performing (t-test, P=<0.0001) (Fig. 5.25B), based on the interquartile ranges of their body weight.

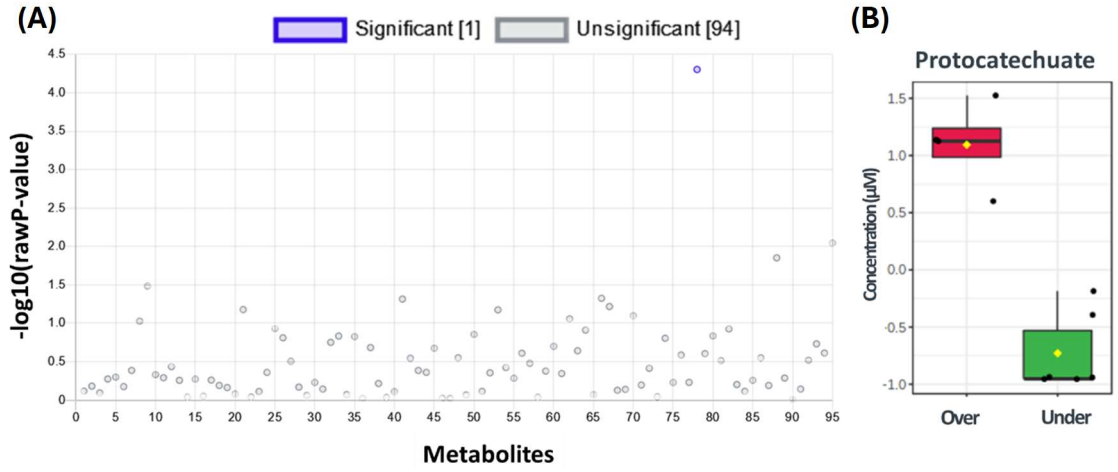


Figure 5.25 Untargeted ^{1}H-NMR identified 95 metabolites, one of which had significant differences in concentration when comparing 'over' and 'under' performing broilers.

Covariate statistical analysis using a fixed-effect linear regression model based on limma (Linear Models for Microarray and RNA-Seq Data) with body weight as a continuous value identified four serum compounds with a significant difference in concentration, correlating to body weight at 13 dph (Fig. 5.26).

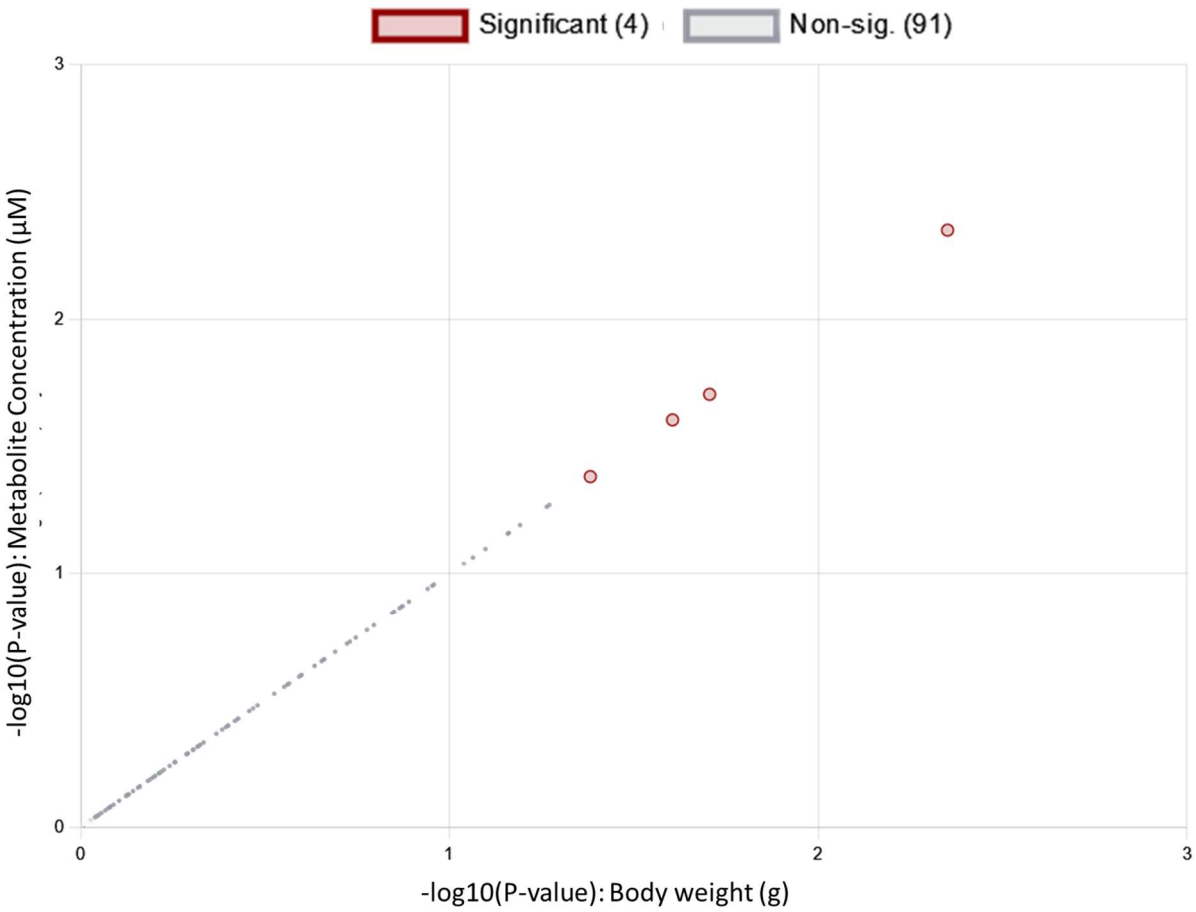


Figure 5.26 Mixed linear regression model (limma) generated in MetaboAnalyst 5.0.

The greatest significance in the linear regression model was with protocatechuate, which is one of the key intermediate metabolites in the microbial degradation of various compounds and has antioxidant properties (Liu et al., 2002) (Fig. 5.26A). Protocatechuate was also the only metabolite with a significantly higher concentration when comparing 'over' and 'under' performing birds based on their weight (Fig. 5.25). Concentrations of vanillate, a precursor of protocatechuate, also correlated with higher weight in the linear regression model (Fig. 5.26B).

Concentrations of τ-methylhistidine (3MH), which is a biomarker for skeletal muscle protein breakdown (Hayashi et al., 1985), were significantly correlated to body weight at 13 dph (Fig. 5.27C). 2-Methylglutarate was the final serum metabolite to correlate with body weight (Fig. 5.27A). It is an α, omega-dicarboxylic acid, and a lipid derived from a glutaric acid and is a metabolite of succinic acid and a citric acid cycle intermediate (Hastings et al., 2012).

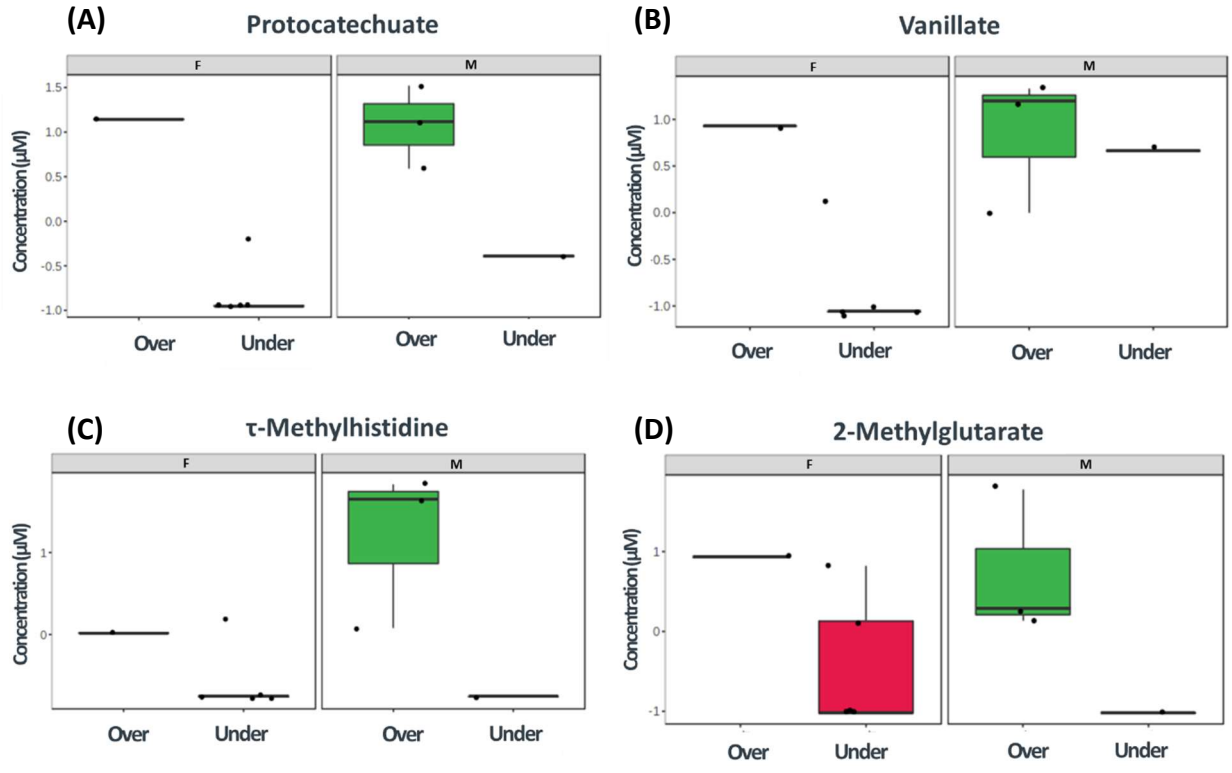


Figure 5.27 Four metabolite concentrations that showed a significant correlation between body weight, separated by sex and performance categories (t-test): **(A)** Protocatechuate (P = <0.0001), **(B)** Vanillate (P = 0.02), **(C)** τ-Methylhistidine (P = 0.01) and **(D)** 2-Methylglutarate (P = 0.04).

5.3 DISCUSSION

Bias in the literature towards the caecum is evident, primarily attributed to its high density and diversity of bacteria (Glendinning et al., 2019, Ocejo et al., 2019b, Richards et al., 2019), which is particularly noticeable when relying solely on culturable techniques. However, next-generation sequencing has facilitated a more comprehensive examination of the entire GI microbiome. By integrating shotgun metagenomic sequencing with parallel ITS1 metabarcoding, a detailed overview of all microbiome components can be attained, thereby alleviating previous biases towards the caecum. Moreover, this approach presents an opportunity to examine faeces as a potential marker source. Notably, faecal sampling offers a more ethical collection method, eliminating the need for bird stress or sacrifice.

No significant differences emerged between genders. Likewise, no significant differences were observed between performance groups when examining the caecal, faecal, and jejunal microbiome and metabolome. However, one metabolite in the serum metabolome exhibited a significant increase in 'over' performing broilers. It reinforces the introduction's premise that a combination of sample types may be better to characterise gut health and performance. Despite some shared resemblances, the physiological and functional disparities across GI tract locations contribute to distinct microbial and metabolic compositions. Having no significant sex-based differences observed but significant variations evident between 'under' and 'over' performing broilers at 13 dph determines the disparity in performance is attributed to the uneven distribution of sexes across weight categories. Given that male broilers typically exhibit higher body weight than females, the observed differences are indicative of weight-based distinctions rather than sex-based ones.

Research demonstrates a greater diversity of bacterial species in the gut than fungi (Robinson et al., 2022), evidenced by the number of reads in shotgun metagenomic sequencing in these results. In the literature, this observation primarily relies on 16S and ITS rRNA metabarcoding (Robinson et al., 2022a), overlooking the fact that many fungal taxonomic groups, such as *Aspergillus*, possess multiple copies of the gene (Bradshaw et al., 2023), unlike most bacteria species. Despite their lower numerical presence, fungi contribute to gut microbiome biomass due to their larger size. Unfortunately, the poultry industry tends to overlook fungal pathogens, reacting rather than proactively addressing

them. Yet, with rising global temperatures altering microbial selection pressures, fungal pathogens could become a future concern (Byrd et al., 2017, Dhama et al., 2013). The mycobiome is described as transient and variable, influenced mainly by environment, diet, and age (Robinson et al., 2022a, Davies et al., 2022). In broilers at 13 dph, there was no significant difference in fungal species richness and evenness between the caecum, jejunum, and faeces. However, contrary to the literature, the caecum had significantly higher fungal β-diversity than the SI and colon.

Comparatively, the metabolomes of faeces and jejunum display striking similarities, contrasting with the distinct profile of the caecum. For instance, lactate, the second most abundant compound in faeces at 13 dph, exhibits higher concentrations than caecal and jejunal contents. Similarly, *Lactobacilli* dominates the faecal microbiome at 13 dph, as indicated by shotgun metagenomic data and demonstrated in the literature (Glendinning et al., 2019, Jurburg et al., 2019, Mohd Shaufi et al., 2015). Populations of *Lactobacilli* in the chicken GI tract have been extensively investigated for probiotics (Adhikari and Kwon, 2017, Gonmei et al., 2019, Pokorná et al., 2019) and the composition of these taxa are known to influence the microbiome development and structure of broiler chicks (Zou et al., 2018, Angelakis and Raoult, 2010). Butyrate, proposed as a faecal biomarker for poultry intestinal health (Ducatelle et al., 2018a), is highest in the caecum, the site of SCFA production. Like the microbiome and mycobiome, the metabolome underscores greater similarities between the jejunum and faecal content than with the caecum, which had significant differences. The anticipated outcome was for faeces to serve as a composite of all three areas. However, it does not fulfil this expectation. Nonetheless, the inability to achieve this does not affect the identification of faecal-based markers. The study reveals that the caecum, commonly studied by many, does not accurately reflect the contents of the jejunum and colon, highlighting a significant observation.

Like bacteria and fungi, viruses exhibit comparable microbiome compositions in the jejunum and faeces. Correlations between the bacteria and viruses in the broiler gut microbiome have been identified to understand enteric viral infections and dysbacteriosis better (Clavijo et al., 2022). Quantifying these relationships requires understanding their roles in modulating gut health and performance. Bacteriophages have been proposed as potential alternative antibiotics for managing poultry intestinal health (Eid et al., 2022). Understanding the viral composition of the broiler GI tract at 13 dph will contribute to

elucidating their role in modulating the microbiome for gut health and performance, however Low yields suggest the need for more precise extraction methods to investigate the virome component effectively.

The serum analysis, albeit conducted on a small sample size of 10 birds, indicates significant metabolite differences, highlighting the potential to identify biomarkers related to bird body weight and performance. Chickens' inherent intestinal permeability, exacerbated by conditions like heat stress or poor enteric health (Barekatain et al., 2019), warrants further investigation into compounds like protocatechuate, vanillate, τ-methylhistidine, and 2-methylglutarate as potential biomarkers of performance at 13 dph. Future studies should encompass different ages along the broiler life cycle and additional performance parameters such as feed efficiency for validation.

In line with existing literature, the caecum exhibits the highest bacterial density and diversity, contrasting notably with the small and large intestine. While faeces sampling fails to fully capture the species richness of the caecum, it aligns closely with the SI, potentially providing deeper insights into digestive function. Continuous faecal sampling from live birds facilitates monitoring. Moreover, understanding the relationship between bacterial and fungal diversity remains pivotal, with limited studies exploring this aspect.

5.3.1 Summary & Concluding Remarks

The primary objective was to assess the efficacy of faecal sampling in characterising the performance of broilers. This involved analysing the variability of the microbiome, mycobiome, and metabolome across three GI tract locations in 10 broilers at 13 dph. Through analysis of shotgun metagenomic sequencing and untargeted ^{1}H-NMR, it was observed that faeces exhibited the least variability among the sample types for both microbiome and metabolome. This GI tract location displayed the lowest variation in α-diversity. Additionally, faeces demonstrated the least variation in the untargeted metabolome among individual broilers. Interestingly, faeces showed similarities to the jejunum in microbiome and metabolome composition, with the top 10 species collectively accounting for 75-95% of the total bacterial microbiome composition, while the caecum exhibited distinct differences. These findings diverge from the existing literature, which

typically portrays faeces as variable. Instead, this study reveals that the caecum and jejunum exhibit greater variability than faeces.

The next objective aimed to examine the interaction between the fungal and bacterial components within the broiler microbiome to gain insights into their relationship and potential effects on performance. A significant correlation in β-diversity was observed between bacteria and fungi across all sample types, indicating that broilers with a diverse bacterial population also tend to have a diverse fungal population in their microbiome. This correlation is crucial for understanding broiler microbiome, as previous assessments primarily focused on bacterial diversity, neglecting the potential influence of fungi. However, my findings demonstrate that fungal diversity is also affected by bacterial diversity.

The final objective aimed to delineate differences in broiler performance through assessments of body weight and serum metabolome profiles at 13 dph, aiming to uncover potential health and performance indicators in broiler production systems. Among the metabolites examined, four showed positive correlations with body weight. Notably, protocatechuate exhibited the strongest correlation with body weight and remained significant when broilers were categorised as 'over' and 'under' performing, indicating a robust finding. Bacteria produces and metabolises protocatechuate (Upadhyay and Lali, 2021). Additionally, serum vanillate concentrations, a protocatechuate precursor, were significantly correlated with body weight. Vanillate is produced following microbial degradation of lignin, which is abundant in plant cell walls and demethylated to protocatechuate ((Kamimura et al., 2017). These compounds are likely generated by the gut microbiome and later detected in the serum. These findings suggest a more metabolically productive microbiome indicates better gut health and performance, as evidenced by increased body weight.

The initial hypothesis posited that faecal sampling could be a dependable proxy for representing the whole gut microbiome and metabolome and for evaluating broilers' performance at 13 dph. It was anticipated that the variations observed in the microbiome, mycobiome, and metabolome across three GI sites—jejunum, caecum, and faeces—at 13 dph would reflect the overall performance. Additionally, it was hypothesised that disparities in broiler performance, as determined by body weight and serum metabolome at 13 dph, could be discerned. The findings revealed that although the caecum hosts the richest

bacterial and metabolic communities, the composition of the faecal and jejunal microbiomes exhibited greater similarity than that of the caecal microbiome concerning bacterial genera, fungal order, and metabolome profiles. There was no correlation between bacterial, fungal or metabolome composition and performance from any GI tract sites; however, serum analysis proved effective in distinguishing between underperforming and overperforming broilers based on body weight at 13 dph, warranting further exploration.

CHAPTER 6

APPLICATIONS OF SERUM TO DEFINE BROILER PERFORMANCE AND GUT HEALTH AT 35-DAYS POST HATCHING

6.1 INTRODUCTION

The collection of blood samples from poultry, specifically broilers, serves as a standard procedure for evaluating the genetic integrity of breeding stock and monitoring overall health status (Ducatelle et al., 2018a). This established practice enables veterinarians to conduct comprehensive examinations for the presence of various diseases, including avian influenza, and identify pathogens that cause intestinal inflammation (Chen et al., 2015), such as *Salmonella* and parasites like *Eimeria*.

Automatic analysers are commonly used in the routine assessment of blood biochemistry. These analysers are crucial in monitoring, diagnosing, treating, and characterising diseases (Rezende et al., 2017). They enable the measurement of various parameters, including total protein, serum lipids such as triglycerides, and metabolites such as urea. Integrating applications to characterise broiler performance and assess gut health can seamlessly be introduced into the industry in conjunction with existing routine serum tests.

In chickens, the inherent permeability of the gut allows for the transport of metabolites or compounds produced by the intestinal microbiome into the bloodstream, facilitating their detection (Tellez et al., 2014). Notably, the identification of D-lactate (Tellez et al., 2014) and fluorescein isothiocyanate (FITC) (Vicuna et al.) in broiler serum has proven indicative of intestinal permeability. It is essential to acknowledge that the concentration of D-lactate in serum lacks a predefined parameter and is contingent upon knowledge of the initial host concentration. Furthermore, detecting FITC in chicken serum necessitates the administration of the fluorescent compound through oral gavage to enable subsequent identification in the serum. Hence, these markers are unsuitable for regularly assessing gut health and performance in a commercial poultry production environment.

In nutritional intervention trials, serum markers often exhibit significant effects, while overall performance or carcass traits may not show substantial differences (Lopes et al., 2013). Crude protein supplementation does not impact feed efficiency (Dehghani-Tafti and Jahanian, 2016), generally categorised as the relative ability to convert feed to the product in production animals. Still, the addition of organic acids (such as citric and butyric acids) does enhance Feed Conversion Ratio (FCR) and average daily growth (Dehghani-Tafti and Jahanian, 2016). Specifically, in this study, organic acid supplementation, particularly

with butyric acid, decreased serum triglyceride content (Dehghani-Tafti and Jahanian, 2016). Triglycerides, synthesised in the liver and intestinal mucosa from digested dietary components and absorbed fatty acids, play a crucial role in gut health (Ravindran et al., 2016, Rezende et al., 2017).

Butyrate, a short-chain fatty acid produced in the caeca through carbohydrate fermentation by resident bacterial communities, is essential for maintaining a healthy gut (Yang et al., 2018). Broilers fed with dietary butyrate glycerides exhibited alterations in ileal and caecal microbiome composition and increased serum concentrations of various metabolites. These metabolites, including choline, glycerophosphorylcholine, dimethylamine, trimethylamine, trimethylamine-N-oxide, and succinate, were suggested to originate from intestinal bacteria. However, no significant changes in performance parameters, such as body weight or feed efficiency, were reported in this study (Yang et al., 2018).

In another study, broilers fed with fermented cottonseed meal showed significant differences in 20 serum metabolites despite no observed effects on bird growth. However, a notable improvement in feed efficiency and reduced abdominal fat was observed (Niu et al., 2019). The serum from these broilers exhibited lower glucose and triglyceride content compared to control groups (Niu et al., 2019). Grapeseed extract supplementation improved weight gain while reducing daily feed intake (Cao et al., 2020). This supplementation led to observable changes in the caecal microbiome, significantly increased caecal butyrate, and a notable difference in 23 serum metabolites related to lipid, amino acid, and alkaloid metabolism (Cao et al., 2020).

Dietary supplementation with ginger root extract in broilers increased total protein in their serum without significantly affecting overall growth or feed efficiency; however, it did improve carcass yield (Zhang et al., 2009). These studies highlight the potential for utilising serum markers to assess the impacts of dietary interventions on performance. While many nutritional interventions may not directly influence performance metrics, they often induce significant changes in serum metabolic profiles, indicating substantial alterations in metabolism. Identifying a serum metabolite that could predict performance or gut health when applied to a longitudinal study would be valuable for commercial poultry production and research.

The chicken immune system relies on antibodies, specifically Ig, to recognise and counteract foreign entities, such as pathogens (Broom and Kogut, 2018). Chickens possess three distinct serum antibodies: IgG, IgM, and IgA (Kogut et al., 2017). Antigen-binding natural antibodies (NAbs) exhibit lower specificity than other antibody types as a crucial link between the innate and adaptive immune systems (Haghighi et al., 2006). Isotypes of Ig are differentiated based on the heavy chain type present in the molecules. IgG (sometimes referred to in the literature as IgY because it is the avian homologue of mammalian IgG) predominates as the principal serum Ig in chickens (Lebacq-Verheyden et al., 1974). IgG is essential to the chicken immune system, providing defence against various infectious agents (Kaspers and Schat, 2012). IgM, associated with B cell production, has been correlated with heat stress, where decreased IgM levels indicate this condition (Honda et al., 2015). IgA, linked to the microbiome, plays a pivotal role as the least abundant serum Ig, protecting mucosal tissues from microbial intrusion and contributing to immune homeostasis with the microbiome (Pabst and Slack, 2019). This interplay is essential for bolstering disease and pathogen resistance. Berghof and colleagues developed a method for quantifying NAbs (Berghof et al., 2019, Berghof et al., 2015b, Van Der Klein et al., 2015) with a primary focus on layers. However, the investigation of NAbs in broilers has not been undertaken. The abbreviated 35-day life cycle of broilers implies a shorter period for developing their immune system than the 16-week-old layers from the original study. As NAbs are inheritable (Berghof et al., 2015b), identifying broilers with higher antibody titres would benefit commercial breeding practices to improve broiler performance and gut health.

Previous studies have established a correlation between poor gut health in chickens and the loss of tight claudin junctions in the intestinal epithelial wall (Chen et al., 2015 2018, Baxter et al., 2019). This loss results in heightened gut permeability, facilitating the transportation of metabolites produced by the gut microbiome into the bloodstream, where they become measurable in the serum. To empirically test this hypothesis, it is proposed that birds with suboptimal performance parameters, characterised by low body weight and high Feed Conversion Ratio (FCR), will demonstrate an increased presence of detectable metabolites in the serum due to heightened gut permeability.

6.1.2 Aims, Objectives, and Hypothesis of this Chapter

The primary aims and objectives of this thesis chapter are as follows:

1. Utilise the broiler serum metabolome to identify compounds consistently detectable, aiming to differentiate between the most and least productive birds at 35 dph, with a focus on body weight and feed efficiency metrics.
2. Determine whether NAb titres can be used to differentiate between the most productive and efficient birds and the least productive ones.
3. Examine the relationship between NAb titres, the serum metabolome, and overall production parameters, aiming to elucidate potential connections and correlations among these factors.

Hypothesis

The hypothesis posits that broilers exhibiting inferior performance will demonstrate compromised gut health, leading to increased intestinal permeability. This increase can be quantified by either a surge in the number or concentration of metabolites in the serum. In contrast, broilers displaying superior performance are anticipated to exhibit higher NAb titres, indicative of a more robust immune system.

6.2 METHODS

6.2.1 Sample Collection and Storage

In brief, as described in **Chapter 2: Methods**, shotgun metagenomic sequencing (**Methods 2.3–2.4**), statistical analyses (**Methods 2.5**), and metabolomic analyses (**Methods 2.6**) were conducted.

Aviagen provided feed conversion ratio (FCR) and body weight data for all broilers, which were of the same genotype. Additionally, serum samples were collected from 200 female broilers at 35 dph, stored at **-20°C**, and transported to QIB on dry ice. To prevent degradation from freeze-thaw cycles, serum was defrosted and immediately aliquoted for future use.

6.3 RESULTS

6.3.1 Determining Broiler Performance According to the Body Weight and Feed Efficiency at 35 dph

The body weight and feed efficiency metrics were used to verify the performance of the 200 broilers, enabling better statistical quantification of metabolic and immunological differences between the serum samples. The birds were grouped according to the interquartile ranges of the sample metadata, as shown in Table 6.1.

Table 6.1. Distribution of broilers according to their body weight and feed efficiency. Performance group 3 represents the most productive and efficient birds, whilst cohort 4 represents those that are the least commercially desirable.

Performance Group No.	Body weight Range (g)	FCR Range	No. of Broilers	Description
1	1910- 2310	1.312 – 1.214	24	Low body weight and low FCR
2	2320- 2650	1.319 – 1.397	31	High body weight and high FCR
3	2330- 2880	1.16 – 1.317	21	High body weight and low FCR (*most efficient*)
4	1910- 2290	1.318 – 1.432	18	Low body weight and high FCR (*least efficient*)
5	2420- 2220	1.351 – 1.286	53	Mid-range

The most significant proportion of broilers (27%) were grouped as mid-ranged (performance group 5) as they did not meet the requirements for the other four cohorts (Fig. 6.1). In performance group 1, 12% of the broilers had low body weight and low FCR. In performance group 2, 16% of broilers had high body weight and FCR. In performance group 3, 11% of broilers had high body weight and low FCR (the most efficient). Finally, in performance group 4, 9% of the broilers had low body weight and high FCR (the least efficient). Body weight and feed efficiency data can still be used as continuous values, but the grouping permits more robust statistical analysis to differentiate between broiler performance groups.

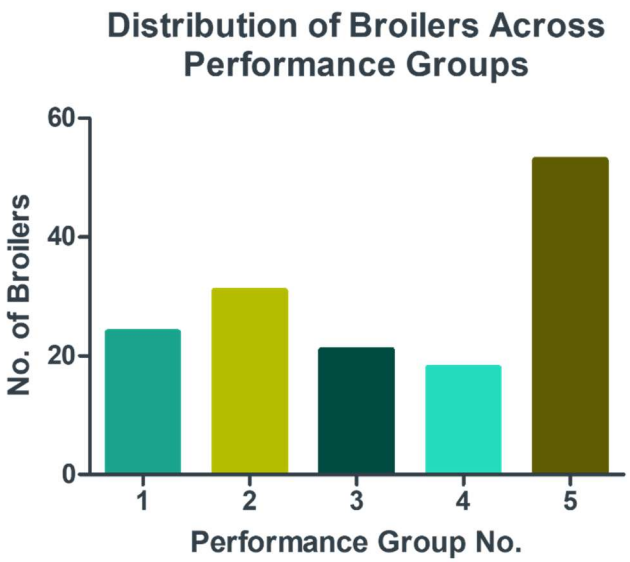


Figure 6.1 Bar graph depicting the distribution of broilers between five cohorts according to body weight and feed efficiency. Performance group 1 has low body weight and low FCR, group 2 has high body weight and high FCR, group 3 has high body weight and low FCR (the most efficient), group 4 has low body weight and high FCR (the least efficient), and group 5 are mid-range birds that fall outside of the interquartile ranges.

6.3.2 Sex, weight and FCR distribution of 200 broilers at 35 days post-hatching

The 200 female broilers at 35 dph weighed from 1910 g to 2880 g, and feed efficiency ranged from 1.27 FCR to 1.43 FCR. The interquartile ranges of both body weight (Fig. 6.2A) and FCR (Fig. 6.2B) were calculated so that broilers could be grouped according to performance efficiency and desirable production qualities: high body weight and low FCR.

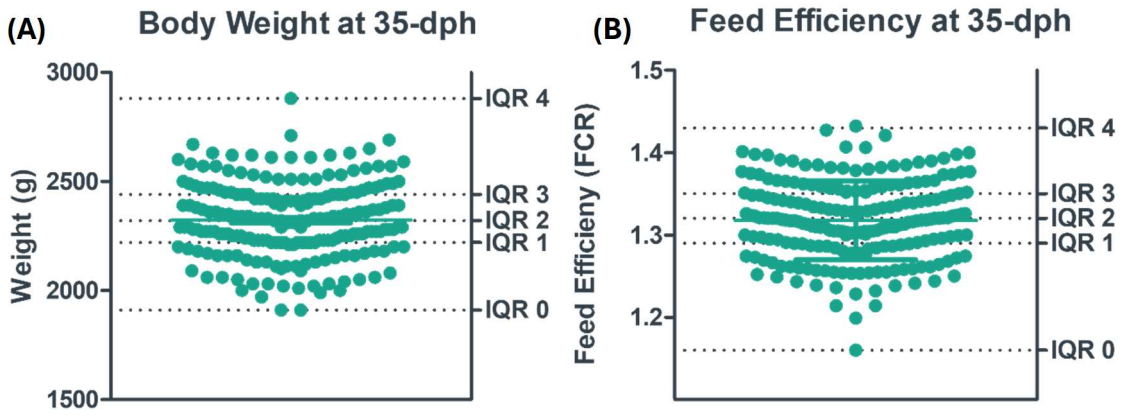


Figure 6.2 Vertical scatter plot graph depicting distribution and interquartile ranges of **(A)** total body weight (g) and **(B)** feed efficiency (FCR) at 35 dph in 200 female broilers.

Feed efficiency uses body weight to calculate the FCR value, which is why body weight and FCR have a significant relationship as determined by $P < 0.005$. However, the correlation effect was not significant as determined by the $R^2 < 0.7$ (Fig. 6.3A). It is essential to recognise that although the FCR and body weight values are intrinsic to each other, they capture different performance metrics. The most efficient broilers for commercial poultry production have a high body weight but low FCR. This demonstrates that the broiler can gain weight with reduced food consumption, making them more desirable for production.

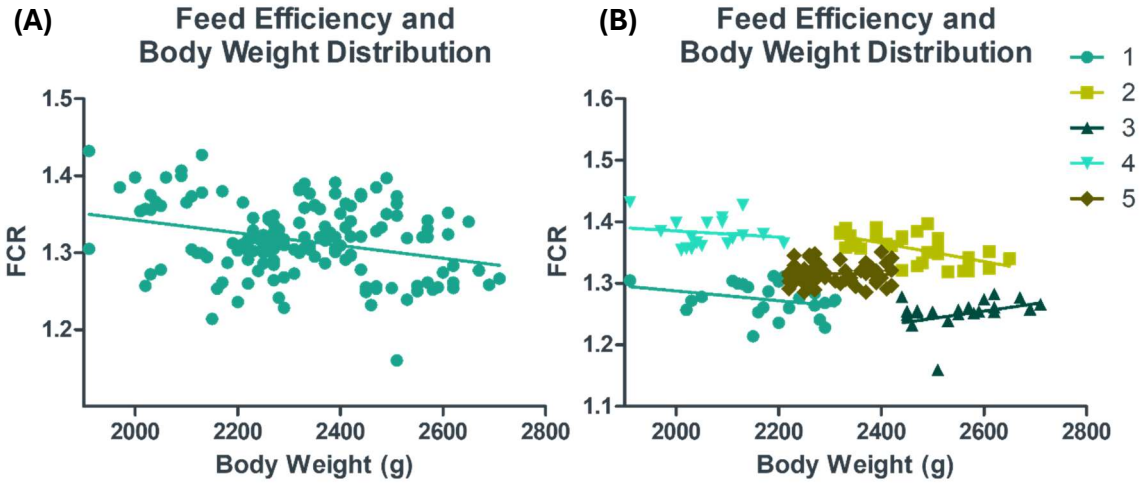


Figure 6.3 Scatter plot graph and linear regression to show **(A)** the significant relationship (P=<0.001) between body weight and feed efficiency but not a significant effect (R^2= 0.1104) and **(B)** feed efficiency and body weight distribution between the five performance groups (no relationship or effect).

6.3.3 Investigating the Untargeted Serum Metabolome Using ^{1}H-NMR

6.3.3.1 *Determining Sample Quality and Differences in Profiled Compounds in Serum Between Performance Groups*

All samples had a signal-to-noise ratio greater than 10, passing quality control. There was no difference between the performance groups and the signal-to-noise ratios (Fig. 6.4) demonstrating similar sample quality regardless of performance.

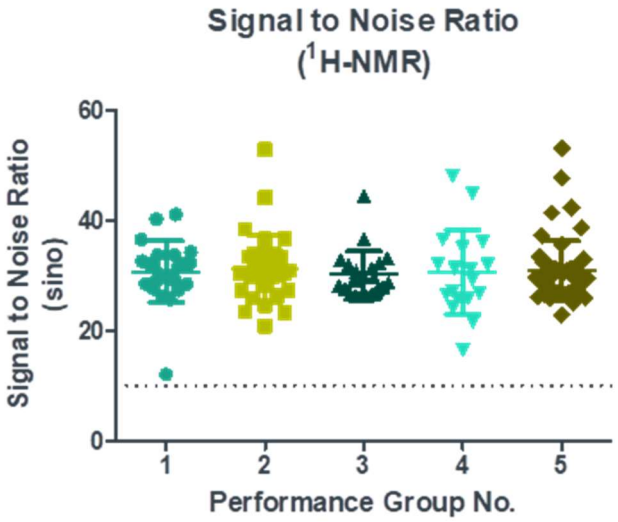


Figure 6.4. Horizontal scatter graph exhibiting the signal-to-noise ratio for each serum sample when acquired on the 600 Hz Bruker NEO at 32 scans. The line on the Y-axis at 10 represents the minimum signal-to-noise ratio requirement to pass quality control.

Between 5 and 122 compounds were profiled, averaging 71 (±SD 21.7) metabolites per sample (Fig. 6.5A). The total profiled compound concentrations ranged from 1,965 µM to 93,198.3 µM per sample, with an average of 5821.6 µM (±SD 9554.7) per sample (Fig. 6.5B). There were no significant differences (1-way ANOVA, Kruskal-Wallis test) between the number of profiled compounds nor the total concentration of all profiled compounds between the five performance groups. The greatest variation in the total concentration of profiled compounds was within performance group 2 (high body weight and high FCR) and performance group 5 (mid-range), highlighting the disparity between individual broilers.

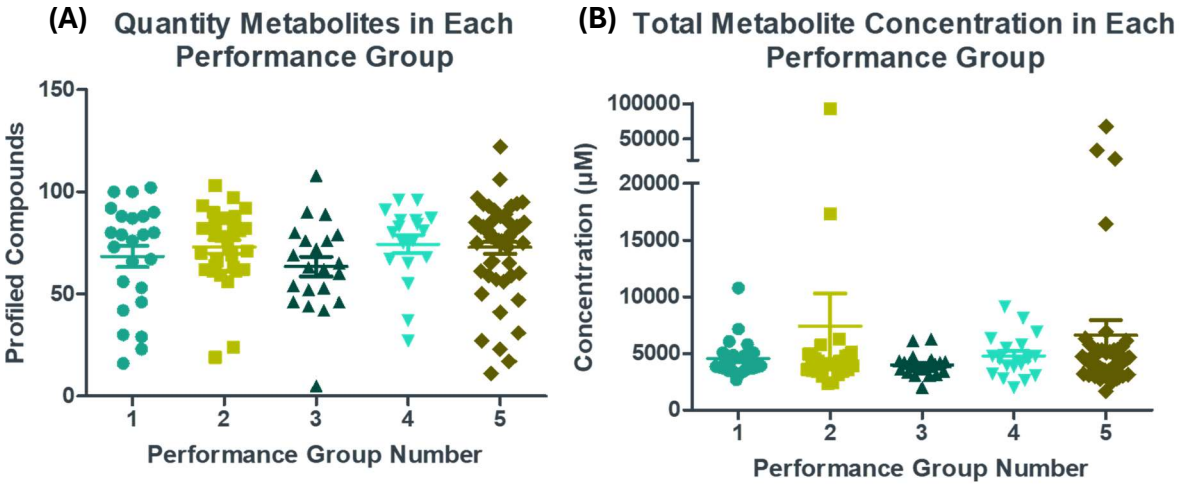


Figure 6.5. Horizontal scatter graph exhibiting (A) the number of profiled compounds and (B) the total concentration (µM) of all profiled compounds between the different performance groups.

There was a positive relationship (P = 0.02) between feed efficiency and the number of profiled compounds (Fig. 6.6A), suggesting that more metabolites are detectable in the serum in birds with worse feed efficiency (i.e. higher FCR). However, the effect (R^2=0.03) was not significant. There was no significant relationship or effect between the body weight and the number of profiled compounds (**Fig. 6.6B**). Furthermore, there was no significant relationship or effect between the total concentration of serum metabolites with either feed efficiency (Fig. 6C) or body weight (Fig. 6D).

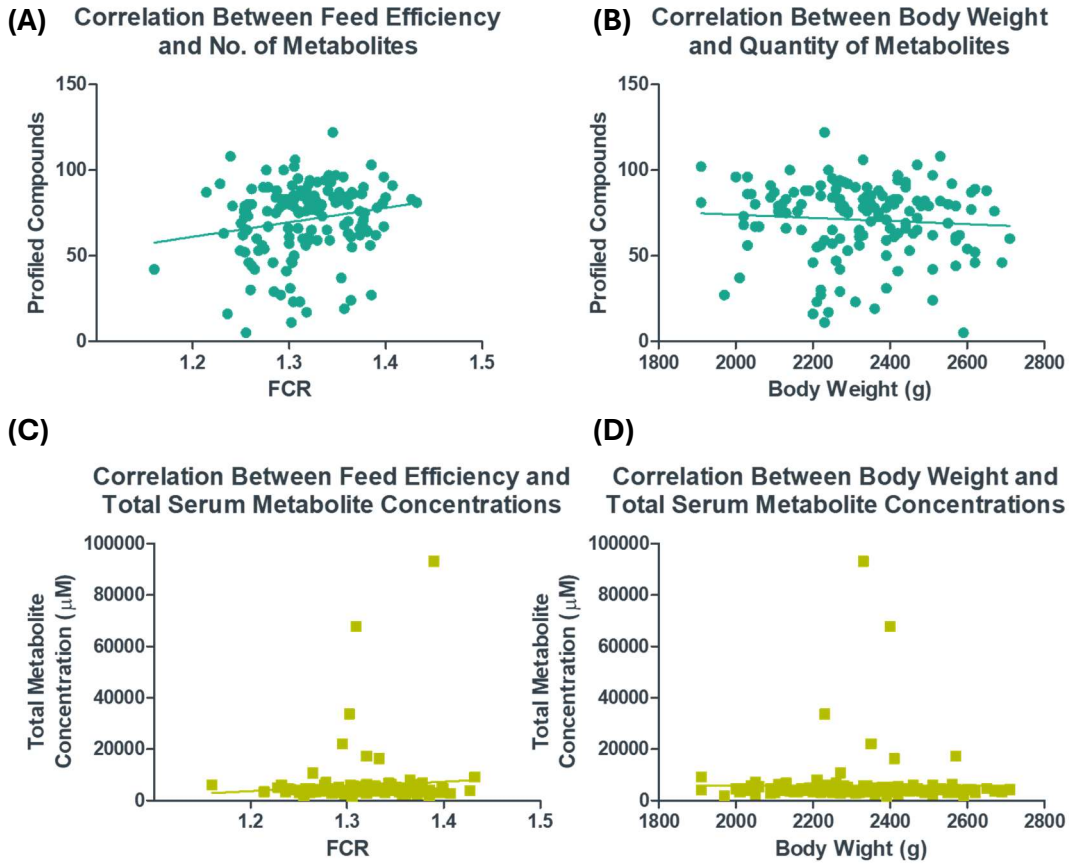


Figure 6.6 Scatter plot and linear regression models correlating the number of profiled metabolites to (A) feed efficiency (FCR) and (B) body weight and total serum metabolite concentration to (C) feed efficiency and body weight (D).

6.3.3.2 *Determining Differences in the Serum Metabolome Concentrations and Metabolic Pathways in Broilers at 35 dph with Different Performance Parameters*

Metabolites in broiler serum at 35 dph were successfully identified, and their respective metabolic families and pathways were elucidated. Analysis showed no significant differences in the number of profiled compounds, total serum metabolite concentration, or individual metabolite concentrations among the five performance groups of broilers.

Two hundred forty-seven metabolites were profiled using the Chenomx NMR Suite v9.0 compound reference library. There were no significant differences between the 247 metabolite concentrations and the five performance groups (Fig. 6.7), with 29 metabolites consistently appearing within all serum samples (Table 6.2).

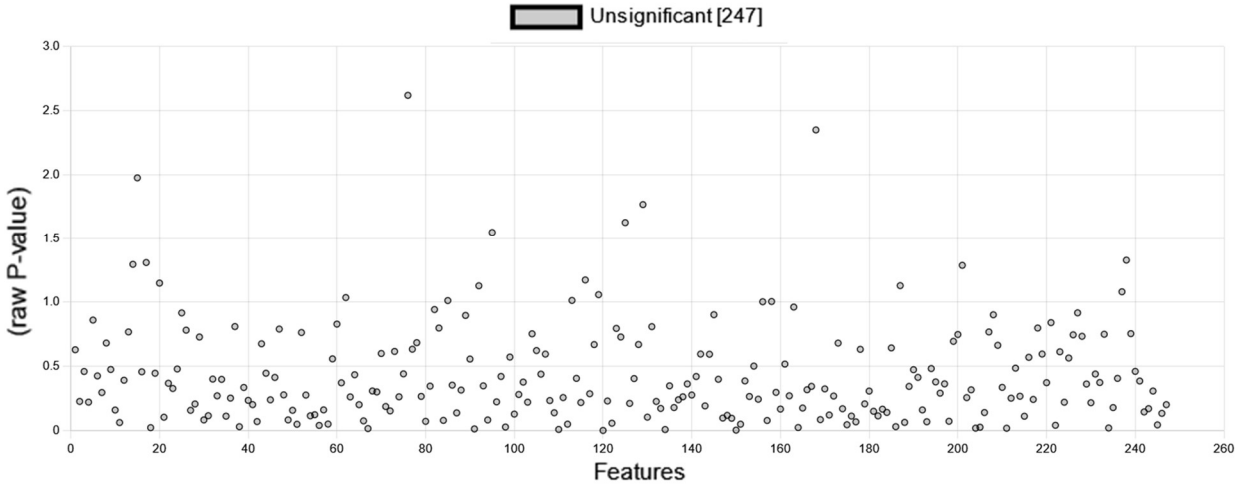


Figure 6.7. Scatter plot visualisation showed no significant differences (ANOVA) between metabolite concentrations and the five performance groups after data normalisation (auto-scaling) using the MetaboAnalyst 5.0 R package.

Table 6.2 Details of the 29 metabolites that were consistently profiled in all 200 broiler serum samples with metabolic families, presented with descending concentrations (µM) and standard deviation (±SD).

Compound Name	Average Concentration (µM)	Standard Deviation (±SD)	Metabolic Families
Glucose	671.57	278.44	Monosaccharides and Disaccharides
Lactate	520.44	110.25	Organic Acids
Homoserine	263.59	146.68	Amino Acids, Organic Acids
Caprylate	134.48	129.08	Fatty Acids
Caprate	98.72	46.95	Fatty Acids
Alanine	87.48	28.01	Amino Acids
Propionate	64.98	12.54	Fatty Acids
O-Phosphoserine	62.58	27.26	Amino Acids, Phosphates
4-Carboxyglutamate	61.08	13.31	Amino Acids, Organic Acids
Glycine	54.58	14.20	Amino Acids
5-Aminolevulinate	45.64	27.57	Amines, Keto Acids
Glucose-6-phosphate	45.11	26.89	Monosaccharides and Disaccharides, Phosphates
Glycylproline	41.83	53.50	Amino Acids, Amines

Glutathione	34.74	7.10	Amino Acids, Sulfur Compounds
Galactitol	28.13	9.77	Alcohols and Polyols
Valine	27.85	19.72	Amino Acids
Betaine	23.28	6.11	Amino Acids
N-Methylhydantoin	18.40	5.69	Amines
Melatonin	18.37	7.64	Amines, Aromatics
Glutaric acid monomethyl ester	17.79	11.47	Organic Acids
Trimethylamine N-oxide	16.02	12.64	N-oxides
Sarcosine	13.79	4.24	Amino Acids
N-Phenylacetylphenylalanine	12.45	3.85	Amino Acids, Aromatics
Anserine	11.07	11.60	Amino Acids, Amines, Aromatics
Protocatechuate	10.67	9.33	Organic Acids, Phenols
Desaminotyrosine	9.73	3.12	Organic Acids, Phenols
4-Pyridoxate	8.22	5.96	Alcohols and Polyols, Amines, Aromatics
Carnitine	5.46	2.45	Amines, Organic Acids
Theophylline	3.90	3.32	Purines and Pyrimidines

The most abundant compounds were lactate (**Fig. 6.8A**) and glucose (**Fig. 6.8B**), with an average concentration of 520.4 µM (±SD 110.2) and 671.6 (±SD 278.4), respectively. However, the performance groups had no significant differences in concentration. Understanding the consistent detection of metabolites in broiler serum assists in pinpointing suitable markers that could be routinely utilised in both research and production contexts.

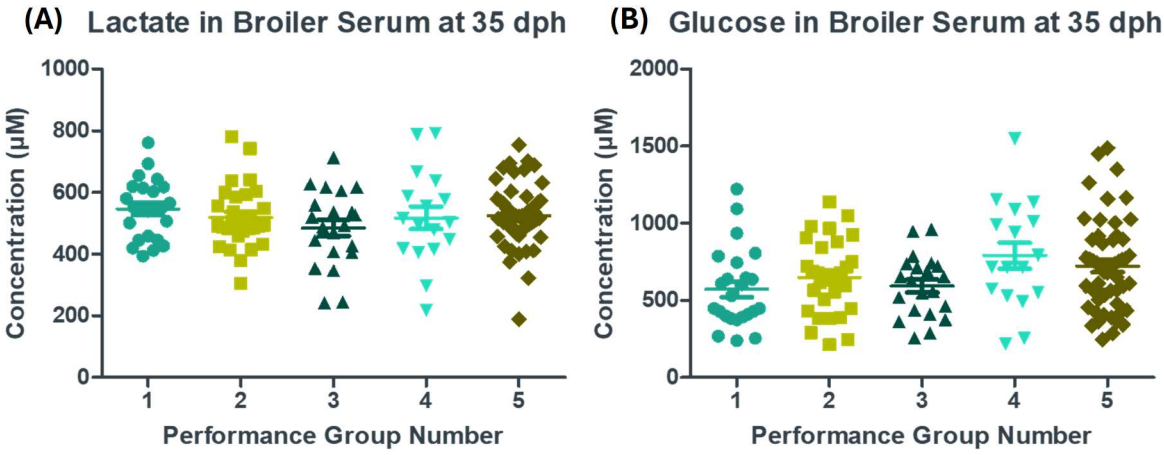


Figure 6.8. Horizontal scatter plots of the two most abundant metabolites found in all broiler serum samples at 35 dph: (A) lactate and (B) glucose.

The 247 compounds identified in all broiler serum samples at 35 dph were attributed to 19 metabolic families (Fig. 6.9A). Some compounds can belong to more than one metabolic family, such as glucose-phosphates, which are simultaneously a saccharide and phosphate. Almost a quarter of the metabolites were organic acids. Given ongoing nutritional intervention studies exploring organic acid supplementation in broiler feed (Dehghani-Tafti and Jahanian, 2016), it is advantageous to acknowledge and comprehend the natural abundance of these compounds in hosts that were not fed supplements.

The 247 compounds identified in all broiler serum samples at 35 dph were attributed to 17 metabolic pathways, identified using the Chenomx NMR Suite v9.0 reference library. The carbohydrate metabolism, phenylalanine and tyrosine metabolism, and the ketone body metabolic pathways had the most compounds (Fig. 6.9B). Carbohydrate metabolism is fundamental to starch digestion in the SI (Moran Jr, 1985). Tyrosine is metabolised by the host to produce acetoacetic and fumaric acid, a process regulated by the gut microbiome (Yoon et al., 2022). The utilisation of ketone body enzymes is higher in broilers than in layers, which is vital for broiler production as skeletal muscles are a primary target for ketone body utilisation (Zheng et al., 2009).

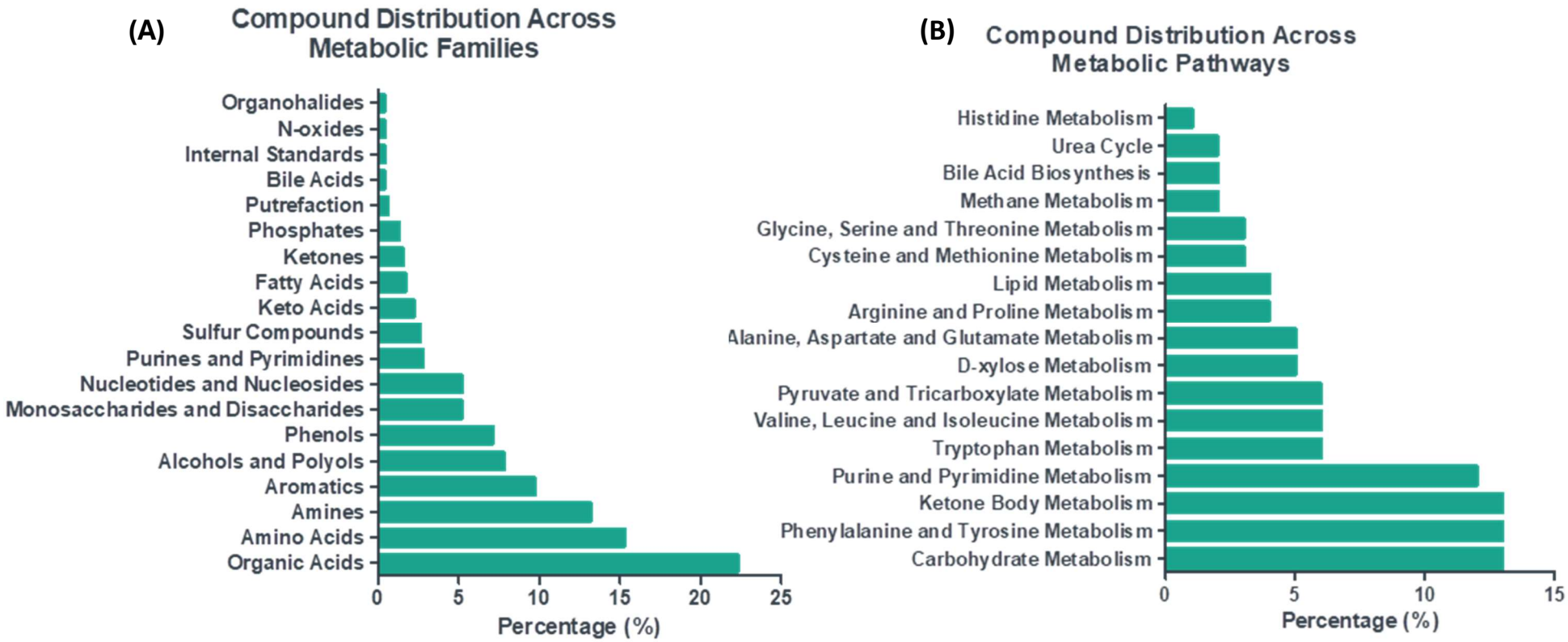


Figure 6.9 Horizontal bar graph of the metabolic families to which the 247 profiled compounds can be attributed **(A)** and, the metabolic pathways to which the 247 profiled compounds can be attributed **(B)**, according to the Chenomx NMR Suite v9.0 reference library.

These findings suggest that performance differentiation may depend on metabolites' collective composition, indicating the potential existence of a distinct serum metabolite profile capable of distinguishing between different performance levels.

6.3.3.3 *Determining Differences in the Serum Metabolite Profile in Broilers at 35 dph with Different Performance Parameters*

A Sparse Partial Least Squared Discriminant Analysis (sPLSDA) is an ordination plot, like a PCoA. However, PCoA is unsupervised and looks at the metabolome profile for each sample. In contrast, the sPLSDA is a supervised method considering the sample groups and performance variables. Therefore, it includes body weight and feed efficiency values from the metadata. Whereas PCoA shows the similarities in variables (e.g., similar metabolite profiles), sPLSDA shows the discrimination between variables (e.g., metabolites that make those profiles distinct).

The metabolite profile composition differences in performance group 3 represent the most productive and efficient broilers. Seven serum samples from this group were distributed between 2 and 8 along the Y-axis (component 2) (Fig. 6.10), suggesting a distinct difference in the serum metabolite profiles of these broilers. Interestingly, a single sample from performance group 5, the mid-range broilers, also appears to have this distinguished profile.

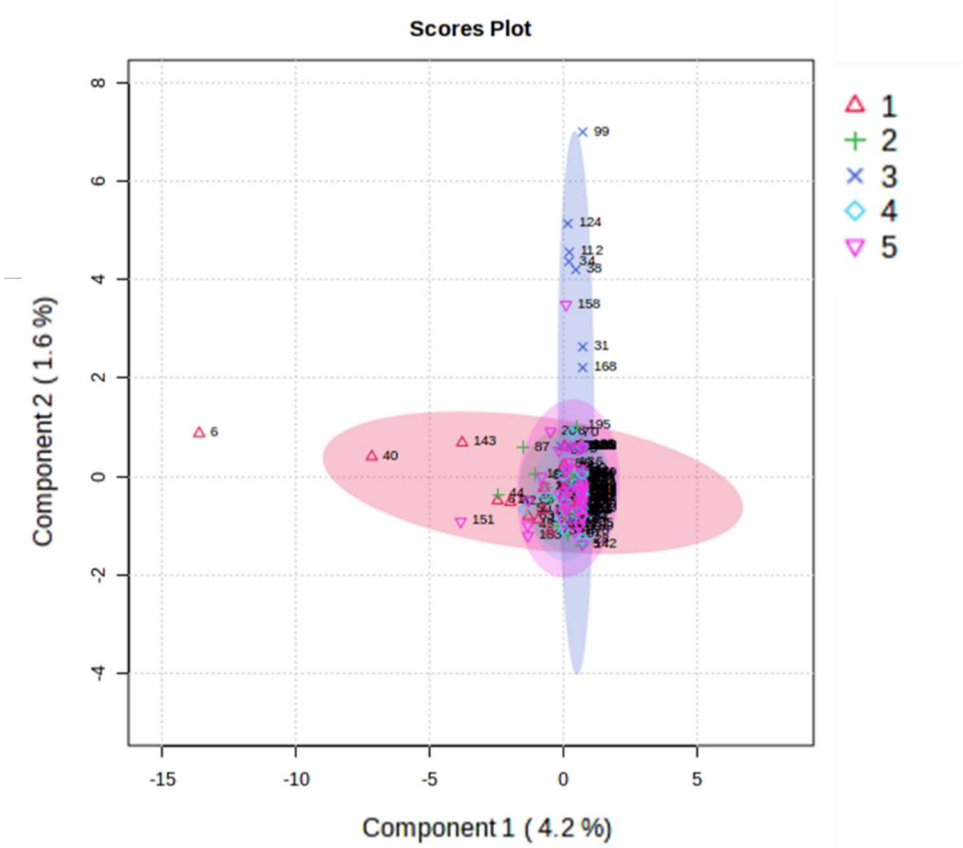


Figure 6.10 shows a sPLSDA generated with the MetaboAnalyst 5.0 R package to visualise differences in metabolite composition between the five performance groups. The ellipses indicate the samples' proximity to each other, indicating their average distribution.

Twenty metabolites contributed to distinguishing differences in the serum metabolome composition, observed in the seven birds from performance group 3 and single bird from performance group 5 (Table 6.3). The loading components only contributed to 4.2% and 1.6% variance, representing subtle changes in the serum metabolic profiles. Component 2 (Y-axis) loadings are particularly interesting. The differentiating metabolites show an increase in catechol, erythritol, chlorogenate, 5,6-dihydrothymine, cytosine and propionate concentrations and a decrease in desaminotyrosine, glutathione, 2-hydroxyphenylacetate and Nα-acetyllysine.

Table 6.3 Loadings for component 1 (X-axis) and component 2 (Y-axis) for the sPLSDA generated using MetaboAnalyst 5.0 R package.

Compound Name	Component 1 (4.2%)	Component 2 (1.6%)	Metabolic Family
N-Acetylglutamate	-0.87	0.00	Amino Acids, Organic Acids
Glucuronate	-0.34	0.00	Monosaccharides and Disaccharides
Kynurenine	-0.23	0.00	Amines, Amino Acids, Aromatics, Keto Acids
Allantoin	-0.19	0.00	Amines
2-Octenoate	-0.16	0.00	Fatty Acids
UDP-glucose	-0.13	0.00	Monosaccharides and Disaccharides, Nucleotides and Nucleosides
Hippurate	-0.06	0.00	Amino Acids, Aromatics
Galactarate	-0.03	0.00	Alcohols and Polyols, Organic Acids
Anserine	-0.01	0.00	Amino Acids, Amines, Aromatics
Citrate	-0.01	0.00	Organic Acids
Catechol	0.00	0.72	Phenols
Erythritol	0.00	0.45	Alcohols and Polyols
Desaminotyrosine	0.00	-0.30	Organic Acids, Phenols
Glutathione	0.00	-0.29	Amino Acids, Sulfur Compounds
Chlorogenate	0.00	0.17	Alcohols and Polyols, Organic Acids, Phenols
5,6-Dihydrothymine	0.00	0.16	Purines and Pyrimidines
Cytosine	0.00	0.13	Purines and Pyrimidines
Propionate	0.00	0.13	Fatty Acids
2-Hydroxyphenylacetate	0.00	-0.11	Organic Acids
Nα-Acetyllysine	0.00	-0.02	Amines, Amino Acids

Correlation coefficient modelling revealed five metabolites to positively correlate with increased body weight (Fig. 6.11A), whilst seven metabolites negatively correlated with FCR (Fig. 6.11B), which are the appropriate correlations for the performance metric. Catechol positively correlated with body weight and negatively correlated to FCR, which may make it a potential serum metabolic biomarker for efficient performance. Catechol was also a metabolite with a higher concentration in the eight broilers with a distinct profile from the sPLSDA analysis (Fig 6.10, Table 6.4). Nα-acetyllysine, glutathione, 2-

hydroxyisocoporate and pyridoxine shared a negative correlation coefficient to body weight and a positive correlation to FCR; therefore, they could also be used as markers of inefficient performance.

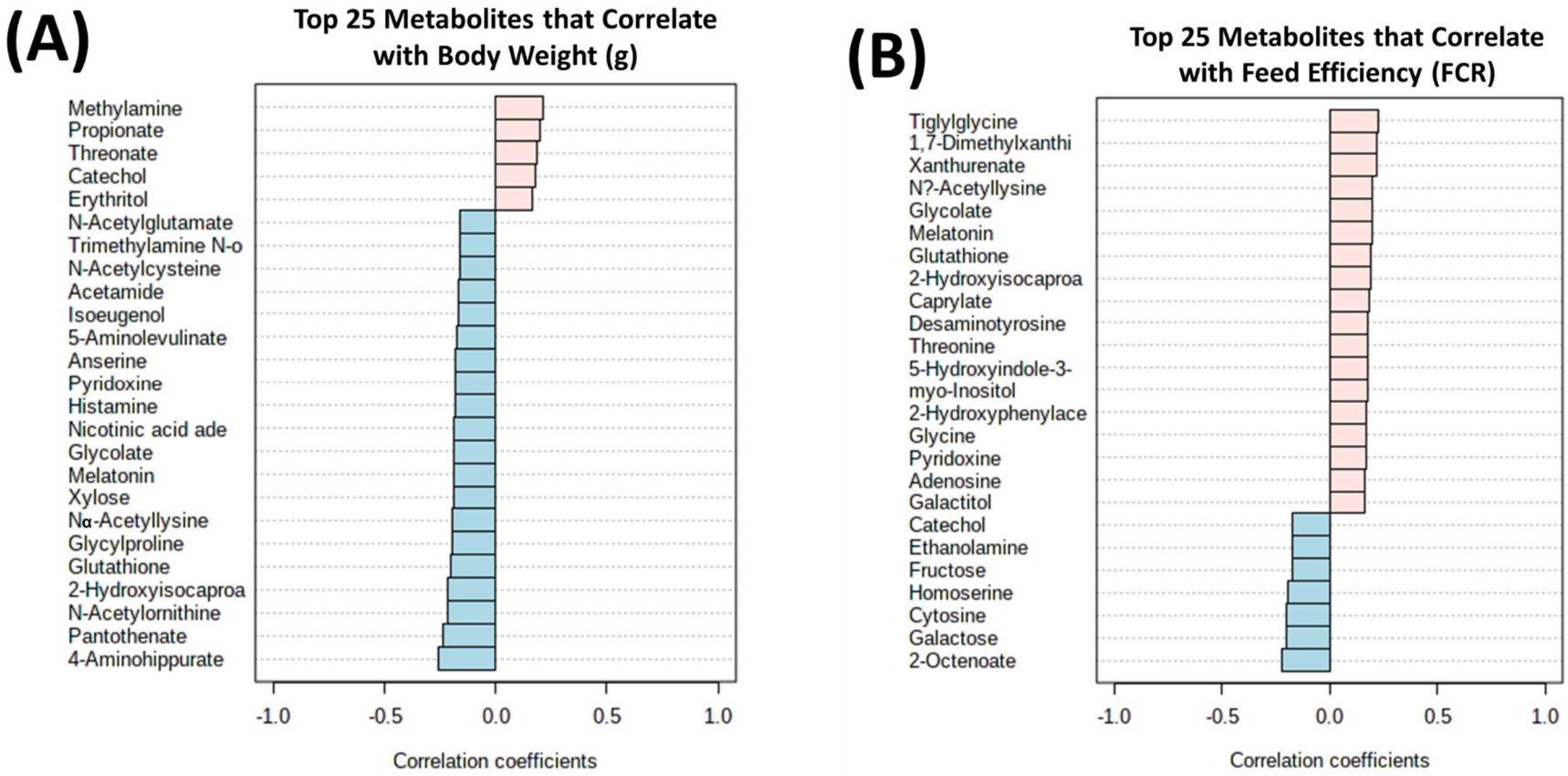


Figure 6.11 Visualisation of normalised (auto-scaling) metabolite concentrations that correlate to **(A)** body weight (g) and **(B)** feed efficiency (FCR), generated with the MetaboAnalyst 5.0 R package. Compound names are on the Y-axis, and Spearman's rank correlation of coefficients is along the X-axis.

To further determine which metabolites were associated with performance parameters, using a mixed linear regression modelling, 24 metabolites significantly correlated with body weight and FCR (Fig 6.12, Table 6.6). Notably, catechol, erythritol, Nα-acetyllysine and glutathione were all significantly correlated, which were four of the metabolites that contribute to the distinct serum metabolite profile from the sPLSDA analyses (Fig. 6.10, Table 6.4) and shared correlation coefficients with body weight and FCR performance metrics (Fig. 6.11). As identified in the correlation coefficients analyses, short-chain fatty acid propionate was increased in the serum metabolite profile from the sPLSDA analyses (Fig. 6.10, Table 6.4) and positively correlated with performance metrics in the mixed linear regression model (Fig. 6.12).

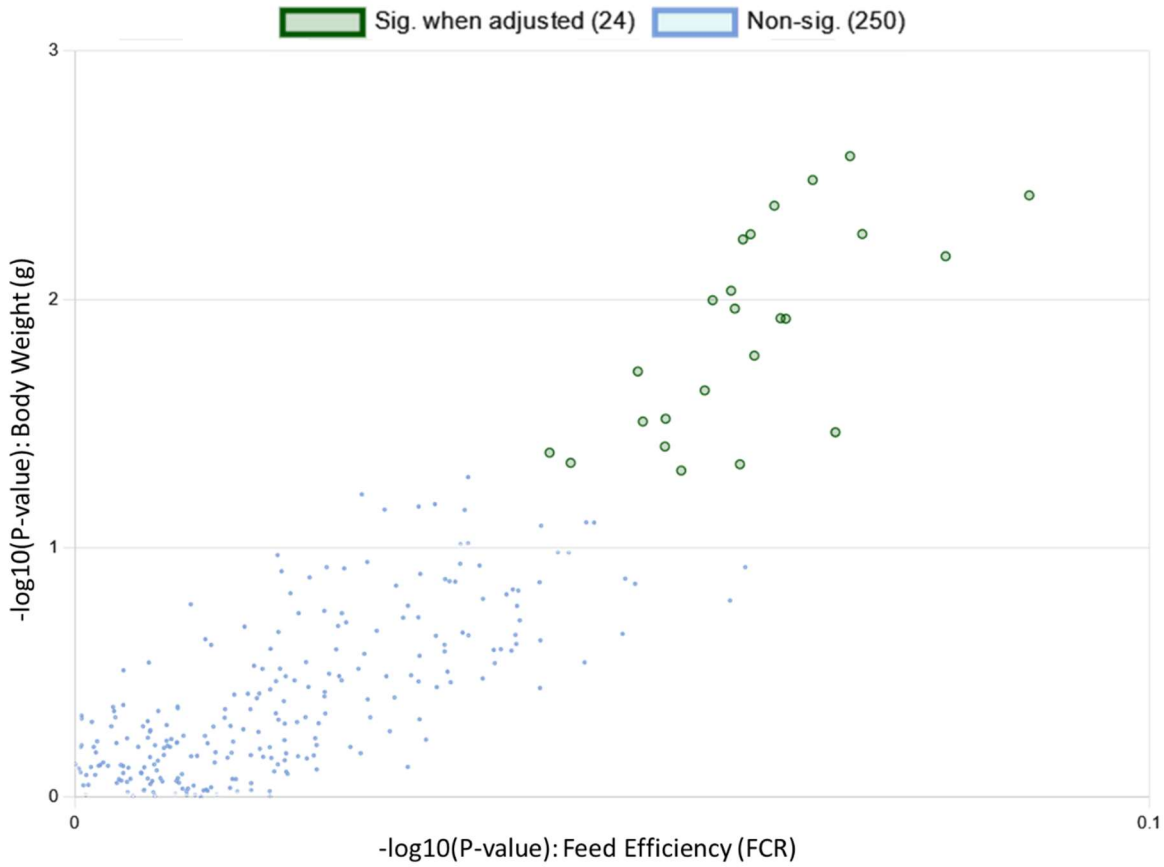


Figure 6.12 Scatter plot exhibiting a mixed linear regression model (limma) based on the body weight (g) along the X-axis and applying feed efficiency (FCR) as the covariate on the Y-axis, generated in MetaboAnalyst 5.0 R package. Green circles highlight the 24 metabolites that correlate to body weight when adjusted for feed efficiency.

Table 6.4 Details the 24 metabolites that show a significant correlation with body weight (g) and feed efficiency (FCR) as a continuous variable, generated using a linear regression model (limma) in the MetaboAnalyst 5.0 R package.

Compound Name	P value	Metabolic Families
2-Hydroxyglutarate	0.045	Organic Acids
2-Hydroxyisocaproate	0.003	Organic Acids
4-Aminohippurate	0.004	Amino Acids, Amines, Aromatics
5-Aminolevulinate	0.023	Amines, Keto Acids
Acetamide	0.030	Amines
Carnitine	0.041	Amines, Organic Acids
Catechol	0.010	Phenols
Erythritol	0.039	Alcohols and Polyols
Glutathione	0.003	Amino Acids, Sulfur Compounds
Glycolate	0.006	Alcohols and Polyols, Organic Acids
Glycylproline	0.012	Amines, Amino Acids
Histamine	0.011	Amines, Aromatics
Isoeugenol	0.049	Phenols
Melatonin	0.005	Amines, Aromatics
Methylamine	0.034	Amines
Nα-Acetyllysine	0.004	Amines, Amino Acids
N-Acetylcysteine	0.031	Amino Acids, Sulfur Compounds
N-Acetylornithine	0.005	Amines, Amino Acids
Nicotinic acid adenine dinucleotide	0.046	Aromatics, Monosaccharides and Disaccharides, Nucleotides and Nucleosides, Organic Acids
Pantothenate	0.007	Alcohols and Polyols, Amino Acids
Propionate	0.012	Fatty Acids
Pyridoxine	0.009	Alcohols and Polyols, Amines, Aromatics
Trimethylamine N-oxide	0.019	N-oxides
Xylose	0.017	Monosaccharides and Disaccharides

Although there were no significant differences observed in metabolite concentrations between groups (Fig. 6.13), employing sPLSDA ordination analyses facilitated the identification of a distinct serum metabolite profile among eight broilers, with seven exhibiting the most productive and efficient performance characterised by low FCR and high body weight. Coefficient analyses pinpointed metabolites correlating with either body weight or FCR. Meanwhile, linear regression modelling correlated metabolites with body weight and FCR as continuous variables, disregarding the performance groups, like the coefficients analyses. By examining the metabolite profiles, five serum compounds

were contributing to a unique serum metabolic profile that has an association with broiler performance. Elevated levels of catechol, erythritol, and propionate, coupled with reduced levels of glutathione and Nα-acetyllysine, may be associated with improved performance, such as increased body weight and decreased FCR (Fig. 6.13).

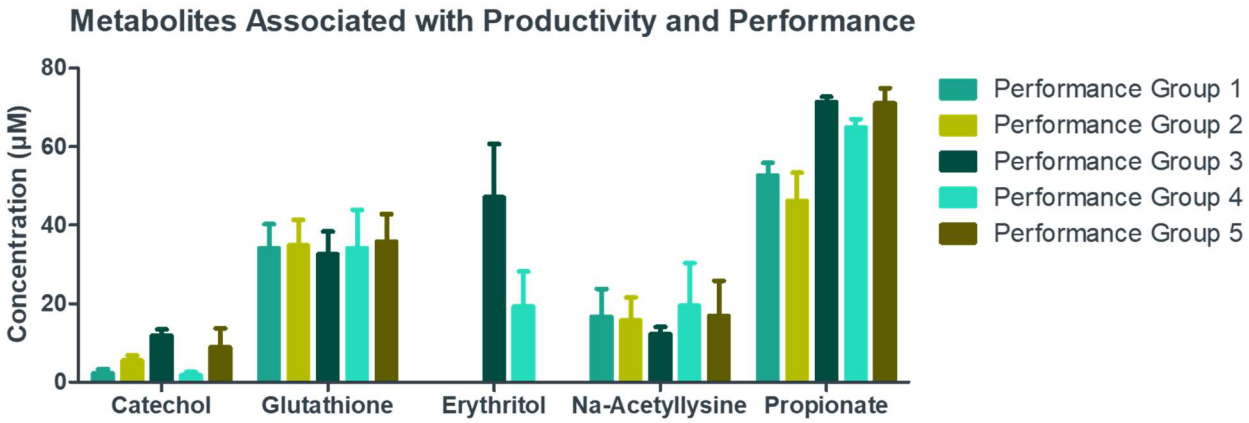


Figure 6.13 Bar graph depicting concentration (uM) and standard deviations of the five metabolites associated with performance. No significant differences were identified (ANOVA, P = >0.05), due to high individual variance between broilers.

Having discerned potential markers in the serum metabolic profile of the most productive and efficient broilers, it is now imperative to investigate the interplay between performance and the serum metabolome concerning the immune system. This entails examining serum natural antibody titres to determine whether the metabolites linked to performance correlate with a more robust immune response by looking at the most and least efficient broilers, performance groups 3 and 4.

6.3.3 Identifying Differences in Natural Antibody Titres of Most and Least Productive, Efficient and Desirable Birds

To assess the effect of performance on serum NAbs, only samples from the most and least efficient broilers (performance groups 3 and 4) were included in this analysis. There was no significant difference in serum antibody titres between the most and least

efficient broilers (Fig. 6.14). Total natural antibody (IgT) titres across all samples had an average of 4.27 (±SD 0.765). Immunoglobin G (IgG) had the highest antibody titres of all NAbs with an average of 1.73 (±SD 0.39), followed by immunoglobin A (IgA) with an average of 1.38 (±SD 0.32). Immunoglobin M (IgM) had the lowest NAb titres, averaging 0.7 (±SD 0.16).

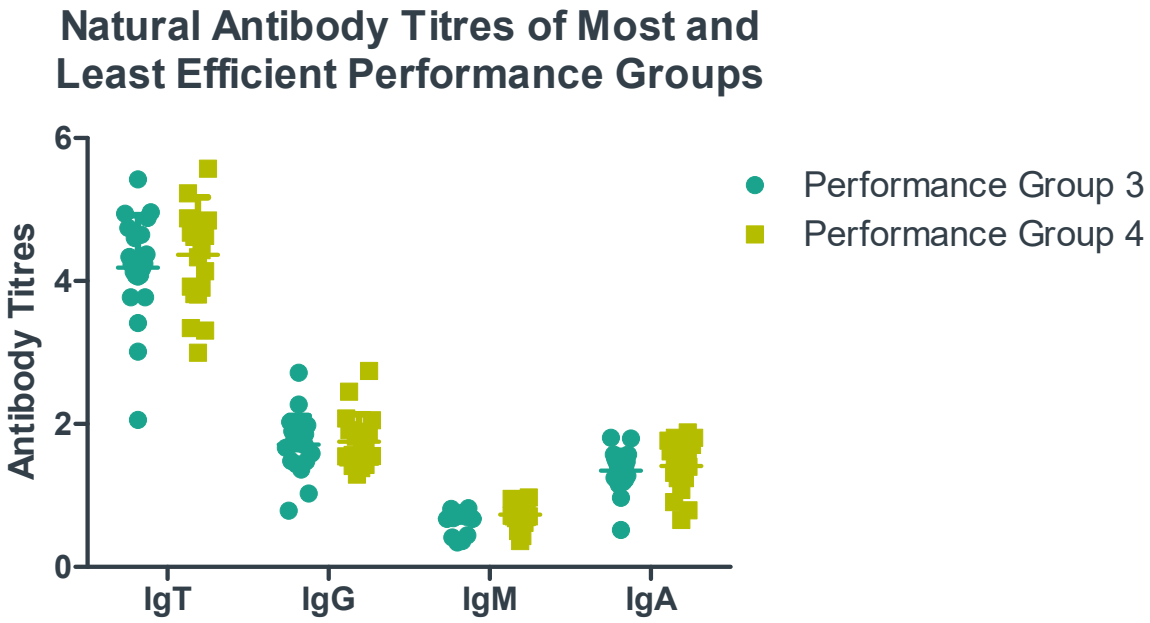
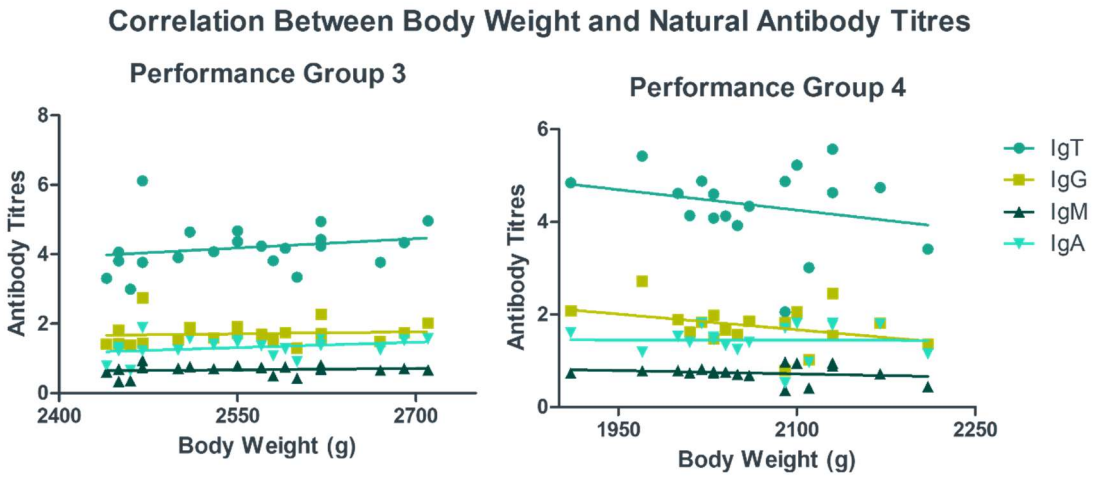


Figure 6.14 Vertical scatter graph of individual antibody titres from performance group 3 and performance group 4.

There was no correlation between the NAb titres and bodyweight (Fig. 6.15A) or feed efficiency (Fig. 6.15B) for either performance group 3 or 4. This suggests no relationship exists between NAb titres and performance parameters in these cohorts.

(A)



(B)

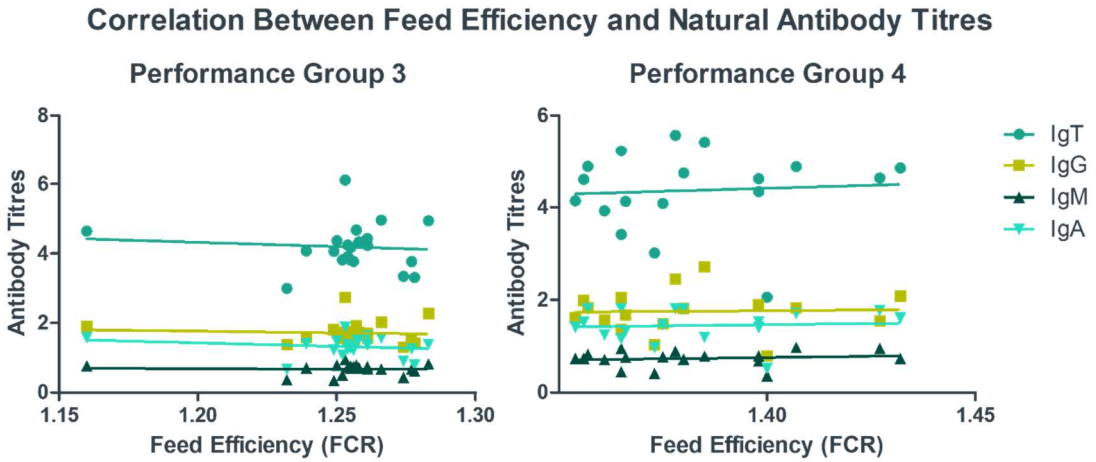


Figure 6.15 Horizontal scatter plot and linear regression models correlating natural antibody titres to (A) feed efficiency (FCR) and (B) body weight.

Each immunoglobin antibody titre showed a significant positive correlation to one another (Fig. 6.16). This implies that individual birds have higher NAb titres, regardless of their performance metrics. Establishing that individual broilers will have all their relative NAb titres elevated is relevant for breeding selection because serum NAb titres are inherited and can be selectively bred for (Berghof et al., 2019).

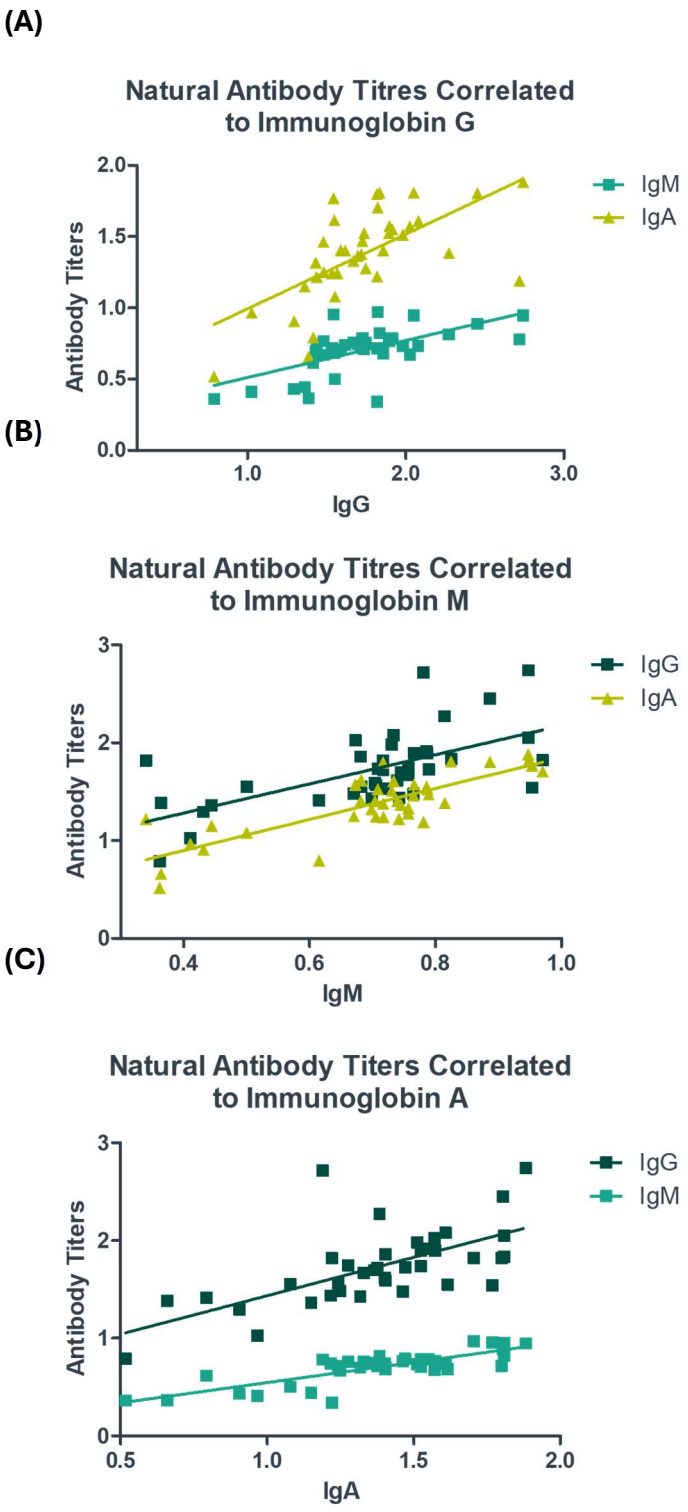


Figure 6.16 Linear regression models correlating individual natural antibody titres: **(A)** IgG significantly correlated to IgM (P=< 0.0001, R^2=0.4) and IgA (P=< 0.0001, R^2=0.4), **(B)** IgM significantly correlated to IgG (P=< 0.0001, R^2=0.4) and IgA (P=< 0.0001, R^2=0.7), **(C)** IgA significantly correlates to IgG (P=< 0.0001, R^2=0.4) and IgM (P=< 0.0001, R^2=0.7).

6.3.4 Correlation of the Serum Metabolites and NAb Titres to Identify Distinguishing Factors Between Most and Least Efficient Broilers at 35 dph

For this segment of the results, focusing on the impact of performance on serum NAbs, only samples from the most and least efficient broilers (performance groups 3 and 4) were incorporated into the analysis. Consequently, the serum metabolomes underwent re-analysis. No significant correlation existed between total serum metabolite concentration and total NAb (IgT) titres (Fig. 6.17A), and no significant correlation was found between any individual (IgG, IgM, IgA) NAb titres and the total serum metabolite concentration (data not shown).

There was no significant correlation between the serum's total number of profile compounds and the IgT titres (Fig. 6.17B). There was also no significant correlation between individual (IgG, IgM, IgA) NAbs titres and the total number of profiled compounds in the serum (data not shown).

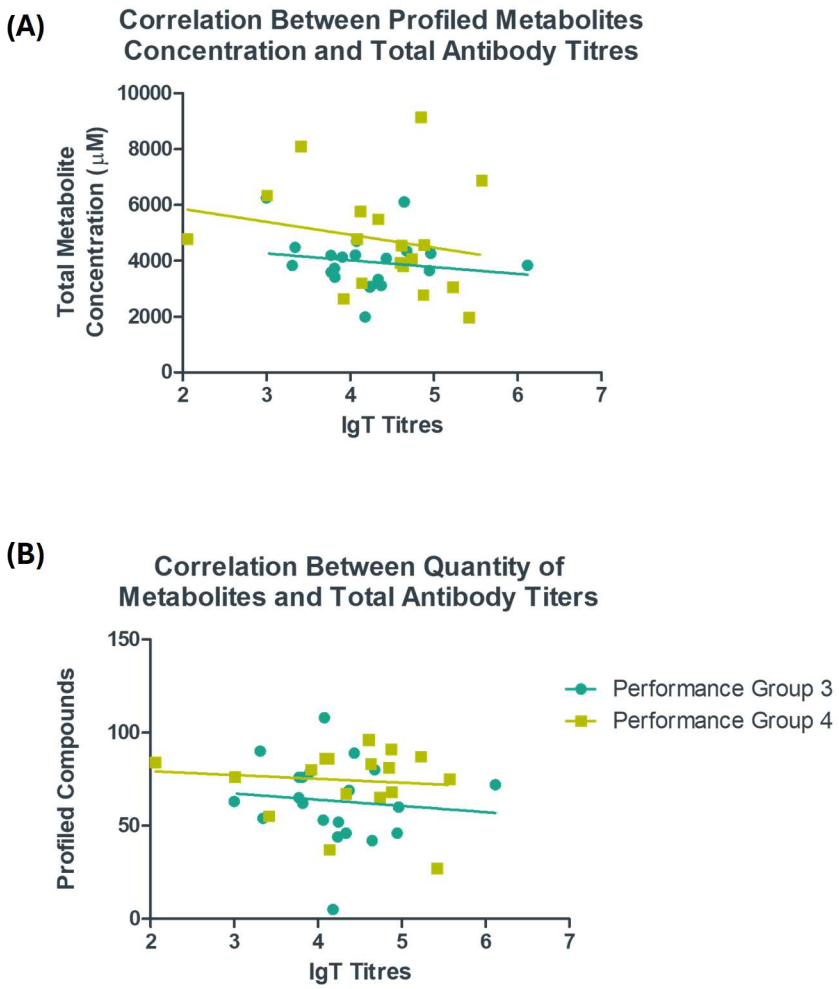


Figure 6.17 Scatter graph and linear regression model assessing the correlation and effect of total natural antibody titres to (A) total metabolite concentration of serum and (B) number of profiled serum metabolites.

There were no significant differences between the five performance groups, as shown in section 6.2.3 Investigating the Untargeted Serum Metabolome Using ^{1}H-NMR. Upon reanalysing the serum metabolomes of performance group 3, representing the most efficient, and performance group 4, representing the least efficient, a total of 209 metabolites were identified in the serum across these 39 samples. In addition to the 29 serum metabolites found in all serum samples of the five performance groups, six additional metabolites are shared between performance groups 3 and 4 (Table 6.5). As with documenting the consistent detection of the 29 metabolites in all broiler serum samples, it is advantageous to identify these six additionally shared metabolites. Identifying these metabolites proves valuable since their consistent detection will be evaluated in upcoming chapters in relation to performance. Moreover, their presence holds significance for fellow

researchers, offering insights into the reliability of detecting these metabolites in broiler serum. Interestingly, three metabolites are sugars utilised by the gut microbiome, and the other three are all nonessential amino acids.

Table 6.5 Details of the additional shared metabolites found in all broiler serum samples at 35 dph in performance groups 3 and 4.

Compound Name	Average Concentration (μM)	±SD	Metabolic Families
Ribose	156.05	69.94	Monosaccharides and Disaccharides
Alanine	82.02	32.38	Amino Acids
Proline	214.56	101.98	Amino Acids
Alloisoleucine	63.32	24.80	Amino Acids, Nucleotides and Nucleosides
Lactose	42.45	25.74	Monosaccharides and Disaccharides
Mannose	43.33	30.84	Monosaccharides and Disaccharides

To analyse the 209 metabolites identified in the serum samples of the most and least efficient broilers, significant differences in the fold change of metabolite concentrations in performance group 3 (most efficient) were compared to performance group 4 (least efficient) to determine any relative differences in individual metabolites. Six metabolites were upregulated, whilst 33 were downregulated when comparing the relative concentration of metabolites in performance group 3 to performance group 4 (Fig. 6.18, Table 6.6), despite the two performance groups having no significant differences in these metabolite concentrations (t-test, P=<0.05). This further supports the idea that the distinctions in the serum metabolome come from the combination of metabolites (i.e., the metabolic profiles) and not the concentration of individual serum compounds. From the five compounds associated with performance (Fig. 6.13), only Nα-acetyllysine was identified as downregulated in group 3 compared to group 4.

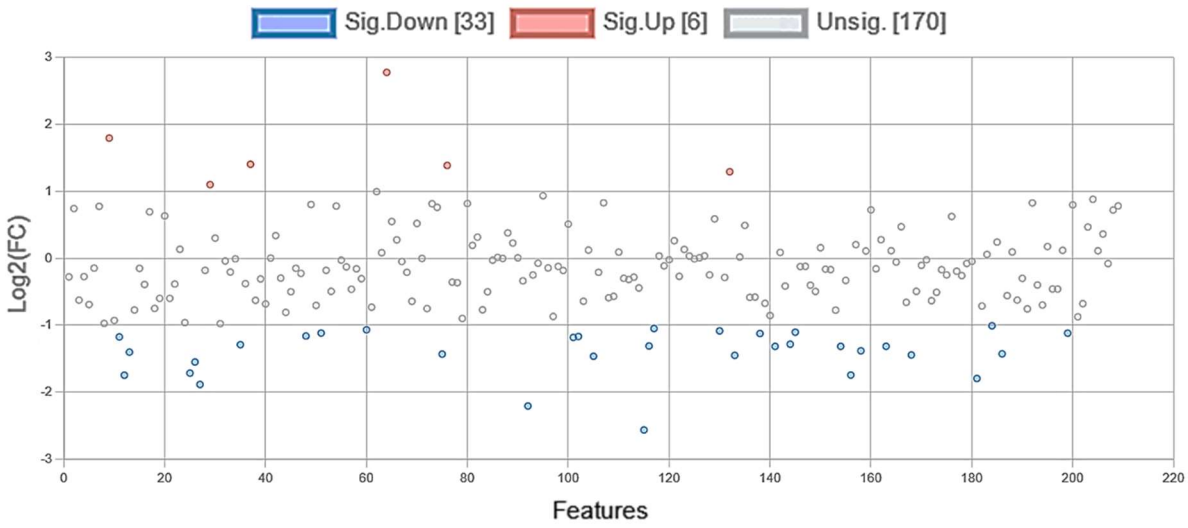


Figure 6.18 Scatter plot visualising the log2-fold change of metabolites from broiler serum of performance groups 3 and 4, generated in MetaboAnalyst 5.0 R package, where red circles are upregulated, and blue circles are downregulated in group 3 compared to group 4.

Table 6.6 List of metabolites and statistical details of fold-change (log2) when comparing the serum metabolome of performance group 3 with performance group 4.

Compound Name	Fold Change	log2(FC)	Trend	Metabolic Families
Chlorogenate	6.86	2.78	Up	Alcohols and Polyols, Organic Acids, Phenols
2-Hydroxy-3-methylvalerate	3.47	1.80	Up	Organic Acids
5,6-Dihydrothymine	2.65	1.41	Up	Purines and Pyrimidines
Ethanolamine	2.62	1.39	Up	Alcohols and Polyols, Amines
Methylamine	2.45	1.29	Up	Amines
3-Methylglutarate	2.15	1.10	Up	Organic Acids
Threonine	0.50	-1.01	Down	Organic Acids, Amino Acids
Isopropanol	0.48	-1.05	Down	Alcohols and Polyols
Carnitine	0.48	-1.07	Down	Amines, Organic Acids
Melatonin	0.47	-1.08	Down	Amines, Aromatics
N-Isovaleroylglycine	0.47	-1.10	Down	Amino Acids
Arabinose	0.46	-1.12	Down	Monosaccharides and Disaccharides
Xanthurenate	0.46	-1.12	Down	Organic Acids, Alcohols and Polyols, Aromatics
N-Acetylglutamate	0.46	-1.12	Down	Amino Acids, Organic Acids
Allantoin	0.45	-1.16	Down	Amines

Glycylproline	0.45	-1.17	Down	Amines, Amino Acids
2-Hydroxyglutarate	0.44	-1.17	Down	Organic Acids
Glycolate	0.44	-1.18	Down	Alcohols and Polyols, Organic Acids
N-Carbamoylaspartate	0.41	-1.28	Down	Amino Acids, Organic Acids
4-Hydroxyphenylacetate	0.41	-1.29	Down	Organic Acids, Phenols
Isoleucine	0.40	-1.31	Down	Amino Acids, Nucleotides and Nucleosides
Pantothenate	0.40	-1.31	Down	Alcohols and Polyols, Amino Acids
N-Acetylornithine	0.40	-1.32	Down	Amines, Amino Acids
Nicotinic acid adenine dinucleotide	0.40	-1.32	Down	Aromatics, Monosaccharides and Disaccharides, Nucleotides and Nucleosides Organic Acids
O-Acetylcholine	0.38	-1.38	Down	Amines
2-Hydroxyisovalerate	0.38	-1.40	Down	Organic Acids
Tiglylglycine	0.37	-1.43	Down	Amino Acids
Ethanol	0.37	-1.43	Down	Alcohols and Polyols
Pyridoxine	0.37	-1.44	Down	Alcohols and Polyols, Amines, Aromatics
Methylsuccinate	0.37	-1.45	Down	Organic Acids
Histamine	0.36	-1.46	Down	Amines, Aromatics
3-Hydroxykynurenine	0.34	-1.55	Down	Amino Acids, Keto Acids, Phenols
3-Hydroxyisobutyrate	0.30	-1.71	Down	Organic Acids
Nα-Acetyllysine	0.30	-1.74	Down	Amines, Amino Acids
2-Hydroxyisocaproate	0.30	-1.75	Down	Organic Acids
Taurine	0.29	-1.79	Down	Amines, Sulfur Compounds
3-Methyl-2-oxovalerate	0.27	-1.88	Down	Keto Acids
Glucose-1-phosphate	0.22	-2.21	Down	Monosaccharides and Disaccharides, Phosphates
Isoeugenol	0.17	-2.56	Down	Phenols

Out of the 209 compounds profiled in the serum of broilers in performance groups 3 and 4, eight metabolites positively correlated to IgT titres (Fig. 6.19). Catechol was the only metabolite negatively correlated to IgT titres (Table 6.7). It was also the only metabolite

to significantly correlate when adjusted for multiple testing, suggesting a robust result. Catechol was previously demonstrated to correlate with efficient performance parameters as part of a profile pattern despite not having a significant fold-change between performance groups 3 and 4. Nα-acetyllysine, positively correlated to IgT, negatively correlated to performance parameters (Fig. 6.11) and was significantly downregulated by a log2 fold change of -1.74 (Fig. 6.18). Another notable metabolite is melatonin, which positively correlates with IgT NAb titres (Fig. 6.19, Table 6.9). This amine was found in all broiler serum samples (Table 6.2), positively correlated with body weight and FCR in a mixed linear regression model (Fig 6.12, Table 6.4) and was significantly downregulated by a log2 fold change of -1.08 (Fig. 6.18, Table 6.6) in performance group 3 compared to performance group 4.

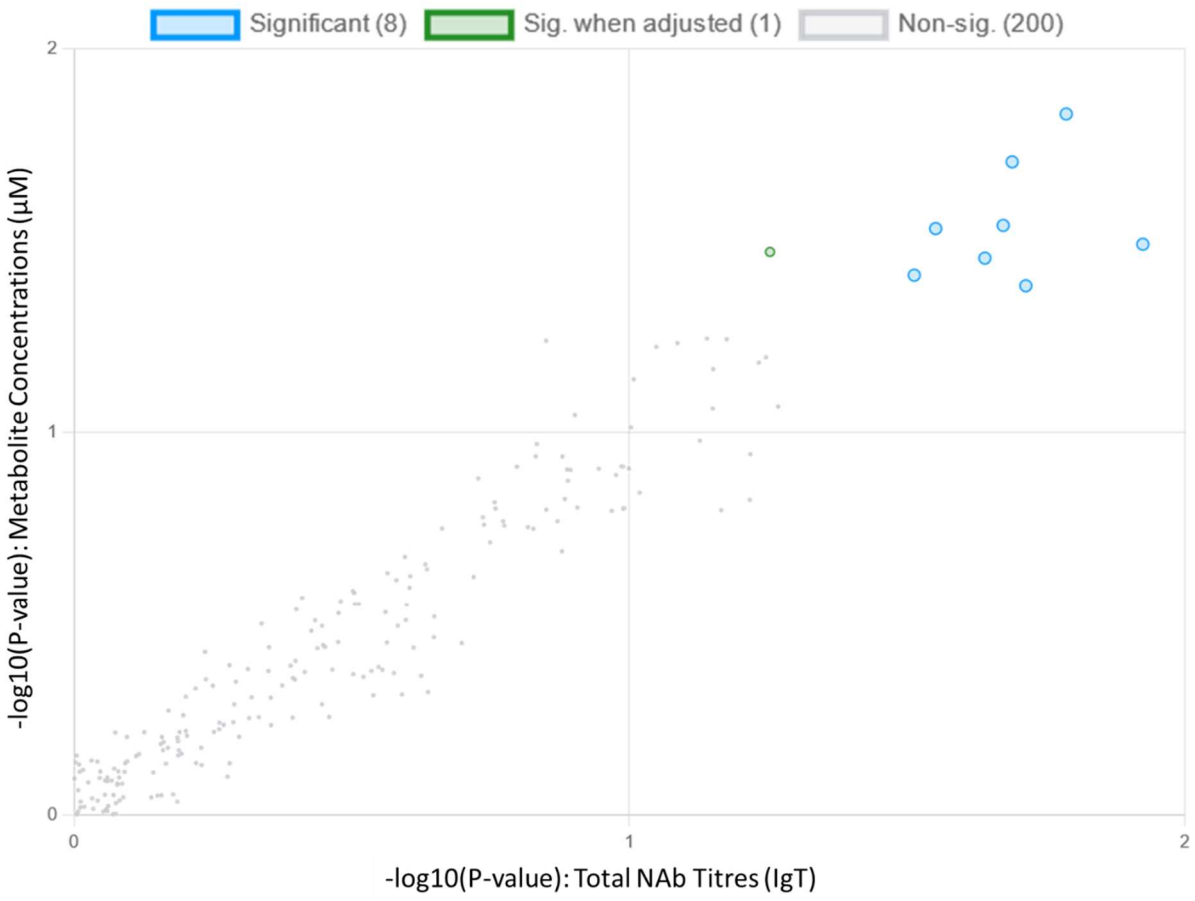


Figure 6.19 Scatter plot exhibiting a mixed linear regression model (limma) based on the significant correlation of serum metabolite concentrations along the Y-axis and total natural antibody (IgT) titres on the X-axis, generated in MetaboAnalyst 5.0 R package. Blue circles highlight the eight metabolites that significantly correlate to elevated IgT titres. The green

circle highlights the single metabolite, demonstrating a significant correlation to IgT titres when adjusted for multiple testing.

Table 6.7 Results from the mixed linear regression model (limma) between individual metabolite concentrations and total natural antibody (IgT) titres, visualised in Fig. 6.19.

Compound Name	Correlation	P Value	Metabolic Families
2-Hydroxyisocaproate	0.79	0.01	Organic Acids
Glycolate	0.76	0.02	Alcohols and Polyols, Organic Acids
Melatonin	0.71	0.03	Amines, Aromatics
Pyridoxine	0.71	0.03	Alcohols and Polyols Amines, Aromatics
Glutathione	0.70	0.03	Amino Acids, Sulfur Compounds
Catechol	-0.69	0.03	Phenols
Nα-Acetyllysine	0.68	0.04	Amines, Amino Acids
Xanthurenate	0.67	0.04	Organic Acids, Alcohols and Polyols, Aromatics
Histamine	0.66	0.04	Amines, Aromatics

The relationship between NAb titres and performance remains ambiguous. Metabolites found to be associated with suboptimal performance metrics, such as low body weight and high FCR (Fig. 6.11), exhibit positive correlations with IgT. These identified metabolites are consistently present in broiler samples, raising the possibility that significant correlations may stem from their abundance, rather than reflecting genuine biological connections. These findings suggest that elevated IgT NAb titres may not support optimal performance.

6.4 DISCUSSION

The most economically advantageous and effective broilers exhibit high body weight and low Feed Conversion Ratio (FCR), indicating their ability to put on weight efficiently with minimal dietary input. Such birds prove cost-effective for farmers, considering that feed constitutes the most considerable expense in poultry farming and delivers a higher meat yield. Conversely, birds with low body weight and high FCR are less desirable, as they consume disproportionate amounts of feed relative to their weight. While FCR is traditionally computed based on body weight, considering both metrics is advantageous for identifying the most efficient and sustainable birds within a flock. Performance group 5, categorised as mid-range broilers, accounted for the highest sample count, indicating that a significant portion of the flock falls outside the optimal productivity and efficiency ranges. Hence, it is crucial to aid flocks in achieving their utmost efficiency and productivity. Identifying alterations in the serum metabolome that differentiate broiler performance will aid commercial production management and enhance breeding selection processes. The standardised practice of collecting blood samples from poultry to evaluate health status and genetic integrity (as well as the increased accessibility to assess blood biochemistry, such as automatic analyser (Rezende et al., 2017, Lebedev et al., 2023) makes serum sampling advantageous to introduce parallel assessments of production parameters, such as body weight and feed efficiency. Evaluating the serum metabolome with untargeted ^{1}H-NMR permits a broad investigation into many compounds relevant to gut health while the same serum samples can be used to investigate natural antibody (Gilroy et al.) titres with performance.

6.4.1 The Broiler Serum Metabolome at 35 dph

The five cohorts had no significant difference between the total number of metabolites or the total metabolite concentration. Furthermore, there was no significant difference in the presence or concentration of individual metabolites between the five performance groups. This was also seen in dietary intervention studies where there was no difference in serum total protein despite observable differences in performance parameters (Zhang et al., 2009). This demonstrates that the serum metabolome is consistent and stable

within a flock at 35 dph. Twenty-nine metabolites were consistently profiled within all 200 broiler serum samples.

Of all 247 profiled compounds, 22.3% were attributed to organic acids. Of the 29 consistently identified metabolites, these were attributed to 13 metabolic families, with 41.1% of the profiled compounds being amino acids, making it the most detected metabolic family. This was followed by amines and organic acids (compromising of hydroxy and carboxyl acids), each responsible for 24.1%. This implies that amino acids can be consistently identified in the serum or broilers, which is beneficial for nutritional studies.

In addition to categorising metabolic families, the serum metabolites were attributed to 17 metabolic pathways, with the majority falling within carbohydrate metabolism and the phenylalanine and tyrosine metabolism pathway. The ketone body metabolism and purine and pyrimidine metabolism pathways were similarly well represented by the serum metabolites. This underscores the potential of using broiler serum to monitor and assess metabolic processes. Other studies that detected metabolic pathways in the serum found significant differences in primary bile acid biosynthesis and taurine metabolism (Zhou et al., 2020). They also revealed that heat dissipation disorder may be due to arginine, proline, and histidine metabolism (Zhou et al., 2020). This suggests the ability to utilise serum metabolome pathways as targets for understanding the impact of adverse environments on broiler performance and health.

Lactate and glucose had the highest compound concentrations found in all 200 serum samples but showed no correlation to performance parameters or NAb titres. Lactate is produced by lactic acid bacteria in the gut, a dominant and essential commensal in the broiler intestinal microbiome that protects against pathogens and stimulates the immune system (Pokorná et al., 2019). It had been hypothesised that elevated lactate in the serum would be a suitable biomarker for intestinal permeability in chickens. However, no significant link was found between serum lactate and intestinal permeability (Gilani et al., 2017). However, elevated serum lactate was detected in broilers supplemented with dietary butyrate glycerides (Yang et al., 2018). Glucose has been suggested as a biomarker to evaluate seasonal variation between male and female broilers (Akhavan-Salamat and Ghasemi, 2016). High-fat diets increase body weight and serum glucose in broilers (Lebedev et al., 2023), whilst feed restriction does not affect serum glucose (Metzler-Zebeli et al., 2019). Broilers fed on fermented cottonseed meal had higher serum glucose concentrations

despite significantly reduced fat content and subcutaneous fat thickness (Niu et al., 2019). Glucose is essential for all cellular processes. Increasing dietary carbohydrates is known to increase the expression of mRNA glucose transmitters, thereby permitting greater glucose absorption in the intestine (Omar et al., 2021).

Although no statistically significant differences were found between the five performance groups, there was a difference in the metabolite profile of seven birds from performance group 3 (the most efficient), which had high body weight and low FCR, and one sample from performance group 5, the mid-range broilers. The differences in the metabolite profiles of these eight broilers only accounted for a subtle change but the pattern provided a potential serum marker for broiler performance and gut health.

6.4.2 Metabolites as Potential Markers of Performance

Catechol serum concentrations were increased in the eight broilers with a distinct metabolic profile. Catechol also positively correlated with body weight and is negatively correlated with FCR coefficients. When corrected for feed efficiency, this phenol was also found to correlate positively with broiler body weight in a mixed linear regression model. Still, it was the only compound negatively correlated with total immunoglobin NAb titres. This suggests that elevated IgT antibody titres may indicate stress, as catechol has been linked to stress and adrenalin in poultry (Cheng and Muir, 2007). Catechol is also a precursor for TMAO (Shehata, 2022). Trimethylamine is produced by the gut microbiome (Cartman et al.), converted to TMAO in the liver (Shehata, 2022), and then detected in the serum. TMAO was found in all 200 serum samples, along with betaine, another dietary precursor for TMAO (Shehata, 2022). There was a significant positive correlation between the serum concentration of TMAO and broiler body weight when FCR is a variable. Increased serum levels of TMAO were detected when the broiler's diet was supplemented with butyrate glycerides (Yang et al., 2018), although no performance parameters were reported.

Propionate is a short-chain fatty acid and was found to be increased in the eight broilers with a distinct metabolic profile. It was one of the metabolites found in all 200 broiler serum samples and significantly correlated to body weight when corrected for feed efficiency in a mixed linear regression model. Dietary supplementation with sodium propionate has been demonstrated to reduce feed and calorific intake and increase

microbial diversity despite elevated propionate not being detectable in the serum (Li et al., 2021a).

Erythritol is a four-carbon (or alcohol) sugar and is resistant to fermentation by gut bacteria (Arrigoni et al., 2005). As well as being increased in the eight broilers with a distinct metabolic profile, erythritol was positively correlated with body weight using feed efficiency as a variable. This polyol is produced by yeast fermenting glucose (Roboz et al., 1984). Minimal research into the effects of erythritol on broiler gut health and performance has occurred except for significant utilisation of i-erythritol by gut microbes of chickens kept in a low altitude environment (Bhagat et al., 2023). Additionally, erythritol in the yolk and albumen content of layers was significantly higher when fed a fermented diet (Goto et al., 2019). When supplemented with erythritol, mice with metabolic disorders demonstrated increased short-chain fatty acid production and modulation of innate immunity, resulting in reduced intestinal inflammation (Kawano et al., 2021).

The serum metabolic profiles of the eight broilers showed an increase in chlorogenate. This has been shown to improve immune and antioxidant capacity and promote animal growth and development in 15 dph chicks (Hu et al., 2021). This phenol was also upregulated when comparing performance groups 3 to 4.

Pyrimidine 5,6-dihydrothymine is an intermediate breakdown product of thymine. This compound was increased in the eight broilers with a distinct metabolite profile and efficient performance characteristics and was upregulated when comparing performance groups 3 to 4. Cytosine is also a pyrimidine and was also increased in the eight broilers with a distinct metabolite profile. Combined with other nucleosides, cytosine supplementation has improved performance and enhanced gut development in broilers (Villavan et al., 2021). Cytosine has also been reported to be downregulated in the caecal metabolome of heat-stressed chickens (Liu et al., 2022).

Desaminotyrosine is an organic acid and phenol found to be reduced in the serum metabolome profile of the distinguishing eight efficient broilers. It was also present in all 200 broiler serum samples. This compound was also downregulated in the caecal metabolome of hydrogen sulfide-exposed 180 dph chickens, which also had significantly lower body weight (Zhou et al., 2023).

Glutathione has been proposed as an antioxidant marker and is known to have a hepatoprotective effect (Mikulková et al., 2019). It has also been demonstrated to positively

correlate with broiler body weight (Enkvetchakul and Bottje, 1995) and heavier broilers, indicating increased glutathione peroxidase activity (Mikulková et al., 2019). This amino acid and sulfur compound was found in all 200 broiler serum samples. Still, it was downregulated in the sPLSDA loadings that helped to distinguish the eight broilers with a distinct metabolic profile. Despite this subtle variation, glutathione was additionally found to correlate positively with body weight, which aligns with what has been published in the literature.

Nα-actellysine is an amino acid, amine, and lysine derivative. It had a lower concentration in the serum metabolic profiles of the eight distinct broilers and was downregulated when comparing performance groups 3 to 4. Like glutathione, it was significantly correlated to increased body weight in the mixed linear regression model but negatively correlated to the body weight coefficient and positively correlated to the FCR coefficient suggesting that it is a potential marker for inefficient performance.

The compound 2-hydroxyphenylacetate is an organic acid. It was found to have a lower concentration in the serum of the eight broilers identified as having a distinct metabolic profile relating to their performance. It was also negatively correlated to the body weight coefficient and positively correlated to the FCR coefficient. It is a naturally occurring product associated with intestinal bacterial overgrowth in humans and has also been proposed as a metabolic marker when examining feather pulp in poultry (Brown et al., 2022). Alongside glutathione and Nα-actellysine, this metabolite may help predict inefficient performance.

Taurine is the most common bile acid conjugate in the chicken intestinal tract and was found to be downregulated when comparing performance groups 3 to 4. Taurine has been identified by ^{1}H-NMR in the caecal content of chickens with dysbacteriosis, suggesting that the taurine travelled from bile acid deconjugation in ilea (where decreased overall bile acid was detected in birds with dysbacteriosis) or was released by further deconjugation of bile acids in the caeca (Bailey, 2010). Taurine was the only serum metabolite detected in chickens with reduced feed intake, independent of individual physiological influences (Metzler-Zebeli et al., 2019). However, I saw no correlation between serum taurine concentrations and performance metrics or NAb titres. The only other bile acid profiled in this study was cholate. There was no statistical significance linking cholate to body weight and feed efficiency.

Melatonin is an amine and an aromatic, but most notably, it is implicated in the chicken circadian rhythm (Apeldoorn et al., 1999). This compound profiled in all 200 serum samples was significantly correlated to body weight when FCR was a variable. This serum metabolite was downregulated when comparing performance groups 3 to 4 and positively correlated with IgT NAb titres. Melatonin supplementation improved broiler growth (Fathi et al., 2023) and intestinal morphology but did not affect feed efficiency (Akbarian et al., 2014).

6.4.3 Describing the Natural Antibody Landscape of 35 dph Female Broilers

There was no significant difference between antibody titres based on performance groups or continuous performance values. As expected, IgG had the highest antibody titre. Unexpectedly, IgA had the second-highest antibody titre, followed by IgM. IgM is linked to B cell development (Kaspers and Schat, 2012); these broilers are younger, at only 35 dph, compared to the 16-week layers used by Berghof et al. (2018) that this assay was developed for, and they may have a less developed adaptive immune system. The IgT titres were lower than reported in layers but still within range of what Berghof et al. reported (summarised in **Table 6.8**). Conversely, another study associated higher IgM titres with slow-growing, high FCR broilers (Sarrigeorgiou et al., 2023), which supports the lower IgM findings in this study of fast-growing broilers.

Table 6.8 Comparison of female chicken serum natural antibody titres in this chapter and published work.

	IgT	IgG	IgM	IgA
Berghof et. al (2015)	7.3 Range: 5.4-9.5	6.9 Range 5.0-9.3	7.9 Range 6.1-9.7	6.6 Range 4.6-8.6
Schuitemaker (2024)	4.27 Range 2.05-6.12	1.73 Range 0.79-2.74	0.7 Range 0.34-0.97	1.38 Range 0.52-1.88

6.4.4 Correlating Natural Antibody Titres and the Serum Metabolome

A total of nine metabolites correlated with the serum IgT NAb titres. These were catechol, xanthurenate, pyridoxine, glutathione, Nα-acetyllysine, 2-hydroxyisocaproate,

glycolate, histamine and melatonin. Other than Xanthurenate, all the metabolites correlated with IgT NAb titres were found to correlate with body weight and FCR in a mixed linear regression model, as discussed above. Eight of these metabolites positively correlated to IgT NAb titres, whilst only catechol demonstrated a negative correlation. All eight metabolites positively correlated with IgT were downregulated in the serum of performance group 3 compared to performance group 4.

Xanthurenate is an intermediate metabolic product excreted in pyridoxine-deficient animals and is known to reduce chickens' appetites (Daghir, 1976). Catechol was the only metabolite to correlate to IgT NAb titres negatively. This metabolite was previously demonstrated to correlate with efficient performance metrics. Increased catechol and decreased glutathione and Nα-acetyllysine contributed to the distinct serum metabolome of the eight efficient broilers.

These results indicate that increased IgT NAb titres are not conducive to broiler performance.

6.4.5 Summary and Concluding Remarks

Multiple metabolic markers, compounds and pathways were identified in broiler serum at 35 dph. Notably, 29 metabolites were consistently profiled in all 200 serum samples. A subtle variation in metabolite profiles of eight birds was linked to efficient performance parameters, achieving the first aim of using the serum metabolome to distinguish between performances. Compounds negatively correlated to FCR and positively correlated to body weight were further identified and validated using mixed linear regression modelling. These results indicated a profile of five metabolites that distinguished the most efficient broilers: increased catechol, erythritol and propionate, reduced Nα-acetyllysine and glutathione.

The second objective was to investigate NAb titres in 35 dph broilers with performance. Relative NAb titres were lower than reported in layers, possibly due to differences in age and subsequent immunological development. However, there were no significant differences between the NAb levels between the most and least efficient broilers, performance groups 3 and 4.

The final objective was to examine the relationship between NAb titres, the serum metabolome, and overall production parameters, aiming to elucidate potential connections and correlations among these factors. Nine metabolites were reported to correlate to IgT NAb titres. Interestingly, elevated IgT NAb positively correlated to metabolites associated with inefficient performance, whilst catechol negatively correlated to IgT but was associated with efficient performance. This demonstrated that IgT NAb titres are not associated with better performance.

Regarding gut health, the prominent serum metabolites included catechol, betaine, and TMAO, all linked to the microbial metabolism of TMA. Additionally, propionate, a short-chain fatty acid generated through bacterial fermentation in the caeca, and erythritol, a carbon sugar resistant to bacterial fermentation but produced via yeast fermentation of glucose, are relevant to poultry gut health management. Future research endeavours should delve into detecting these metabolites in the serum alongside alterations in the bacterial and fungal compartments of the gut microbiome.

Additional serum metabolites deserving deeper scrutiny include desaminotyrosine, glutathione, Nα-acetyllysine, 2-hydroxyphenylacetate, taurine, and melatonin. These serum markers, alongside their correlations with IgT NAb titres, may hold greater significance in selecting breeding stock rather than in commercial broiler production because they are an inheritable trait (Berghof et al., 2015b).

I hypothesised that I would see increased NAb titres in the most efficient birds, but the hypothesis was disproven. It may be a case of energy usage; for example, if the bird's body uses energy to generate NAbs, less power can be directed to performance metrics to improve efficiency. The other hypothesis was that either a high concentration or more profiled metabolites would be detectable in the serum of the least efficient birds due to increased gut permeability. Still, there was no significant correlation, disproving the hypothesis.

Despite no clear relationship between NAb titres and performance, Ig have a significant positive correlation. This demonstrates that individual birds in a flock can have higher NAb titres, regardless of performance. Natural antibody titres are genetically linked and inheritable traits (Berghof et al., 2015b), making them a potential marker for breed selection to introduce more immunologically robust stock.

The following chapters will integrate these serum metabolites with the gut microbiome composition and faecal metabolome to better understand the metabolic processes between the gut, serum, and performance. Also, looking over time to see how the serum metabolome, NAb titres, and gut microbiome interact and develop alongside performance will provide a more robust application as a marker for performance and gut health.

CHAPTER 7

DEFINING PERFORMANCE AND GUT HEALTH OF TWO GENOTYPES THROUGHOUT THE BROILER LIFE CYCLE

7.1 INTRODUCTION

In **CHAPTER 4**, two distinct genotypes are examined at 7 dph. Such genotype comparisons offer invaluable insights into scientific inquiry, providing clear and well-defined contrasts that facilitate the identification of underlying factors contributing to variations in these traits. Genetics plays a significant role in shaping GI microbial communities (Chintoan-Uta et al., 2020, Butler, 2010) influencing immunity (Van Der Klein et al., 2015) and productivity (Kapell et al., 2012a, Barbosa et al., 2017). While environmental factors have the greatest influence over the gut microbiome (Rothschild et al., 2018), commercial chicken production typically controls variables such as temperature, lighting, diet, and bedding materials. These comparisons provide essential insights into the genetic basis of performance and gut health, informing breeding programs and genetic selection strategies.

The broiler life cycle begins with hatchlings emerging in a controlled hatchery environment and being transferred to the farm at 1 dph (Jurburg et al., 2019). Commercial broilers follow a carefully curated 3-staged feeding program, tailored with varying nutrient compositions to support each stage of the life cycle (Fig. 7.1). For instance, at 7 dph broiler are fed a starter diet. Their GI microbiome features composition dominated by *Lactobacilli* and *Enterococci* succeeded by rapid colonisers such as *Escherichia/Shigella* and *Streptococcus* from 4-10 dph, followed by the introduction of fast-growing taxa like *Ruminococcus*-like species (Jurburg et al., 2019). These well-documented successional changes in the microbiome play a crucial role in immunological development (Bar-Shira et al., 2003), with early microbial colonisation exerting a lasting impact on microbiome structure (Awad et al., 2008, Ramírez et al., 2020, Richards-Rios et al., 2020b). Transitioning to the grower diet at 10 dph marks a pivotal stage, with a generally stable microbiome and metabolome observed from around 20 dph (Jurburg et al., 2019), coinciding with the introduction of the finisher diet at approximately 23 dph. Understanding the rapid and essential successional changes of the microbiome is paramount. Yet, it remains unclear at what age it is optimal to introduce markers for performance or gut health or when interventions would be most effective. Clarifying these aspects holds significant implications for optimising broiler health and productivity throughout the life cycle.

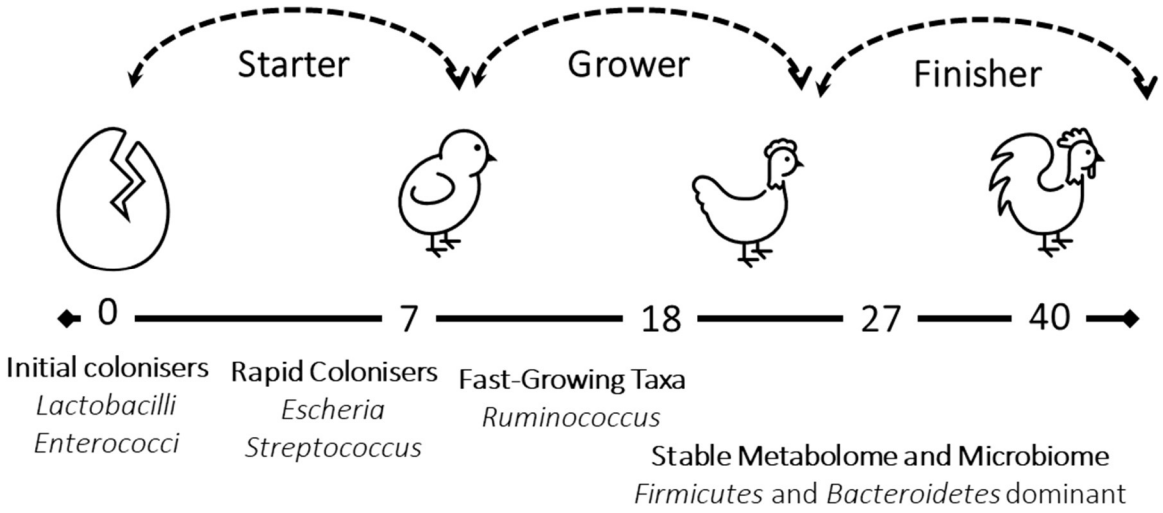


Figure 7.1 Illustration depicting the broiler life cycle, highlighting the staged feed program and well-documented microbiome successional changes.

Body weight and weight gain (BWG) are metrics for assessing broiler biological efficiency in this thesis, yet they do not fully capture feed efficiency. Evaluation of SI morphological structures can contribute to determining gut health (Zhang et al., 2015(De Verdal et al., 2010), development, and function (Yamauchi and Tarachai, 2000, YAMAUCHI, 2007). High faecal water retention can signify diarrhoea, leading to dehydration, electrolyte imbalance and nutrient malabsorption (Pan and Yu, 2014, Roberts et al., 2015). Diarrhoea models, though old-fashioned, remain a well-established method, especially for studying pathogens causing foodborne illnesses (Ruiz-Palacios et al., 1981, Sanyal et al., 1984). However, factors not relating to gut health, such as increased water intake, may also be responsible for faecal water content. Maintaining a balanced water content in faeces is insightful for overall GI health. Combining these biological efficiency metrics with analyses of faecal microbiome, mycobiome, metabolome compositions, and serum Ig and serum metabolome profiles will help to better understand the underlying causes of gut health and performance differences between individual broilers or genetic lines.

Conducting a serial time point study involving repeated collections from the same chickens at different ages offers numerous advantages in scientific research. Firstly, it provides valuable longitudinal insights by allowing researchers to observe changes over time within individual subjects, enabling the detection of temporal trends and age-related variations. Additionally, this approach reduces variability between subjects, enhancing the statistical power of the study and facilitating the detection of subtle changes or

associations. Serial sampling enables the examination of individual-specific responses and dynamics, offering insights into personalised variations in biological processes. Furthermore, it allows for exploring temporal relationships between genotype, age, and diet, elucidating potential causal relationships or dynamic interactions. These serial time point studies provide a comprehensive and dynamic approach to investigating biological processes, optimising resource allocation, and facilitating a deeper understanding of developmental trajectories, temporal dynamics, and individual variations.

7.1.2 Aims and Objectives

The primary aims and objectives of this chapter are as follows:

1. Investigate bacterial successional changes described in the literature, analyse their correlation with faecal and serum metabolites, and explore their relationship with the faecal mycobiome throughout the broiler life cycle.
2. Identify performance markers utilising weight measurements and BWG assessments.
3. Examine markers of gut health by evaluating intestinal structure and morphology and faecal water retention.
4. Determine the optimal age for detecting markers indicative of performance.
5. Assess the applicability of performance markers identified in previous chapters.

Hypotheses

The hypotheses address broiler performance and gut health facets, integrating literature findings with empirical data. Firstly, bacterial successional changes in broilers are hypothesised to align with established patterns, accompanied by alterations in faecal metabolites and correlated variations in the faecal mycobiome. Performance markers identified in previous chapters are anticipated to be applicable and consistent when tested in a new cohort of broilers. Additionally, faecal bacterial microbiome diversity (shotgun metagenomics) is expected to correlate with fungal mycobiome diversity (ITS1 rRNA metabarcoding), reflecting the complex interplay between microbial communities in the

broiler gut. Broilers with good performance are hypothesised to exhibit favourable gut health characteristics, including increased body weight, BWG, a high villus-to-crypt ratio, and low water retention. Similarly, broilers with good performance and gut health are expected to demonstrate higher microbial richness and diversity.

7.2 METHODS

7.2.1 Study Design and Sample Collection

In brief, as described in Chapter 2: General Methods, shotgun metagenomic sequencing for microbiome and functional analysis, metabarcoding sequencing for mycobiome (Methods 2.3–2.4), statistical analyses (Methods 2.5), and metabolomic analyses on faeces and serum (Methods 2.6) were conducted.

Fifty broilers of mixed sex, representing two genotypes previously examined in CHAPTER 4, were randomly chosen at 7 dph. Weight measurements were recorded throughout the broiler life cycle at specified intervals. A staged feeding regimen was implemented, wherein broilers were fed a starter diet from 0 to 10 dph, a grower diet from 10 to 23 dph, and finisher diet from 23 to 40 dph. Sample collection was conducted at least 4 days after each dietary transition to allow for microbiome and metabolome adaptation and minimise dietary changes' effects.

The twenty-five broilers from each genetic strain were housed in an open shed environment and randomly selected for sample collection in October 2022. Faecal collections were performed at 7 dph, 18 dph, and 27 dph, with blood collections conducted by Aviagen at 18 dph, 27 dph, and 35 dph (summarised in Table 7.1). At 35 dph, all broilers were euthanised by Aviagen, and I collected samples of SI, caecal and colon contents, as well as tissues. Body weight measurements were taken at each collection time point. Blood samples were centrifuged at 7,500 × g for 15 minutes, and 1 mL of serum was extracted and transported on ice to the QIB before storage at -20°C. Faecal and GI content samples were flash-frozen on dry ice immediately upon collection and transported to QIB. Gastrointestinal tissues (SI, caecum, and colon) were fixed in ethanol, shipped, and stored in 10% Neutral Buffered Formalin (NBF).

Table 7.1 Summary of study design and sample collection.

AGE (DPH)	FEED STAGE	SAMPLE TYPES	ANALYSES
7	Starter	Faeces	Faecal microbiome, mycobiome metabolome
18	Grower	Faeces and serum	Faecal microbiome, mycobiome metabolome and serum metabolome
27	Finisher	Faeces and serum	Faecal microbiome, mycobiome metabolome and serum metabolome
40	Finisher	Small intestine tissues, faeces, and serum	Faecal microbiome, mycobiome metabolome, serum metabolome

7.3 RESULTS

7.3.1 Defining Broiler Performance

Of the 50 broilers, genotype A had 17 females and 8 males whilst genotype B had 18 females and 7 males. The average growth of all broilers from 7 to 40 dph was 93%, with 3 birds gaining 94% weight as the most productive and two birds with just 91% at the lowest. There was no significant difference between growth between genotypes or sex (data not shown); however, genotype A had significantly greater body weight at all time points despite substantial (Fig. 7.2A). To identify a performance-based marker, using the top and bottom IQR of BWG, 13 broilers were classed as high performers and 12 as low performers (Fig. 7.2B).

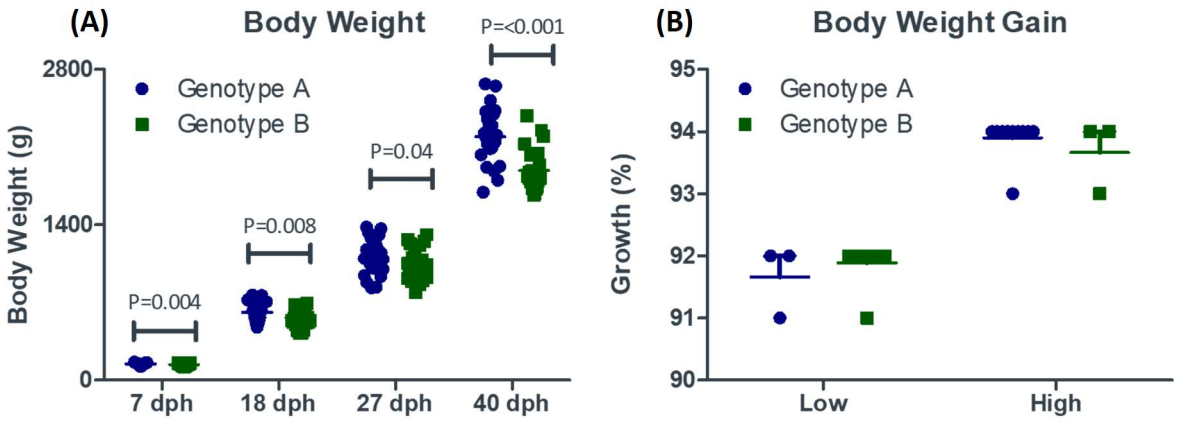


Figure 7.2 Broiler body weight across four time points (non-parametric t-test) (A) and BWG distribution of high and low-performing broiler (B).

7.3.2 Defining Broiler Gut Health

7.3.2.1 *Faecal Water Retention*

The average faecal water retention at 7 dph was 0.08 ml/g (SD 0.07), 0.04 ml/g (SD ± 0.07) at 18 dph, 0.06 ml/g (SD ± 0.08) at 27 dph and 0.1 ml/g (SD ± 0.1) at 40 dph (Fig. 7.3). With the SD being a similar value to the averages demonstrates substantial variability between faecal water content at each age. There was no significant difference in water retention between genotypes at any time. There was no significant correlation between growth and water retention (data not shown). Still, a significant negative relationship existed between water retention and body weight at 7 dph although only representing a weak 10% correlation (Fig. 7.3B).

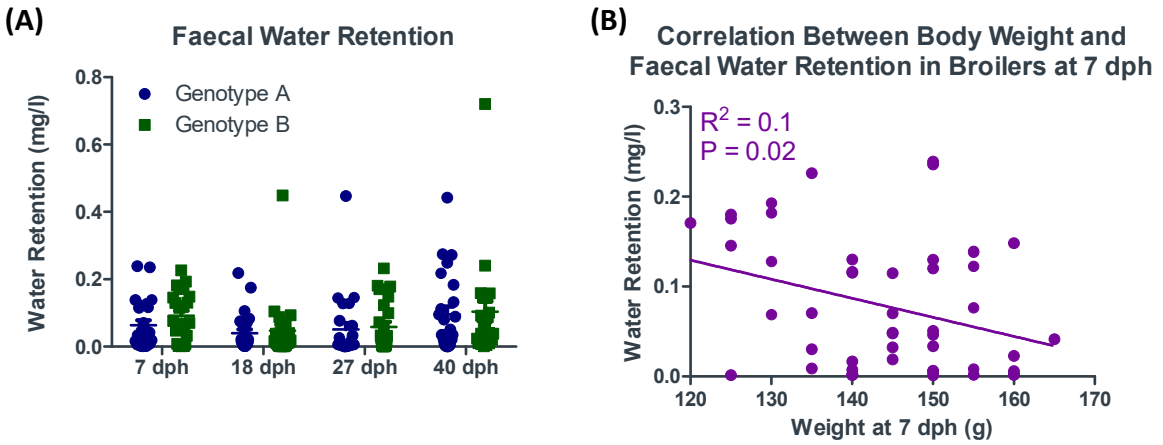


Figure 7.3 Vertical scatter plot of faecal water content across four time points in two genotypes (A) and linear regression model of body weight at 7 dph and faecal water retention (B).

While water retention does not directly indicate performance or gut health, as it could result from excessive water intake and thus may not reflect biological efficiency, it can still offer biological insights into the results of the faecal microbiome, mycobiome, and metabolome analyses.

7.3.2.2 *Histology of the Small Intestine at 40 Days post Hatching*

Each bird underwent an average of 63 measurements for villus height and crypt depth, averaging 21 per section, surpassing the minimum requirement of 10 measurements per section. An illustration of the measurement process is provided in Figure 7.4.

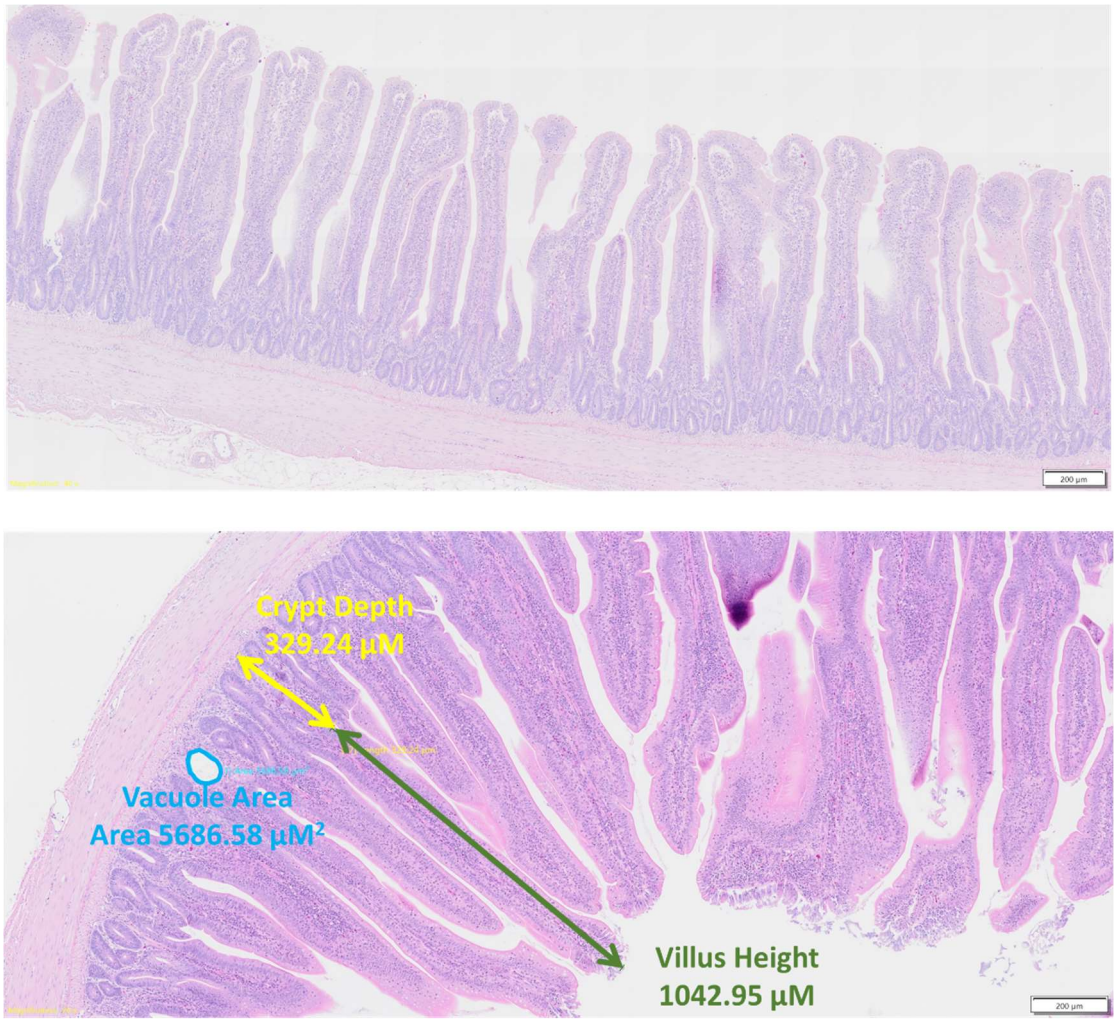


Figure 7.4 Representative images of H&E-stained broiler SI for recording measurement of morphological structures. Acquired and analysed on ZEISS Axioscan 7 Microscope Slide Scanner at x20 to x40 magnification, fitted with automatic stitching of images greater than the microscope's field of view. (**Top**) Example of healthy SI and (**Bottom**) measurements of crypt depth (**yellow**), villus height (**green**) and vacuole (**blue**).

At 40 dph, the average broiler villus height was 826.9 µM (SD ± 169.6), and the average crypt depth was 162.7 µM (SD ± 113.0). No significant differences in villus height (Fig. 7.5A) or crypt depth (Fig. 7.5B) was seen between genotypes. Although the total length of the combined villi height and crypt depth was higher in genotype A (t-test, P = 0.04) (Fig. 7.5C), the villus height to crypt depth ratio was high in genotype B (t-test, P = 0.004) (Fig. 7.5D) suggesting better gut health in genotype B. This contradicts performance, where genotype A demonstrated a greater body weight at all ages.

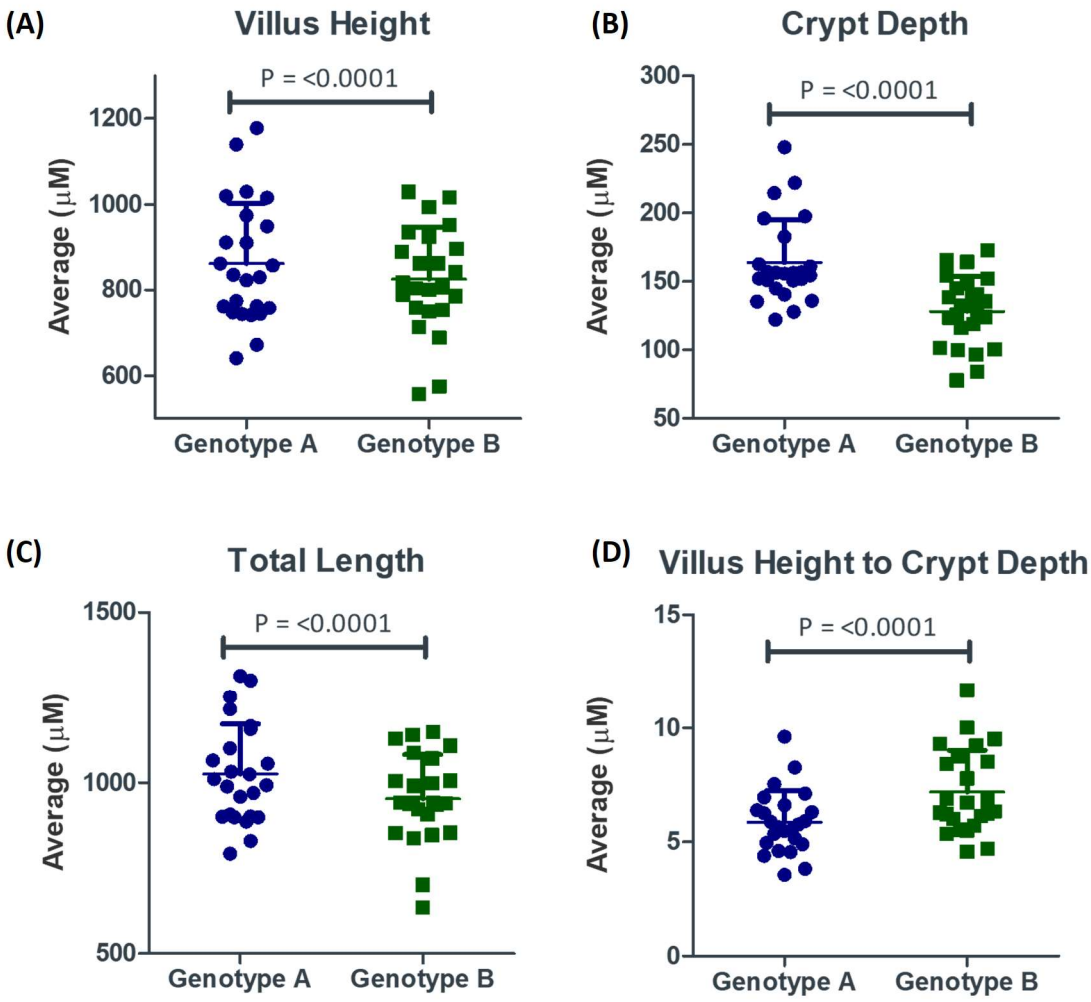


Figure 7.5 Differences in the average villus height (A), crypt depth (B), total length (C) and villus height to crypt depth ratio (D) of the broiler SI at 40 dph, determined with a non-parametric t-test.

Nine broilers had at least one vacuole present in the SI (Fig. 7.6). Other than chicken #33, all broilers with vacuoles were from genotype A. Vacuole areas had an average area of 5,087.2 μM^2 (perimeter of 261.5 μM^2), varying between 955.4 μM^2 and 27,891 μM^2. This is consistent with the villus-to-crypt analysis demonstrating better gut health in genotype B. Vacuoles in the SI lining, particularly below the crypts, can be indicative of various pathological conditions or physiological processes such as infections, cellular degeneration, or inflammation (Yamauchi and Tarachai, 2000, YAMAUCHI, 2007).

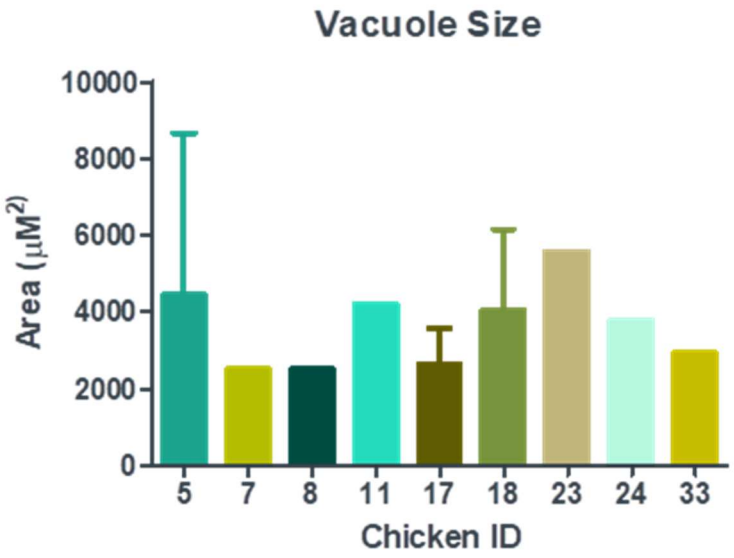


Figure 7.6 Horizontal bar graph of SI vacuole areas in broilers at 40 dph. Chickens #5, #17 and #18 had 3, 2 and 8 vacuoles, respectively, with all other samples having one.

7.3.2 Successional Changes of the Faecal Microbiome

Quality filtering of the sequencing data resulted in the exclusion of one sample at 7 dph, twenty samples at 27 dph, and six samples at 40 dph due to insufficient read counts. Throughout the broilers' lifecycle, 433 taxa were identified in the faecal microbiota. At 7 dph, a total of 173 species were detected. This number remained constant at 18 dph, with 173 species and 27 dph at 171 species. By 40 dph, there was a slight reduction to 169 species.

Lactobacillaceae was the dominant family in broiler faeces, prevalent in most samples across all time points. However, by 40 dph, the 20 most dominant families

represented less than 50% of the total relative abundance (Fig. 7.7). *Ruminococcaceae* consistently appeared in low abundances at all time points. *Enterobacteriaceae* and *Enterococcaceae* were more prevalent at 7 dph than at any other time point, seemingly replaced by *Corynebacteriaceae* at 18 dph. *Akkermansiaceae* began to appear in higher abundance at 27 dph. By 40 dph, *Clostridia*, *Lachnospiraceae*, and *Akkermansiaceae* all showed increased abundance.

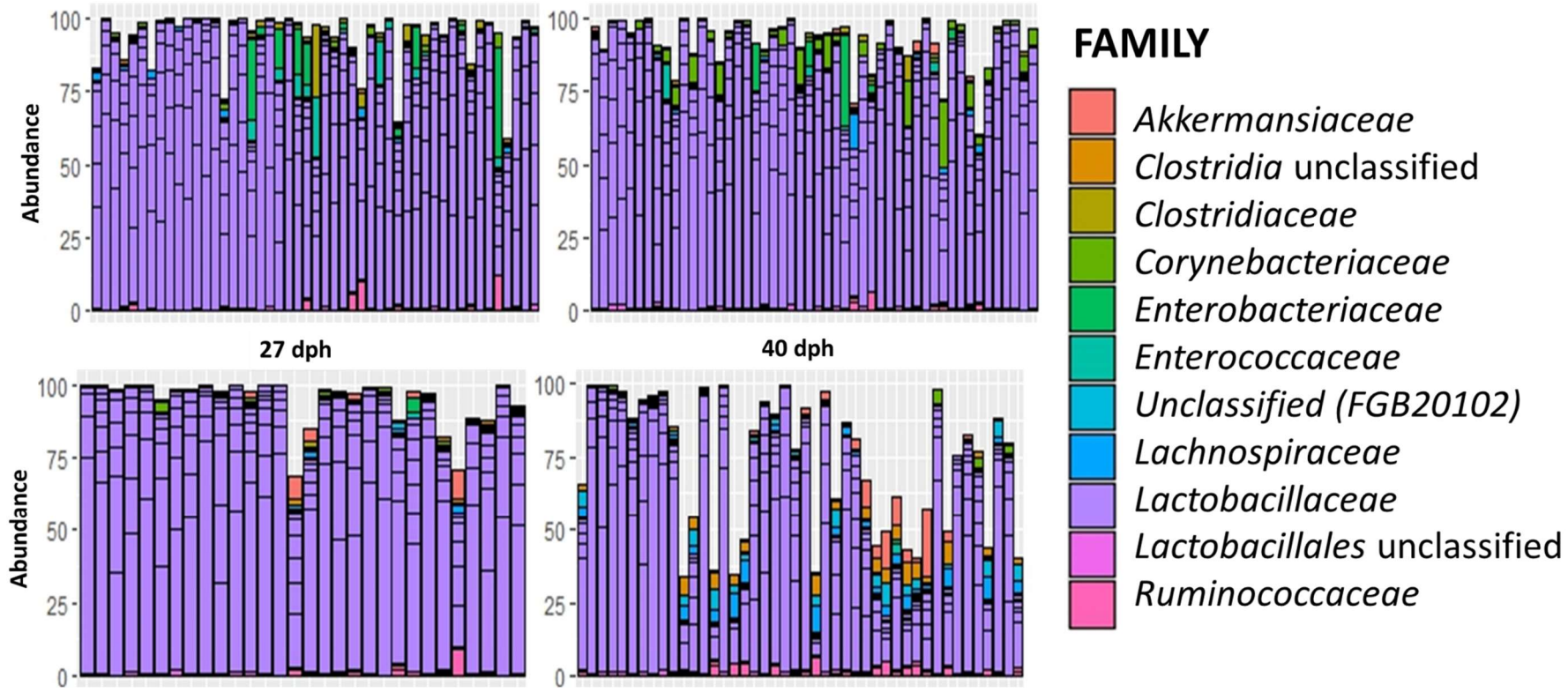


Figure 7.7 Stacked bar graphs illustrating the relative abundance of the top 20 most abundant families in broiler faeces at 7, 18, 27, and 40 dph. The data were generated using shotgun metagenomic sequencing and analysed with MetaPhlAn 4. Visualisation was performed using the Phyloseq and ggplot2 packages in R.

A significant difference in the abundance of 21 species was observed when comparing different time points (Fig. 7.8). Four of these were unclassified microorganisms (GGB9734 SGB15304, GGB42690 SG59894, GGB42674 SGB66070, GGB48305 SGB66329), all of which were significantly more abundant at 40 dph. The abundance of *Candidatus Arthromitus spp.* (SFB turkey), an uncultured segmented filamentous bacterium with a unique relationship to turkey immune development (Hedblom et al., 2018), significantly decreased over time, with the highest abundance at 7 dph. Similarly, decreases were noted in *Escherichia coli* and several lactate-producing bacteria (*Lactobacillus crispatus, Limosilactobacillus reuteri, Lactobacillus johnsonii*, and *Enterococcus hirae*) over time. *Lactobacillus kitasatonis* also showed a significant decrease in abundance over time, present at 18 dph but absent at 7 dph.

Unlike the other *Lactobacilli*, *Lactobacillus gallinarum* increased in abundance from 7 to 27 dph, with a lower but more variable abundance among broilers at 40 dph. *Ligilactobacillus aviarius* also showed a significant increase from 7 to 40 dph. Additionally, *Alistipes*, *Akkermansia*, *Pseudoflavonifractor spp.* (An176), and *Lachnospiraceae* significantly increased in abundance over time.

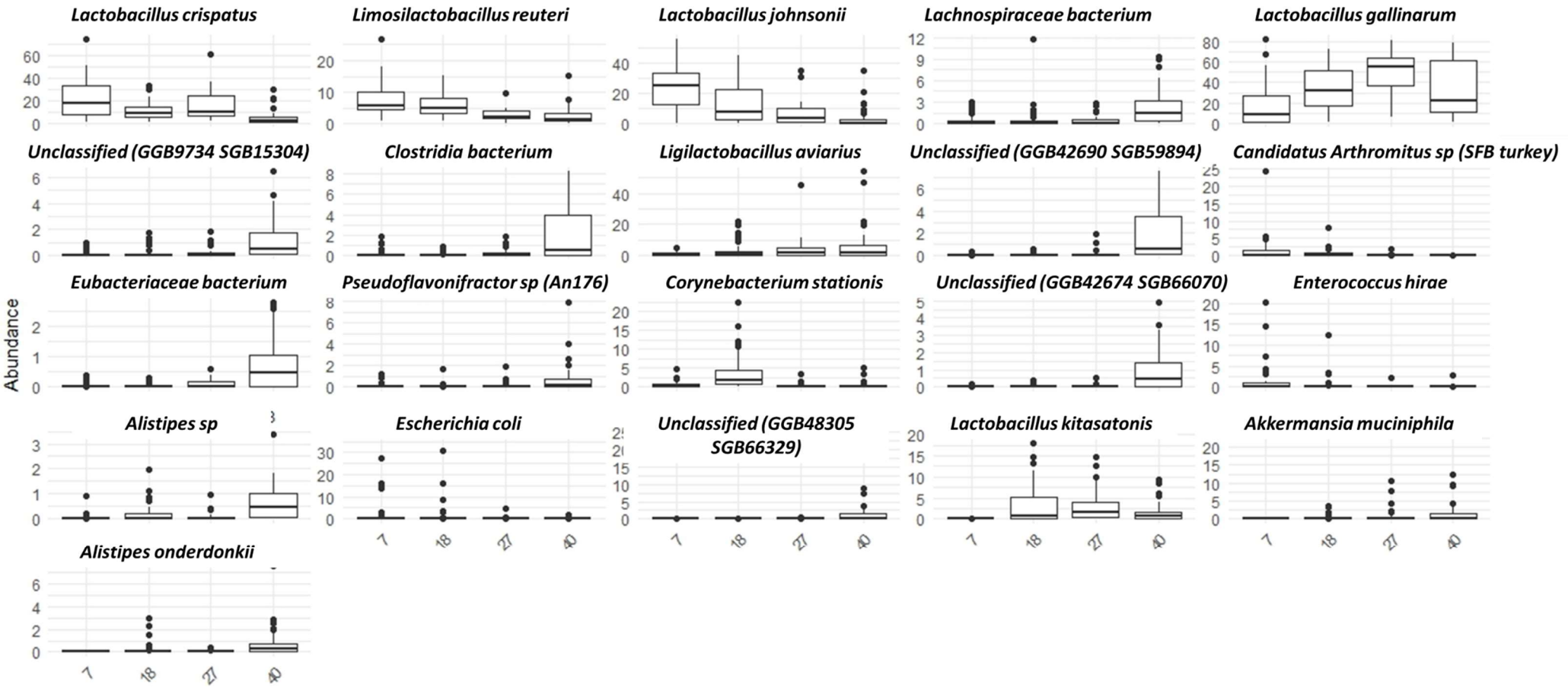


Figure 7.8 Whisker plots showing the relative abundance of species with significant differences across various time points (ANOVA, $P < 0.05$). The data were generated using shotgun metagenomic sequencing and analysed with MetaPhlAn 4. Visualisation was performed using the Phyloseq and ggplot2 packages in R.

Within each age group, 58 significant differences were observed between genotypes, underscoring the variability within faecal microbiome communities despite their shared environment. Appendix 3 provides a comprehensive list of P values and average relative abundances at each time point.

Ligilactobacillus aviarius exhibited consistently higher abundance in genotype B across all time points (Fig. 7.9A), representing the most significant difference observed. Conversely, *Limosilactobacillus vaginalis* showed higher abundance in genotype A from 7 to 27 dph onwards (Fig. 7.9B). A significant difference in *Akkermansia muciniphila* abundance between genotypes emerged from 18 dph onwards, with significantly higher abundance observed in genotype B (Fig. 7.9C).

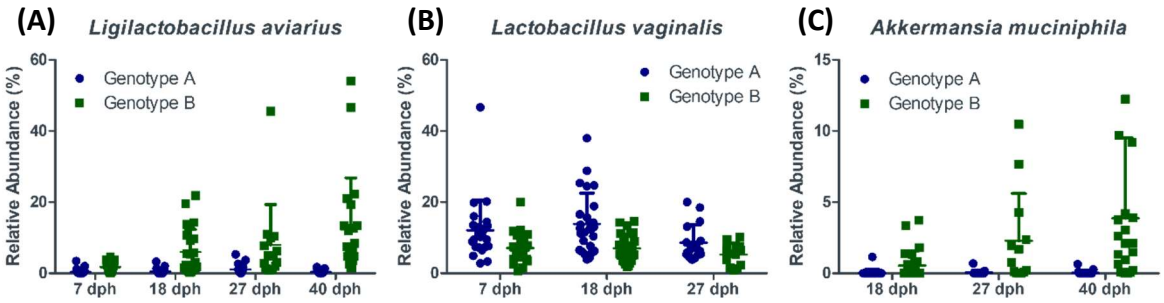


Figure 7.9 Vertical scatter plots depicting the relative abundance of bacterial species in broiler faeces that exhibit significant differences in abundance between genotypes across all time points: (A) *Ligilactobacillus aviarius*, (B) *Limosilactobacillus vaginalis*, and (C) *Akkermansia muciniphila*.

A comparison of the faecal microbiome composition between genotypes at each time point revealed distinct patterns (Fig. 7.10). At 7 dph, 14 significant differences in taxa relative abundance were observed, with 11 species exhibiting significantly greater abundance in genotype B compared to genotype A. Similarly, at 18 dph, 19 differences were noted, with 14 species significantly higher in abundance in genotype B. Notably, 27 dph exhibited fewer differences between genotypes, likely due to a smaller sample size resulting from failed quality control. Nevertheless, 8 significant differences were still observed, with each genotype showing 4 species as significantly more abundant. At 40 dph, the most significant differences in species between genotypes were observed, with 22 species having significantly higher abundance in genotype B and 8 significantly higher in genotype A

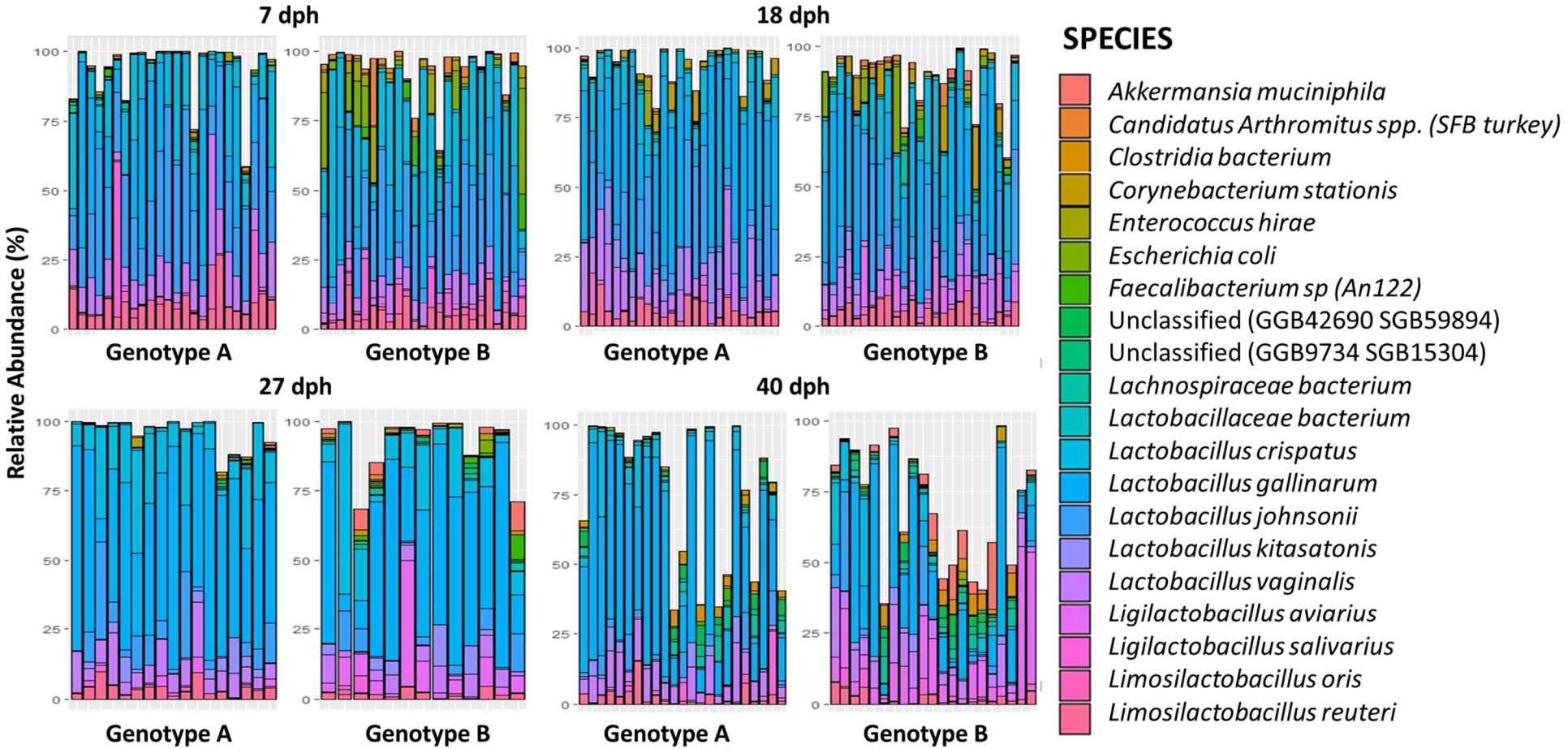


Figure 7.10 Stacked bar graph illustrating the top 20 most abundant species across all time points, with data segmented by genotype. Visualisation was performed using Metaphlan4, Phyloseq, and ggplot2.

Alpha-diversity was significantly higher at 40 dph compared to 7 and 27 dph (Fig. 7.11A). Conversely, no significant differences were observed in β diversity, indicating similar faecal microbiome compositions at each time point (Fig. 7.11B), a trend consistent with the analysis of the top 20 families and species. However, α and β diversity were most variable at 40 dph, suggesting a disparity among broilers with rich and even faecal microbiomes at this stage.

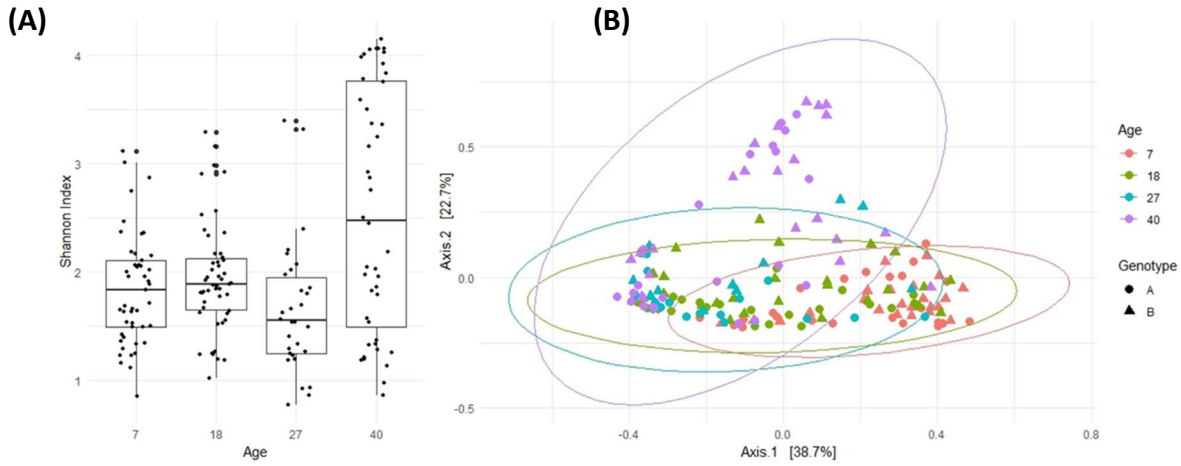


Figure 7.11 Whisker plots illustrating α-diversity using the Shannon index for microbiome shotgun data (A) and a Bray Curtis ordination plot representing β diversity (B). Data visualisation was conducted using MetaPhlAn 4, Phyloseq, and ggplot2 in R.

Contrary to expectations suggested in the literature (Yang et al., 2023) and despite significant differences between time points, no correlation was observed between *Ligilactobacillus salivarius* abundance and performance or gut health (data not shown). However, a significant positive relationship was found between *Alistipes* abundance and body weight (Fig. 7.12A), consistent with previous findings in the chicken caeca (Adewole and Akinyemi, 2021). Furthermore, there was a significantly higher relative abundance of *L. aviarius* in the faeces of low-performing broilers at all time points (Fig. 7.12B).

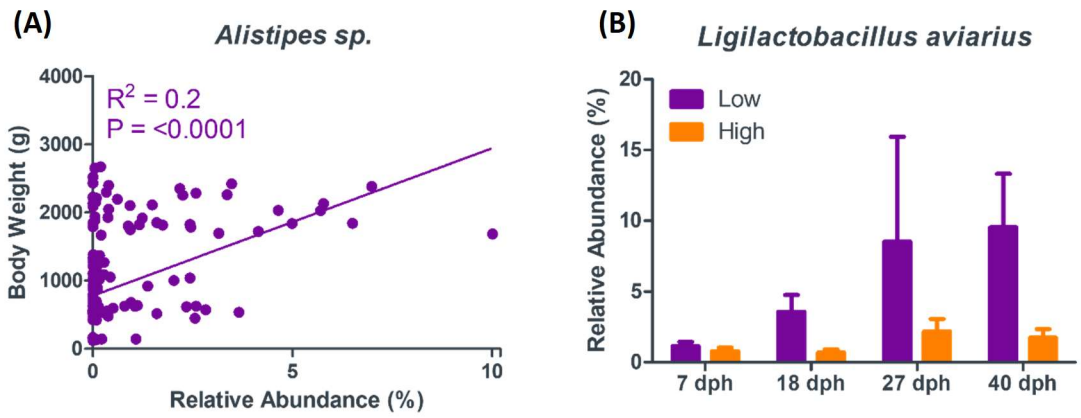


Figure 7.12 Taxa correlating with performance: (A) Linear regression analysis showing the correlation between the relative abundance of genus Alistipes and body weight. (B) Bar graph illustrating the relative abundance of L. aviarius in low and high-performing broilers.

No significant differences were observed in the number of AMR genes found in broiler faeces at each time point (Fig. 7.13A). Across all time points, many AMR genes were detected against specific antibiotics commonly used in poultry farming however, no antibiotics have been used on this farm at any point. Of interest are 506 AMR genes against tetracycline, 450 against lincosamide, and 289 against aminoglycoside antibiotics (Fig. 7.14B). Tetracycline, lincosamide, and aminoglycoside antibiotics are frequently employed in poultry farming for their efficacy in treating bacterial infections (Cervantes, 2015). Despite the presence of AMR genes, their activity is not guaranteed. Often, AMR genes require specific conditions or the presence of companion mutations to confer resistance. The observed low number of AMR genes showed no significant correlation with the broilers' microbiome composition or biological efficiency parameters.

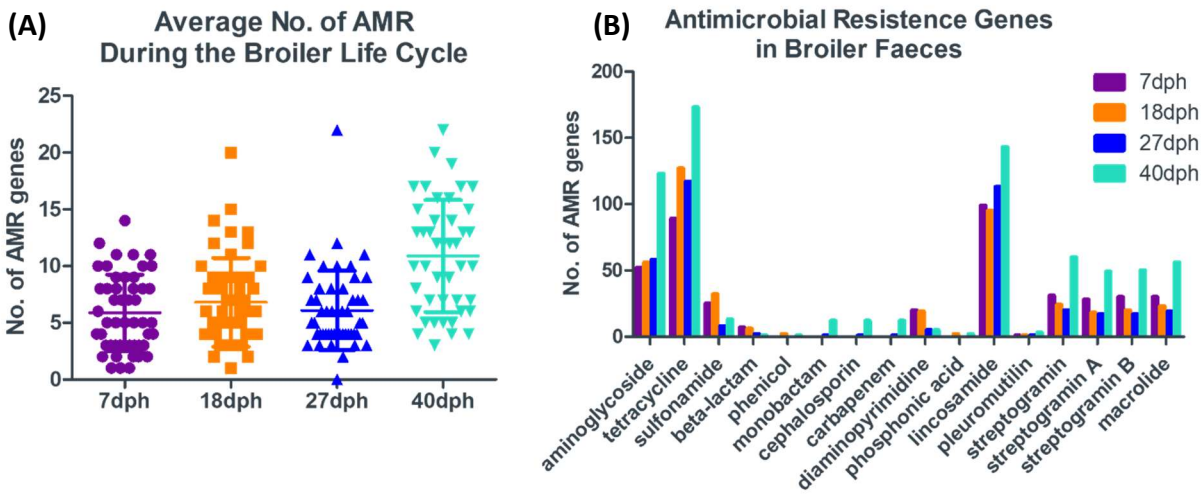


Figure 7.13 Vertical scatter graph of the count of AMR genes detected in broiler faeces (A) and bar graph of antibiotic resistance profile (B) determined using ARIBA and CARD.

Virulence factors were detected in 31 broiler faecal samples (Fig. 7.14). Except for chicken #25 at 27 dph, all virulence factors were found in the faeces of broilers from genotype B. Specifically, 10 were found at 7 dph, 6 at 18 dph, 4 at 27 dph, and only 1 at 40 dph. This observation highlights a decrease in virulence factors in faeces over the broiler life cycle, possibly reflecting microbiome successional changes. At 40 dph, fewer virulence factors were detected when the microbiome was most diverse.

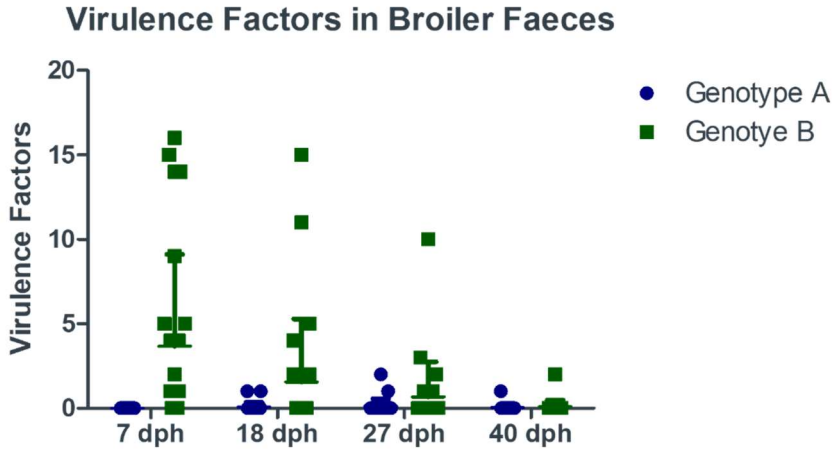


Figure 7.14 Vertical scatter plot illustrating the total number of virulence factors detected in genotypes over time, determined using ARIBA and the Virulence Finder database.

Functional analysis of shotgun metagenomic data was performed using Humann3. Unmapped reads comprised 18% of all reads, with unintegrated reads accounting for 72%,

leaving only 10% available for further analysis. Consequently, the limited proportion of mapped reads restricted the extraction of meaningful results. Examining the 20 most abundant pathways in broiler faeces (Fig. 7.15), 17 pathways remained consistently among the most abundant at all time points, suggesting their widespread presence and likely importance. The most prevalent pathway across all time points was UDP-N-acetyl-D-glucosamine biosynthesis I (UDPNAGSYN-PWY), with an average relative abundance of 0.0003%. UDP-N-acetyl-D-glucosamine biosynthesis I serves as a precursor for the biosynthesis of essential molecules such as peptidoglycan, glycoproteins, glycolipids, and polysaccharides (Kneidinger et al., 2003).

At 40 dph, the dTDP-β-L-rhamnose biosynthesis (DTDPRHAMSYN-PWY) pathway was identified as one of the top 20 most abundant and uniquely present pathways. This pathway is critical for producing dTDP-L-rhamnose, a nucleotide sugar precursor essential for the viability and virulence of pathogens such as *Streptococcus* (van der Beek et al., 2019). Streptococcal pathogens rely on dTDP-L-rhamnose to synthesise structurally similar rhamnose polysaccharides in their cell walls (van der Beek et al., 2019). Pathways associated with glucose metabolism, including glucose and glucose-1-phosphate degradation (GLUCOSE1PMETAB-PWY) and glycolysis IV (PWY-1042), were absent from the top 20 pathways at 7 dph but became prominent from 18 dph onwards

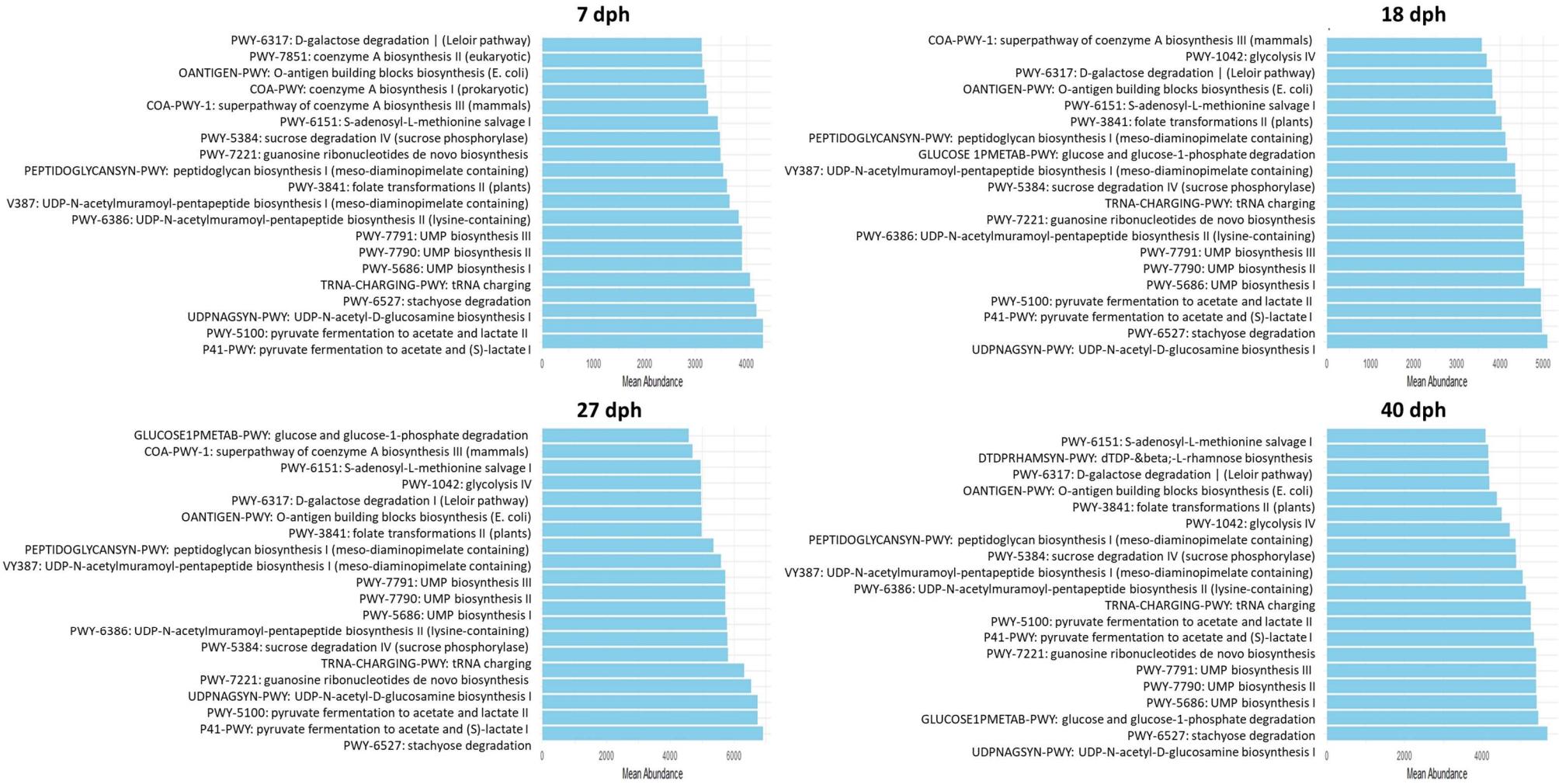


Figure 7.15 The top 20 pathways at each stage of the broiler life cycle. Data were generated using Humann3 and visualised with Phyloseq and ggplot2 in R.

7.3.3 Successional Changes of the Faecal Mycobiome

Some faecal samples failed QC processing after sequencing, with 10 samples failing at 7 dph, 16 samples failing at both 18 and 27 dph, and 8 samples failing at 40 dph. No significant differences in the number of ASVs were identified between time points and genotypes. Unassigned ASVs represented ~30% of the phyla relative abundance (Fig. 7.16); however, this jumps to >50% at the order taxonomic level (Fig. 7.17), attesting to the challenges in assigning taxonomy for ITS1 as it is so highly conserved in other eukaryotes. For example, *Anthrophyta* was discovered in this data but is a phylum for flowering plants (Wu et al., 2022). Furthermore, *Cercozoa* is a phylum for protists, essential for environmental nutrient recycling (Cavalier-Smith and Chao, 2003). Finally, *Rozellomycota* is a basal fungal phylum but generally includes unicellular parasites of animals and the aforementioned protists (Xu et al., 2019).

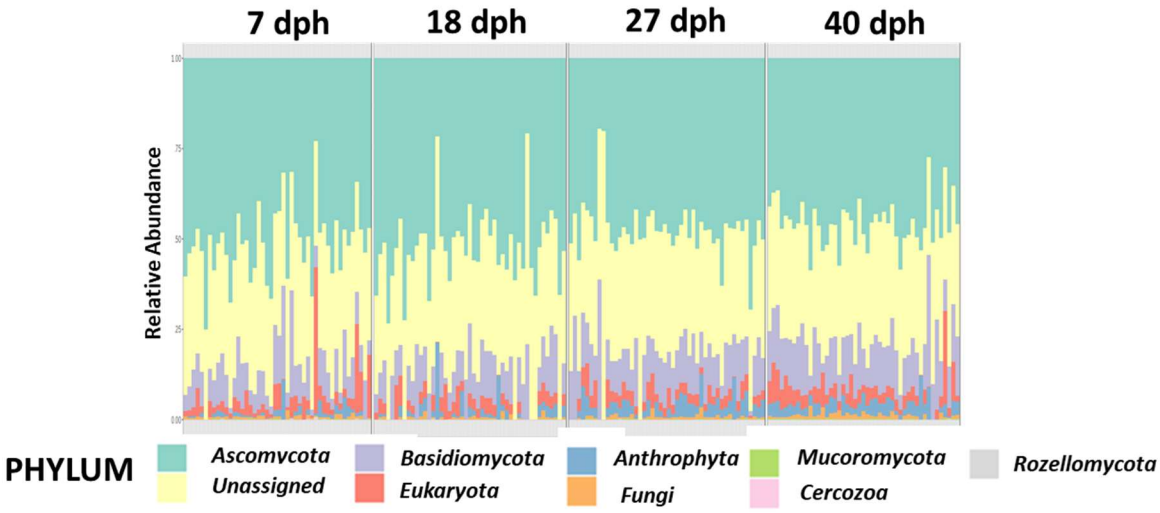


Figure 7.16 Stacked bar graph displaying the relative abundance of taxonomic phyla in the broiler faecal mycobiome at different time points. Data were generated using ITS1 rRNA sequencing and visualised with MicrobiomeAnalyst 2.0 and R.

Saccharomycetales was the most dominant assigned order in broiler faeces, accounting for 11-15% of the relative abundance across all time points, followed by *Pleosporales* and *Eurotiales*, which each accounted for 4-7% of the relative abundance. There were no significant differences between the orders at each time point or between

genotypes. *Saccharomycetales* and *Eurotiales* were the dominant orders in broiler faeces at 13 dph (CHAPTER 5), but there were no *Pleosporales*, which appears unique to this study/chapter. *Pleosporales* is a diverse fungal order primarily known for inhabiting fresh water and being involved in organic decomposition (Zhang et al., 2012).

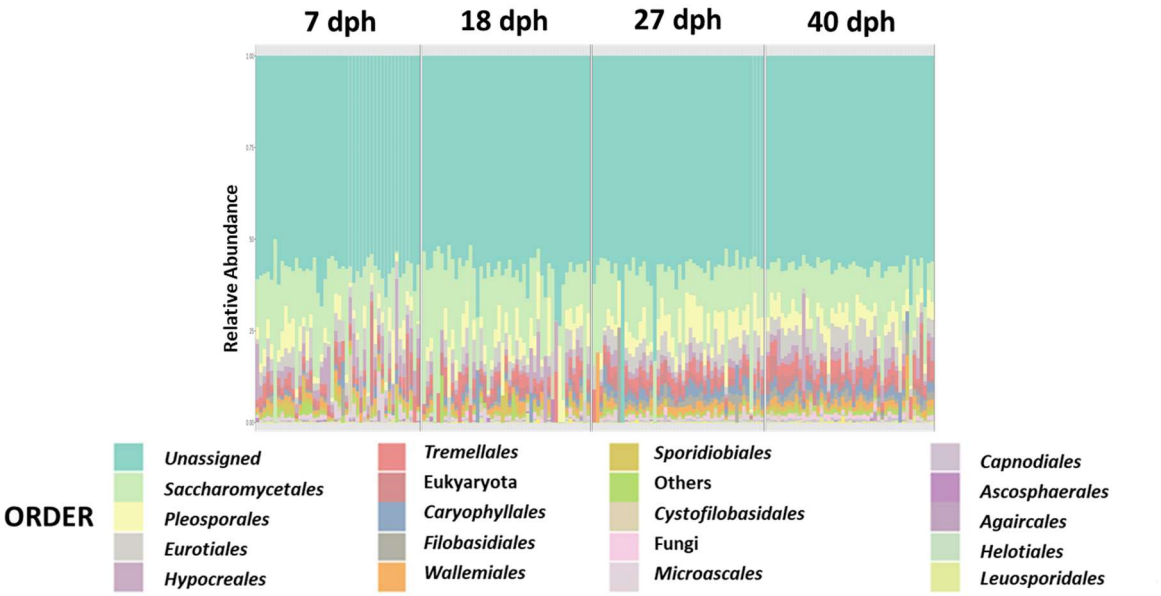


Figure 7.17 Stacked bar graph displaying the relative abundance of taxonomic orders in the broiler faecal mycobiome at different time points. Data were generated using ITS1 rRNA sequencing and visualised with MicrobiomeAnalyst 2.0 and R.

There was a total of three significant differences between genotypes (Fig. 7.18). The filamentous fungi *Nakazawaea* and yeast *Diutina* were both increased in abundance in genotype A, whereas the yeast-like fungus *Leucosporidium* was higher in genotype B.

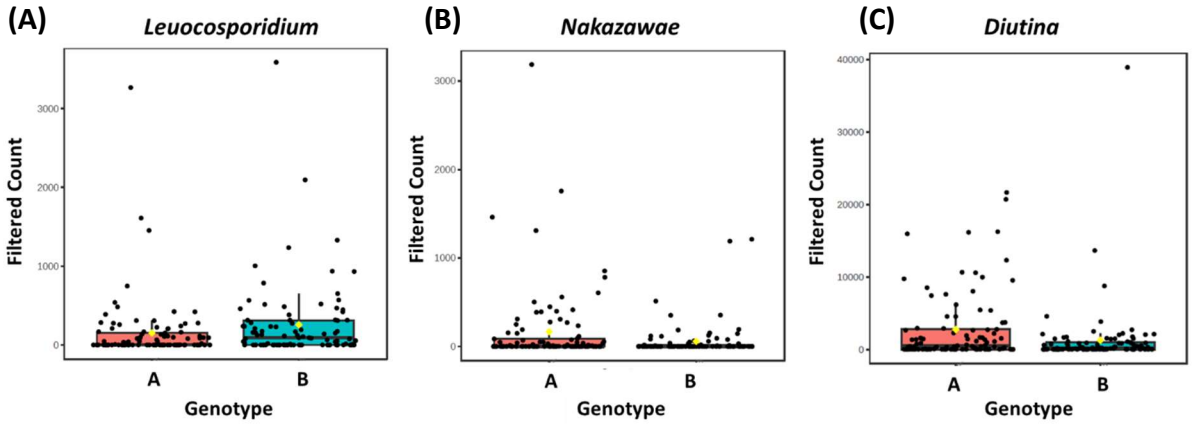


Figure 7.18 Three ITS1 rRNA genera that have a significant difference in abundance between genotypes: (A) *Leucosporidium* (ANOVA, P=0.0085), (B) *Nakazawaea* (ANOVA, P=0.0008) and, (C) *Diutina* (ANOVA, P=0.0007).

There was a total of 23 significant differences between genera at different time points (ANOVA, P<0.0001) (Table 7.2). This included a higher relative abundance of 12 genera at 7 dph, 10 genera at 27 dph, and 40 genera at 40 dph, which is the highest number of differences observed, with no significant differences at 18 dph compared to other time points. Except for the genus *Ustilago*, a plant pathogen belonging to the *Basidiomycota* phylum, all genera with significant differences between time points were from the *Ascomycota* phylum. These findings suggest dynamic shifts in the faecal mycobiome composition over time, particularly at the early and later stages of development, potentially influencing the observed differences in gut health and performance between the two genotypes.

Table 7.2 List of 24 ITS1 rRNA genera that have a significant difference in abundance between time points.

GENUS	P VALUE	GENUS	P VALUE
Melanodothis	<0.0001	*Diutina*	<0.0001
Paraxerochrysium	<0.0001	*Kluyveromyces*	<0.0001
Idriella	<0.0001	*Nakazawaea*	<0.0001
Barnettozyma	<0.0001	*Microdochium*	<0.0001
Acanthotrema	<0.0001	*Mycosphaerella*	<0.0001
Aspergillus	<0.0001	*Elaphomyces*	<0.0001
Sarcoleotia	<0.0001	*Hyphopichia*	<0.0001
Cladosporium	<0.0001	*Ustilago*	<0.0001
Fellomyces	<0.0001	*Cladophialophora*	<0.0001

Kurtzmaniella	<0.0001	*Pachyphlodes*	<0.0001
Octospora	<0.0001	*Monilia*	<0.0001
Filobasidium	<0.0001		

Paraxerochrysium was present at all stages of the broiler lifecycle but was significantly most abundant at 7 dph (**Fig. 7.20A**), suggesting that these fungi could be early colonisers. No correlations were found between this genus and performance or gut health metrics. *Paraxerochrysium* is similar to *Xerochrysium* (Crous et al., 2021) which exists in dry and hot environments, which may explain its lower abundance at a later time point as humidity increases.

Diutina was also abundant at all time points but showed a significant increase at 18 dph (Fig. 7.19B). These yeast-like fungi are capable of metabolising phosphorous (Sun et al., 2021), which is beneficial for phosphorous nutrition in chickens (Wang et al., 2019a) and reduces environmental phosphorous pollution (Sun et al., 2021). The increase at this time point may be due to the grower diet. This is the only timepoint of that diet. At 7 dph the starter diet has 48% available phosphorus, whereas the grower diet has 43.5% and the finisher diet only 39.5%. *Aspergillus*, *Filobasidium* and *Melanodothis* exhibit a significant increase in abundance over time (Fig. 7.19C-F), which may indicate successional changes in the mycobiome or continued existence within the enviroment. *Ustilago*, the only genus from *Basidiomycota* to show a significant difference between time points, and *Barnettazyma*, a yeast (Kurtzman et al., 2011), are significantly increased at 27 and 40 dph compared to earlier time points. This may be attributable to the grower diet.

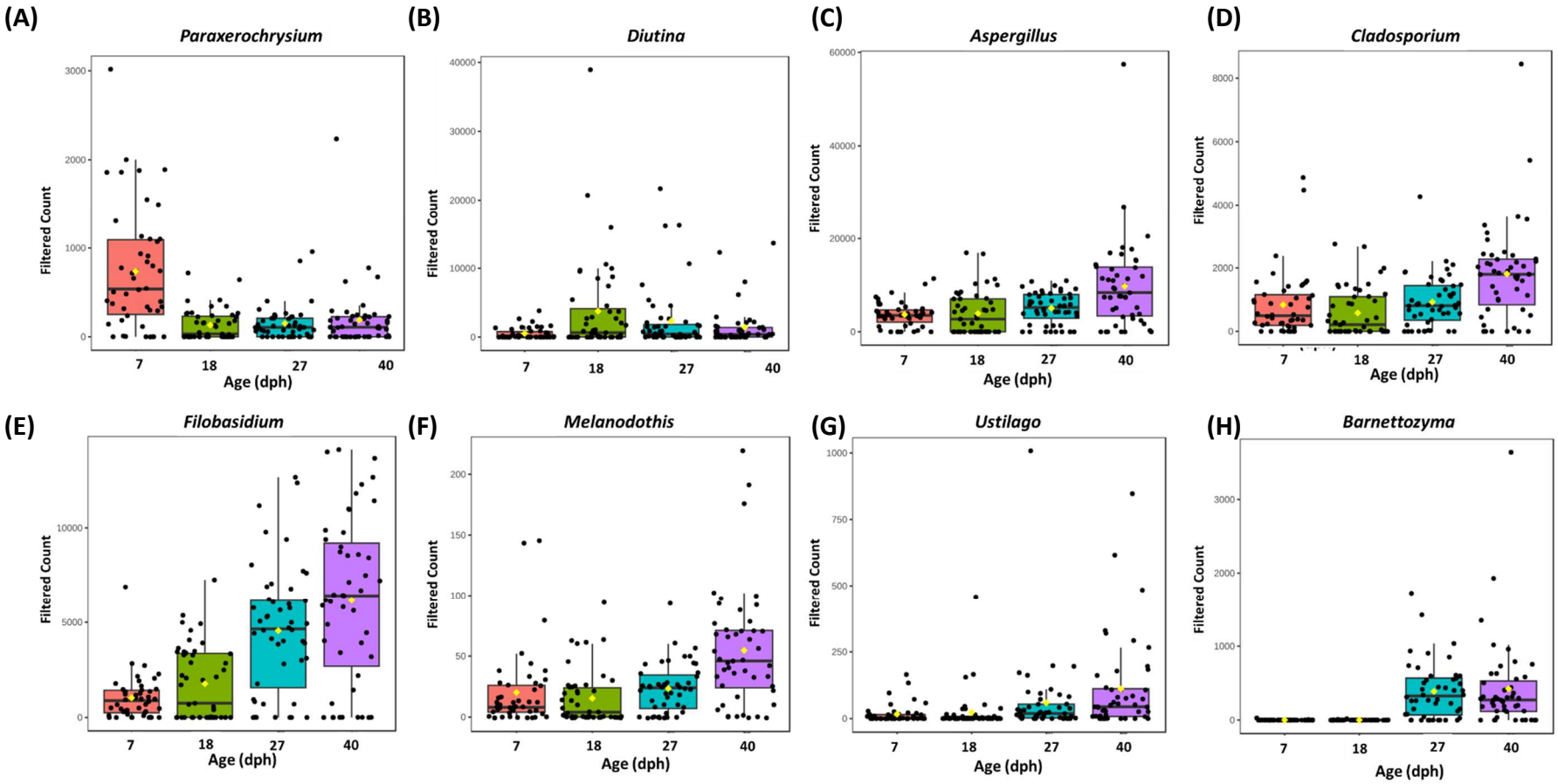


Figure 7.19 Whisker plots showing representative filtered counts of faecal fungal taxa with significant differences in abundance between time points: **(A)** *Paraxerochrysium*, **(B)** *Diutina*, **(C)** *Aspergillus*, **(D)** *Cladosporium*, **(E)** *Filobasidium*, **(F)** *Melanodothis*, **(G)** *Ustilago*, and **(H)** *Barnettazyma*. Data were generated using ITS1 rRNA sequencing, with statistical analysis and visualisation performed using MetaboAnalyst 2.0 in R.

No significant differences in α diversity, as measured by the Shannon index, existed between time points or genotypes (Fig. 7.20A). Bray Curtis β diversity appeared similar in PCoA ordination (Fig. 7.20B). At 40 dph, the broiler faecal mycobiome appeared more similar in species richness and evenness, suggesting a potential trend towards a stabilising mycobiome, though this difference was not statistically significant. The greatest variability in species richness and evenness among broiler faeces was observed at 18 dph.

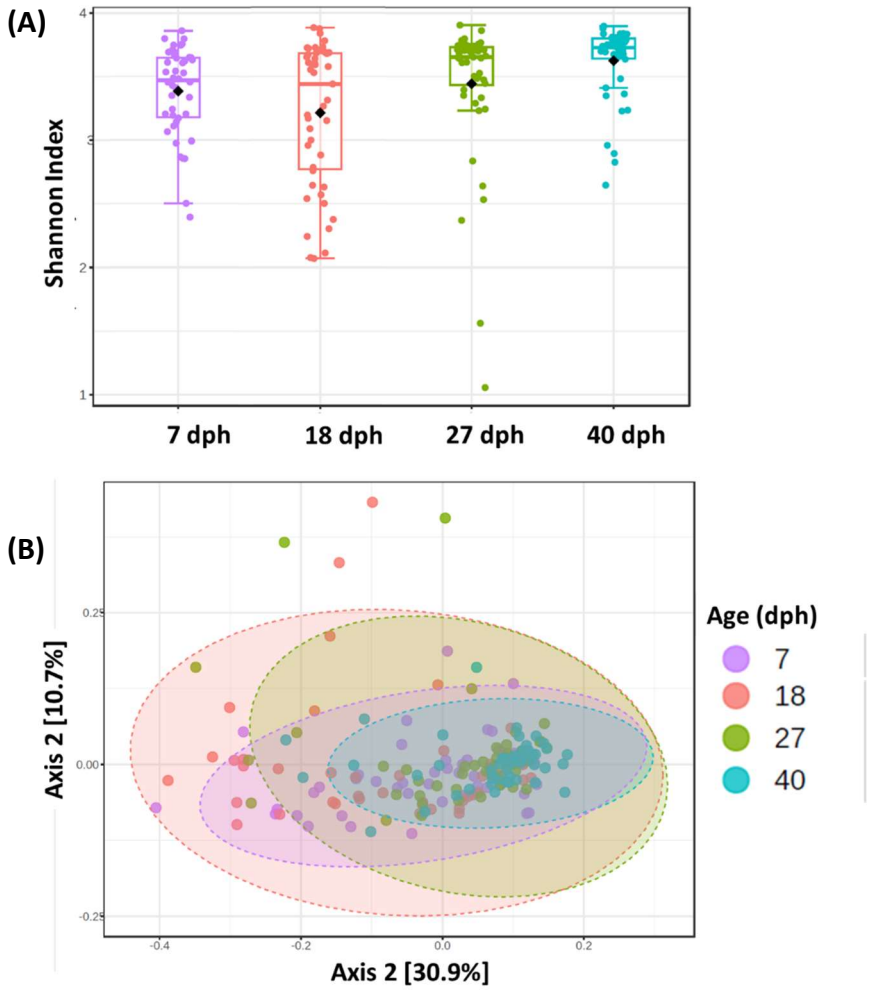


Figure 7.20 Faecal mycobiome α-diversity (Shannon Index) (A) and PCoA β-diversity (Bray Curtis) (B) across the broiler life cycle. Data were generated using ITS1 rRNA sequencing, with statistical analysis and visualisation performed using MetaboAnalyst 2.0 in R.

No taxa correlated with intestinal morphological structures, but *Diutina* demonstrated an association with performance measured by BWG (Fig. 7.21). There was a significantly higher abundance of this genus in high-performing broilers.

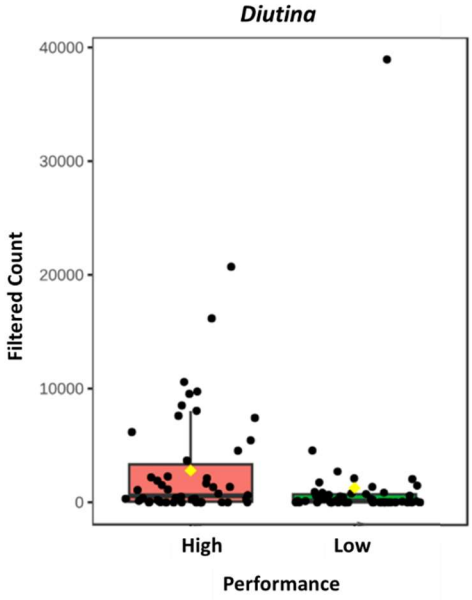


Figure 7.21 The difference in *Diutina* filtered counts between high and low-performing broilers. Data were generated using ITS1 rRNA sequencing, with statistical analysis and visualisation performed using MetaboAnalyst 2.0 in R.

Despite many unassigned ASVs, a core mycobiome could be identified each time. *Wickerhamomyces* was found at a relative abundance of over 0.6% at all time points (**Fig. 7.22A-D**). *Metschnikowia* exhibited a relative abundance of 0.3-0.4% from 7 to 27 dph. Both *Wickerhamomyces* and *Metschnikowia* are genera of yeast-like fungi (Kurtzman et al., 2011). Additionally, *Alternaria* was present within the core mycobiome from 18 dph onwards. This indicates that the broiler mycobiome is not merely transient but includes resident fungi within the GI microbiome. *Alternaria* is a genus of fungi known for its role as a plant pathogen (Thomma, 2003), highlighting the diverse nature of the fungal community in the broiler gut. Identifying these consistent fungal taxa underscores the stability and persistence of specific mycobiome members throughout the broilers' development stages.

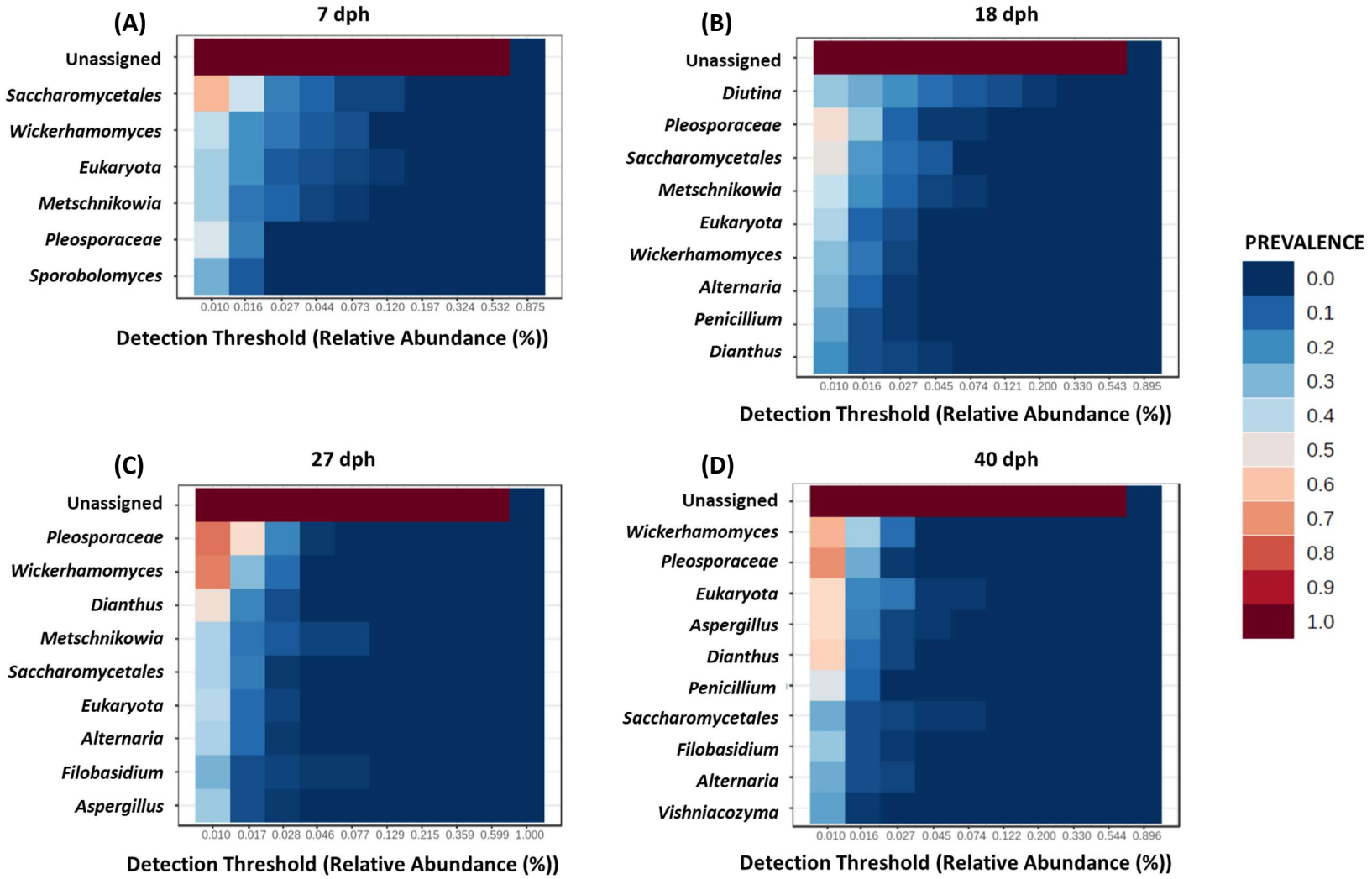


Figure 7.22 Heat maps demonstrating taxa with >0.1% relative abundance across all broiler faeces at (A) 7 dph, (B) 18 dph, (C) 27 dph and (D) 40 dph. Data were generated using ITS1 rRNA sequencing, with statistical analysis and visualisation performed using MetaboAnalyst 2.0 in R.

7.3.4 Correlating the Microbiome and Mycobiome

Utilisation of the Shannon index provides a standardised means of comparison across datasets. The α diversity of the broiler faecal mycobiome exhibited significant elevation compared to the microbiome at 7 and 27 dph (Fig. 7.23A). However, the mycobiome generated fewer sequencing reads and fewer species identified, which may be a feature of the UNITE database. The average β-diversity of the faecal microbiome and mycobiome demonstrated a decrease between 7 and 27 dph (Fig. 7.23B) however this disparity did not reach statistical significance.

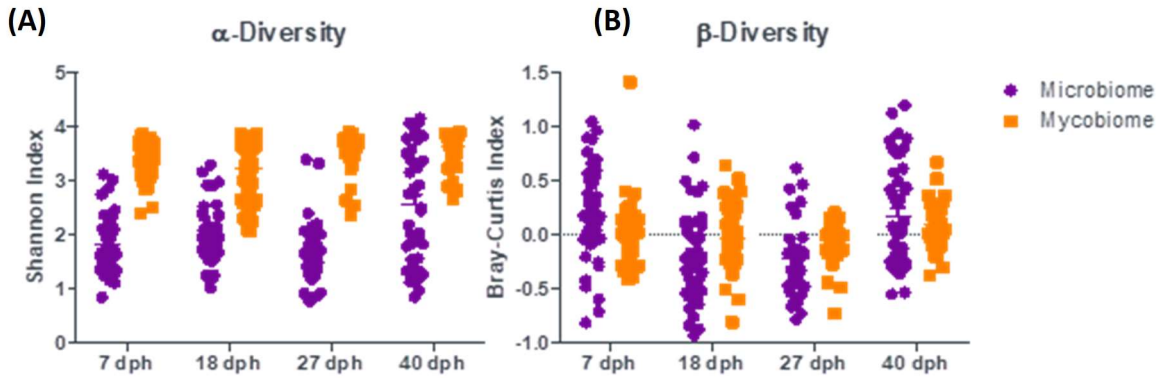


Figure 7.23 Summary and comparison of the broiler faecal microbiome and mycobiome faecal **(A)** α- and **(B)** β-diversity.

A positive trend was observed between the α-diversity of the broiler faecal microbiome and mycobiome at 7, 27 and 40 dph (Fig. 7.24A). There was also a significant positive trend between microbiome and mycobiome β-diversity at all time points (Fig. 7.24B). These observed relationships between fungal and bacterial β diversity in the faecal microbiome may suggest coordinated variability between these microbial communities.

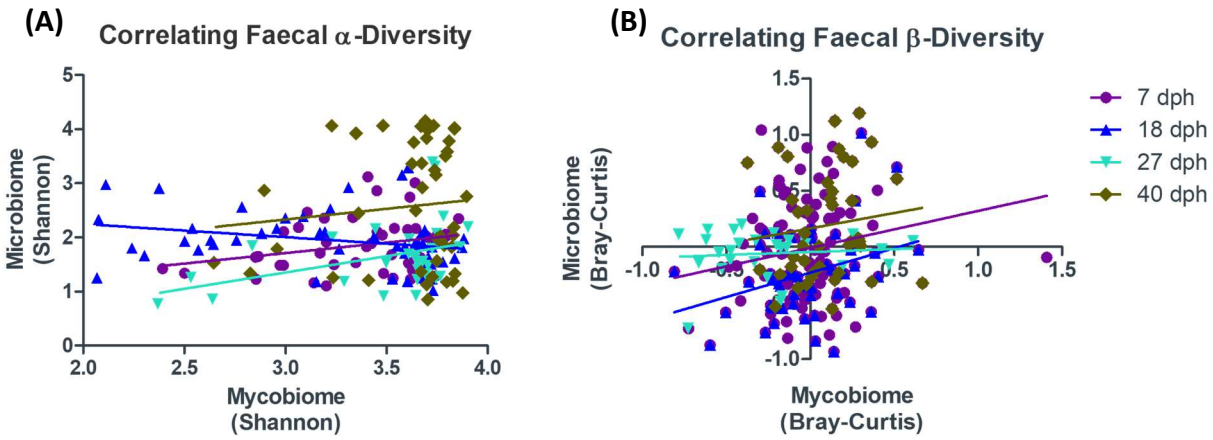


Figure 7.24 Linear regression models correlating microbiome and mycobiome α- (A) and β- (B) diversity during the broiler life cycle.

7.3.5 Successional Changes of the Faecal Metabolome

A total of 308 compounds were identified across three faecal sample time points, corresponding to each dietary change: 7 dph on the starter diet, 18 dph on the grower diet, and 40 dph on the finisher diet. Ninety-five significant differences in metabolite levels between these time points were identified (details provided in Appendix 4).

At 7 dph, 35 metabolites were significantly higher than later timepoints. At 18 dph, 83 metabolites showed significantly higher levels, indicating the most significant number of metabolic differences, and suggesting heightened metabolic activity at this intermediate stage. By 40 dph, 56 metabolites were significantly higher than the other time points, potentially reflecting adaptations to the finisher diet and successional changes in the microbiome. This progression highlights how different broiler lifecycle stages impact their faeces' metabolic profiles. PCoA illustrates the significant differences in faecal metabolic composition at 18 dph, while 7 dph and 40 dph show more similarity, with less variation observed at 7 dph (Fig. 7.25), which may suggest a core metabolome which is overlaid at 18 dph. The variation in metabolic activity at 18 dph may serve as a valuable marker for performance or gut health due to its distinct metabolic profile.

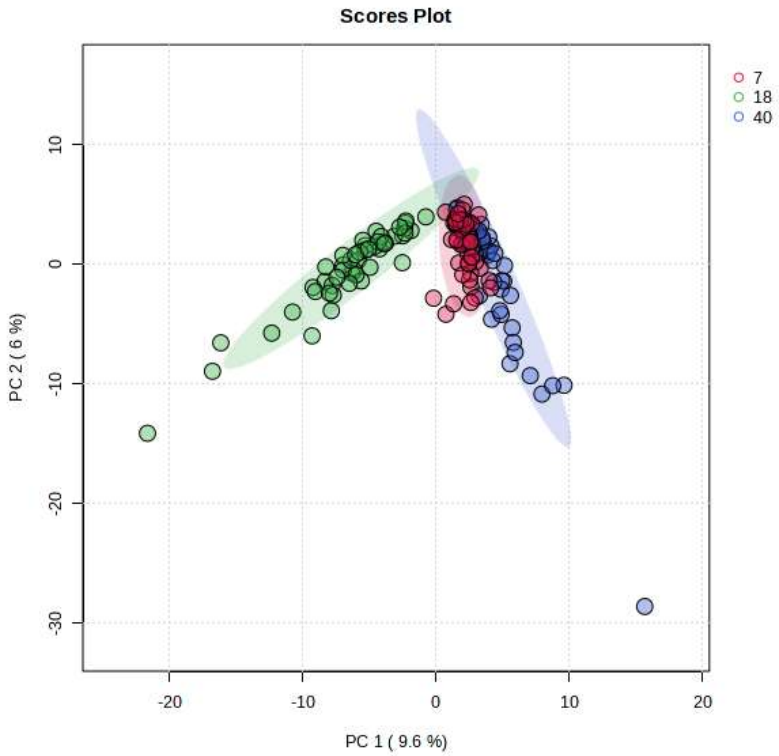


Figure 7.25 PCoA of faecal metabolomes profiles comparing three-time points, 7, 18, and 40 dph. The ellipses represent the average distribution of metabolites within each time point. Distinct clustering patterns indicative of temporal shifts in faecal metabolite composition. Data visualisation and analysis were performed using MetaboAnalyst 5.0.

Acetate concentrations were significantly lower at 7 dph compared to the later time points (**Fig. 7.26A**), but this was the only SCFA to show a difference in the faecal metabolome. Lactate and glucose were the most abundant compounds in all faecal samples, with there being significantly greater concentration at 18 dph (Fig. 7.26B). Two metabolites significantly differed in concentration between genotypes across all time points, although the overlap between groups should be noted. The concentration of sulfur compound dimethyl sulfone (Fig. 7.26C) was significantly higher in genotype A. This compound is anti-inflammatory, an antioxidant, and has potential prebiotic properties (Vitali et al., 2012), suggesting it could positively influence gut health and the composition of the gut microbiome. Galactose concentrations were significantly higher in genotype B (Fig. 7.26D). The monosaccharide is essential for supporting a healthy faecal microbiome, acting as a prebiotic, and promoting the growth of beneficial bacteria (Arnold et al., 2021).

A complete list of differences in faecal metabolite concentrations between genotypes at each age can be found in Appendix 5.

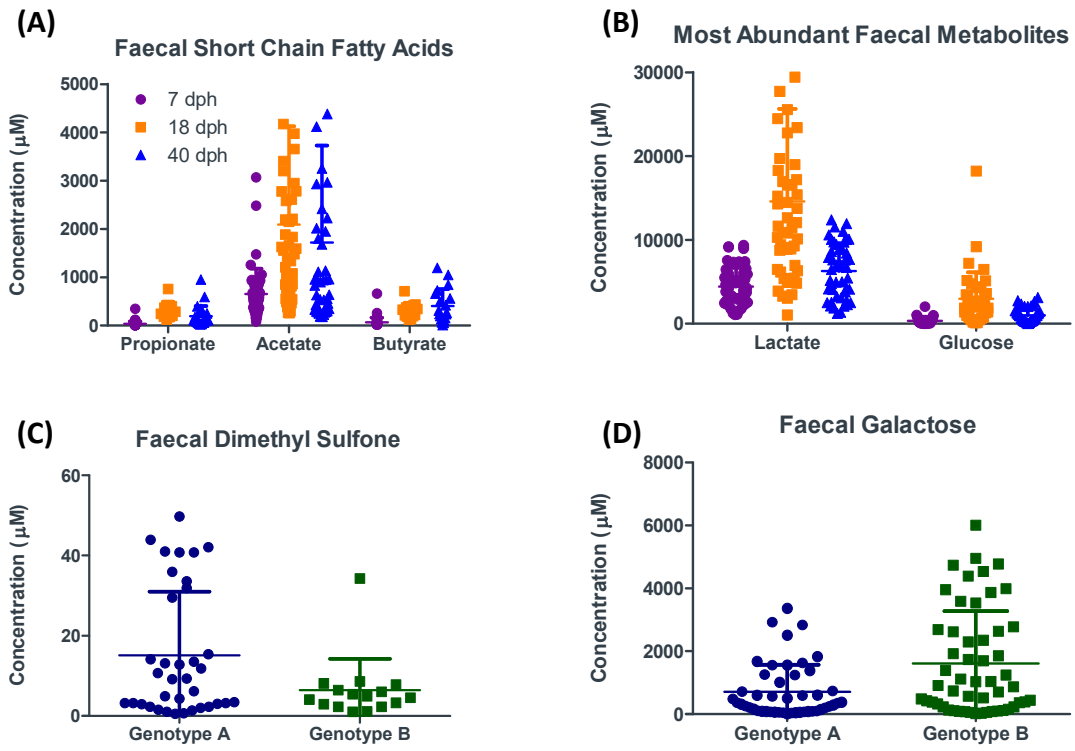


Figure 7.26 Faecal metabolite concentrations of (A) SCFAs, with acetate significantly lower at 7 dph (ANOVA, P=<0.0001) and (B) lactate and glucose at different time points with significantly higher concentrations at 18 dph (ANOVA, P=<0.0001). Faecal metabolites with difference in genotypes throughout the broiler life cycle: (C) dimethyl sulfone and (B) galactose.

No significant correlations were observed between performance metrics and faecal metabolites, consistent with the findings reported in CHAPTER 5 at 13 dph. However, it is noteworthy that the sugar alcohol arabinitol exhibited significantly higher levels (t-test, P= <0.0001) in higher-performing broilers at 18 dph (Fig. 7.27).

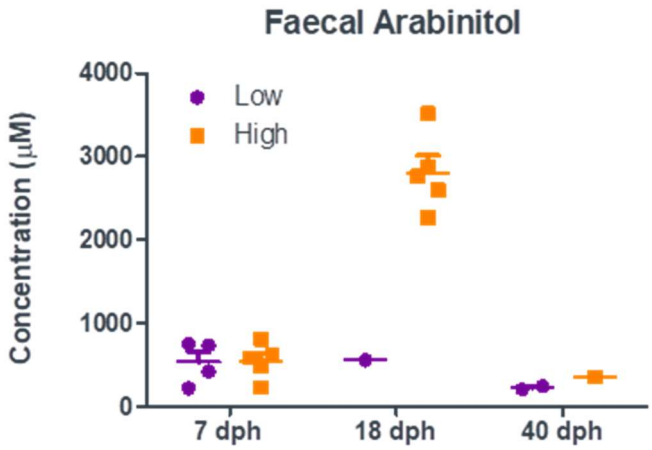


Figure 7.27 The difference in faecal arabinitol concentration between higher and lower performing broilers at different ages.

Identifying the D-arabinose degradation pathway I (DARABCATK12-PWY) in faecal samples at 7 dph, as revealed by Humann3 data, is particularly intriguing. This pathway is associated with yeast (Kordowska-Wiater, 2015). The yeast *Diutina* was significantly more abundant in high-performing broilers however the genus does not appear to have any genes for arabinitol production. It is plausible that other yeast harbouring this specific gene may have contributed to the increased production of arabinitol observed in the faeces of higher-performing birds at 18 dph.

7.3.5 The Serum Metabolome at Difference Stages of the Broiler Life Cycle

A comprehensive analysis of broiler serum revealed the identification of 101 metabolites across three distinct time points, with 47 significant differences observed between these time points. At 18 dph, 53 metabolites exhibited significant variations, followed by 39 differences detected at 40 dph. In contrast, only four metabolites displayed significant differences at 27 dph (refer to table in Appendix 6). These findings mirror the trends observed in the faecal metabolome analysis, wherein the 18 dph time point emerged as the period with the most pronounced metabolic alterations compared to other time points. This highlights the significance of 18 dph as a pivotal time point characterised by dynamic metabolic activity.

The metabolite malonate demonstrated the most significant difference, exhibiting its highest concentration at 40 dph, while melatonin levels were lowest at 27 dph. Melatonin, previously detected in the serum of all broilers in CHAPTER 6, appears to be a host metabolite rather than bacterial, given its well-established role in circadian rhythms (Schwean-Lardner et al., 2012a, Schwean-Lardner et al., 2012b). Additionally, there was a decrease in TMAO concentration from 18 to 27 dph (Fig. 7.28), which was another metabolite found in the serum of all broilers in CHAPTER 6.

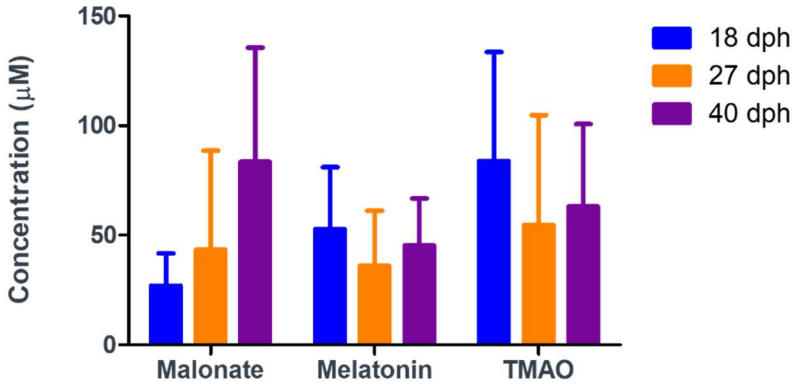


Figure 7.28 Representative serum metabolites with a significant difference in concentration between time points.

Several significant differences in serum metabolite concentrations between low and high-performing broilers were observed however there was some overlap (Fig. 7.29). These discrepancies suggest distinct metabolic adaptations in high-performing birds. Elevated allantoin levels in high-performing broiler hint at heightened purine catabolism (Yamauchi et al., 2020). Increased levels of 4-carboxyglutamate may signal shifts in protein synthesis pathways (Zhang et al., 2022). The metabolites associated with the tricarboxylic acid (TCA) cycle underscore its pivotal role in energy production and metabolic regulation. Higher malonate levels in low-performing birds contrasts with high-performing birds' elevated guanidinosuccinate and malate levels. This may indicate differences associated with and enhanced energy production (Mookerjee et al., 2021). Additionally, heightened concentrations of homovanillate and N-acetylaspartate in high-performing birds which have been linked to neuroendocrine and neurological health (Wang et al., 2009, Janik et al., 2016).

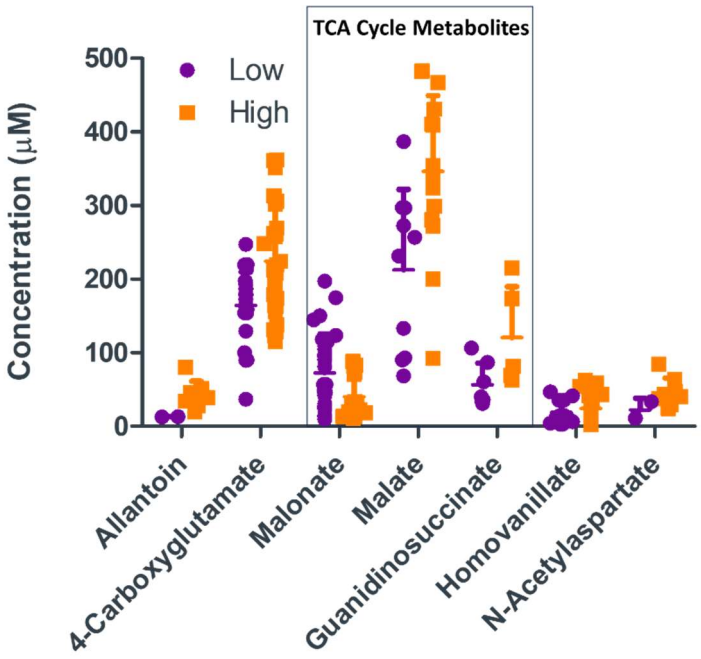


Figure 7.29 Serum metabolites that significantly differ between lower and higher performing broilers.

In this chapter, six metabolites of interest were identified (Fig. 7.30), which had been previously described in broilers at 13 dph in CHAPTER 5 and at 35 dph in CHAPTER 6. However, it should be noted that these metabolites were not consistently found in all broilers and demonstrated no significant relationship to biological efficiency, performance, or gut health. Additionally, the serum of these broilers did not contain propionate, erythritol, or glutathione, which were previously linked to performance in CHAPTER 6.

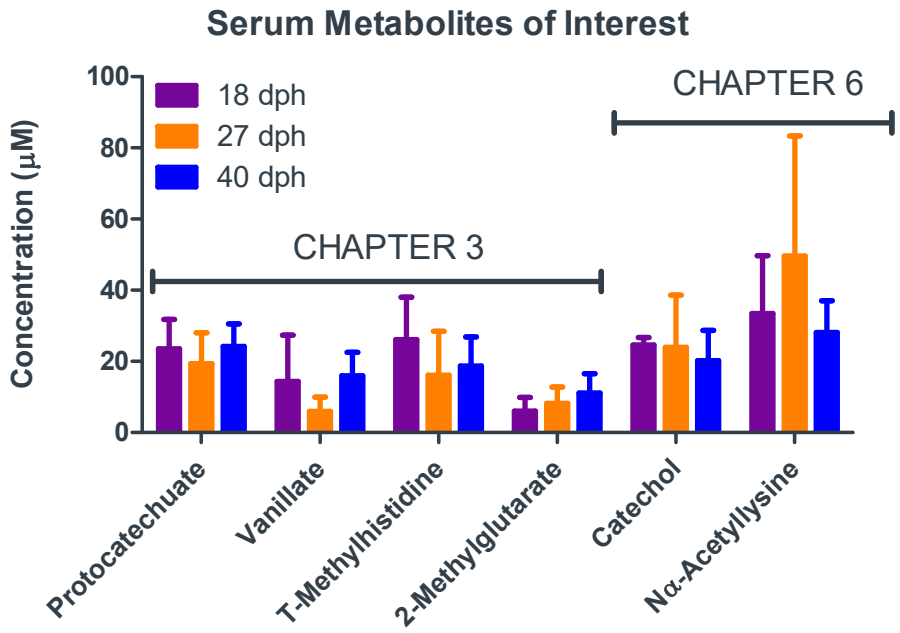


Figure 7.30 Serum metabolite concentrations of potential markers of health identified in previous chapters.

7.3.5.1 *Trimethylamine Oxidation Metabolism in Broiler Serum and Faeces*

A decrease serum in TMAO over time was described in Fig. 7.28. However, no other significant correlations were observed between serum or faecal metabolites and performance or gut health. Despite the declining trend in trimethylamine oxidation compounds, TMAO and betaine, both synthesised from choline, were consistently higher in serum than in faeces across all ages (Fig. 7.31A-C). Conversely, choline levels were significantly higher in faeces at all ages. TMA was elevated in faeces only at 18 dph, coinciding with the heightened metabolic activity observed in both faeces and serum. Additionally, all trimethylamine oxidation compounds were elevated in faeces at 18 dph (Fig. 7.31D), while serum betaine and choline levels remained consistent throughout (Fig. 7.31E).

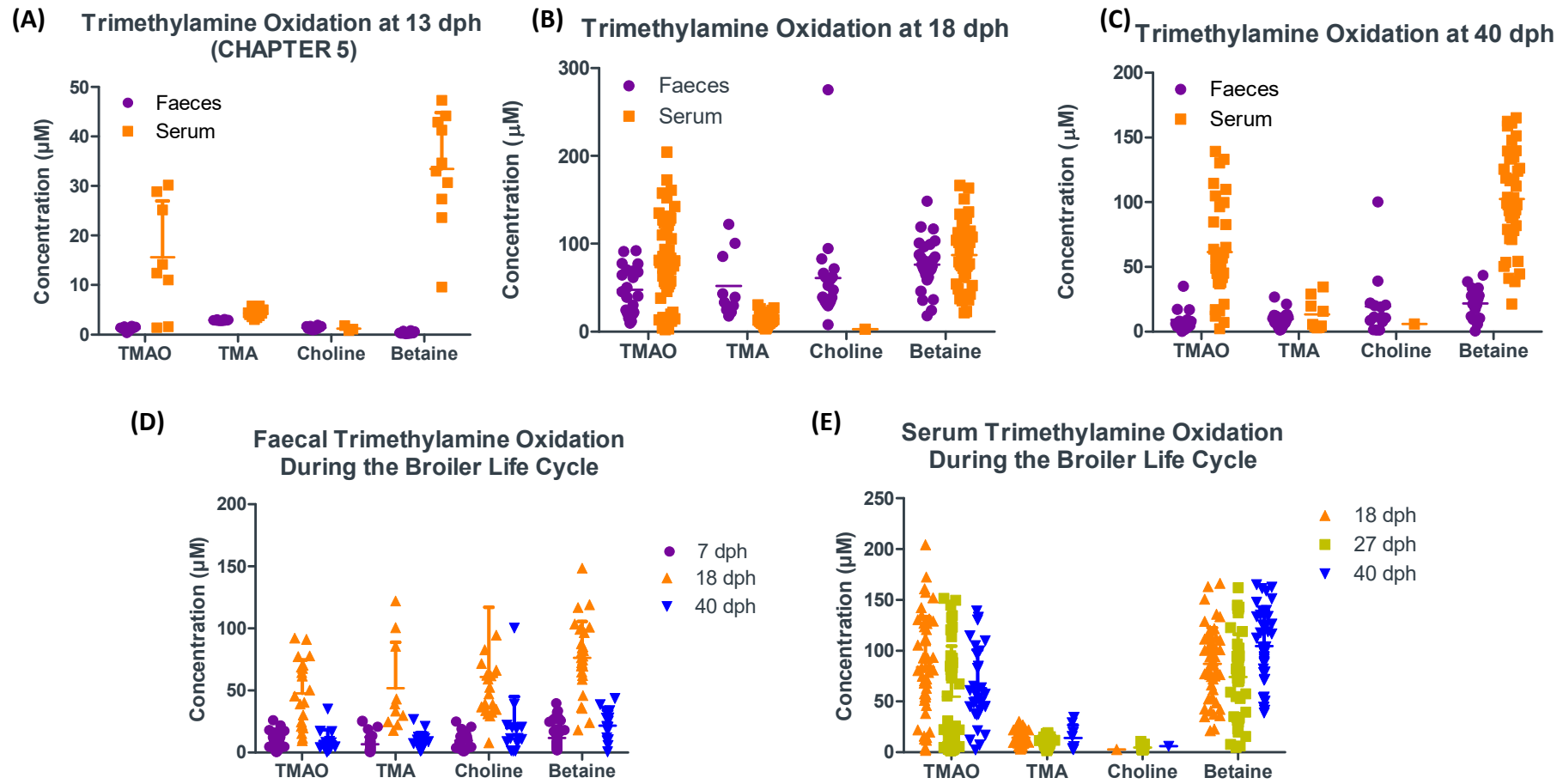


Figure 7.31 Trimethylamine oxidation compounds in broiler faeces and serum at (A) 13 dph (from CHAPTER 5), (B) 18 dph and (C) 40 dph. Trimethylamine oxidation metabolites in broiler faeces (D) and serum (E) over time.

A negative trend between TMAO levels and microbiome β- can be observed (Fig. 7.32). However, these correlations accounted for less than 10% of the variance, suggesting that microbiome diversity has a relatively modest impact on these metabolites.

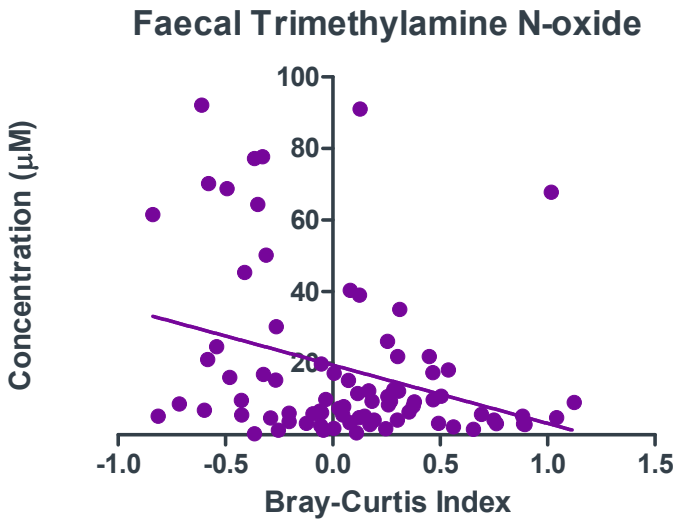


Figure 7.32 Negative trend between microbiome β-diversity (Bray Curtis) and faecal TMAO concentrations (P = 0.003, R^2 = 0.1).

The decline in faecal TMAO concentration over time may be influenced by several factors, including decreased activity of TMA-producing bacteria. Enhanced gut barrier integrity could potentially limit the passage of TMA from the intestines into the bloodstream, where it is subsequently converted to TMAO in the liver. These dynamics suggest interplay between gut microbiota composition and TMAO metabolism in chickens, an area that has not been extensively studied beyond the role of TMA production in the ceca and SI, famously linked to the "fishy egg" consumer issue in the 1970s (March and MacMILLAN, 1979). Managing TMA levels is crucial not only due to their volatile impact on products but also because of their association with cardiovascular health issues in humans, given that animal-protein constitutes a primary source of TMA in human diets (Anderson et al., 2022). Exploring this metabolism within the gut microbiome could potentially provide a foundation for establishing faecal or serum markers. Metabolites from this pathway offer robust detectability across various sample collections, time points, and sample types, highlighting their potential as valuable indicators.

7.3.6 Natural Antibody Titres at Difference Stages of the Broiler Life Cycle

Average IgT titres were 1.5 (SD ± 0.5) at 18 dph, 2.6 (SD ± 0.5) at 27 dph and 13.5 (SD ± 2.0) at 40 dph (**Fig. 7.33A**) with a significant increase at each age (ANOVA, $P < 0.0001$). In contrast to the observed significant age-related increases in other NAb titres, IgA showed a distinctive pattern. Specifically, serum IgA levels we higher at 18 dph compared to 27 and 40 dph (**Fig. 7.33B**). This elevation in IgA aligns with the heightened faecal and serum metabolic activity. Although IgA levels were significantly higher at 40 dph than at 27 dph (ANOVA, $P = 0.04$), they remained lower than those observed at 18 dph.

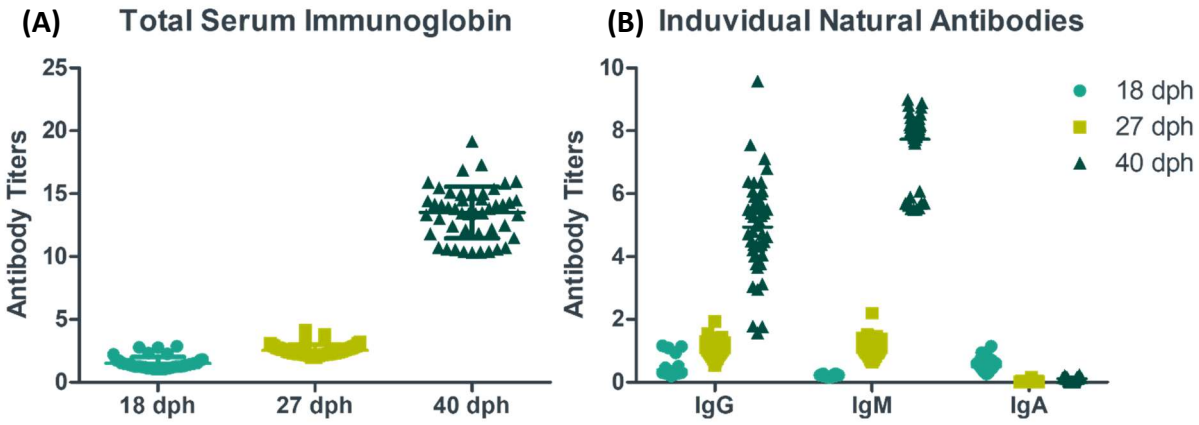


Figure 7.33 Total serum NAb titres (A) and individual NAb titres (B) at three time points.

Several small differences were observed between genotypes (Fig. 7.34) although these did not show a consistent pattern like what is observed over time. In genotype A, IgM and IgA were highest at 27 dph and IgG highest at 40 dph. In genotype B, total immunoglobin and IgG levels were highest at 18 dph and IgM at 40 dph.

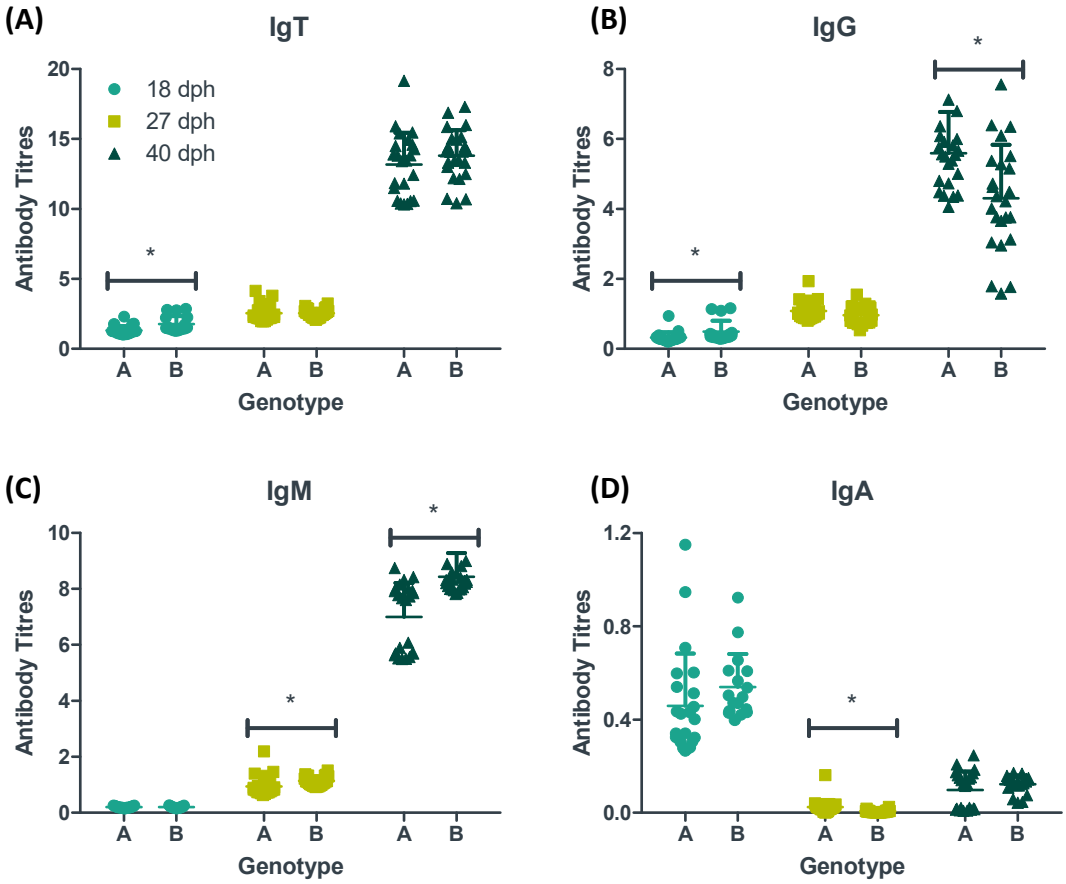


Figure 7.34 Difference in IgT (A), IgG (B), IgM (C) and IgA (D) NAb titres between genotypes at three-time points, determined with a non-parametric t-test.

NAb titres exhibited no correlation with the faecal microbiome, mycobiome, metabolome, morphological structures, or water retention. Linear regression modelling revealed positive relationships between serum IgG, IgM and several serum metabolites (Fig. 7.35A). Specifically, N-methylhydantoin, cis-aconitate, and dimethyl sulfone exhibited increased concentrations in serum at 40 dph compared to 27 dph. Conversely, 1,3-Diaminopropane displayed a significant relationship with serum IgM only. TMA was the sole metabolite demonstrating a negative correlation with IgM. Malonate displayed higher concentrations in low-performing broilers. Additionally, creatinine, citrate, and glycocholate were significantly elevated in serum at 40 dph, showing correlations with IgG. These relationships suggest an age-related effect rather than direct correlations. Unfortunately, none of these correlations aligned with those identified in CHAPTER 6.

The significant relationships observed between serum metabolite concentrations and serum IgA were all positive and distinct to this NAb (Fig. 7.35B). IgA displayed intriguing

correlations with serotonin and TMAO. The correlation between IgA and serotonin is particularly noteworthy, as previous research indicates that IgA levels increase in chicks raised in enriched environments, characterised by high levels of stimulation and welfare considerations (Di Marcantonio et al., 2022). This suggests a potential link between welfare and IgA production. Similarly, the association between IgA and TMAO raises questions, given the observed decrease in TMAO levels in serum over time and its negative relationship with β-diversity.

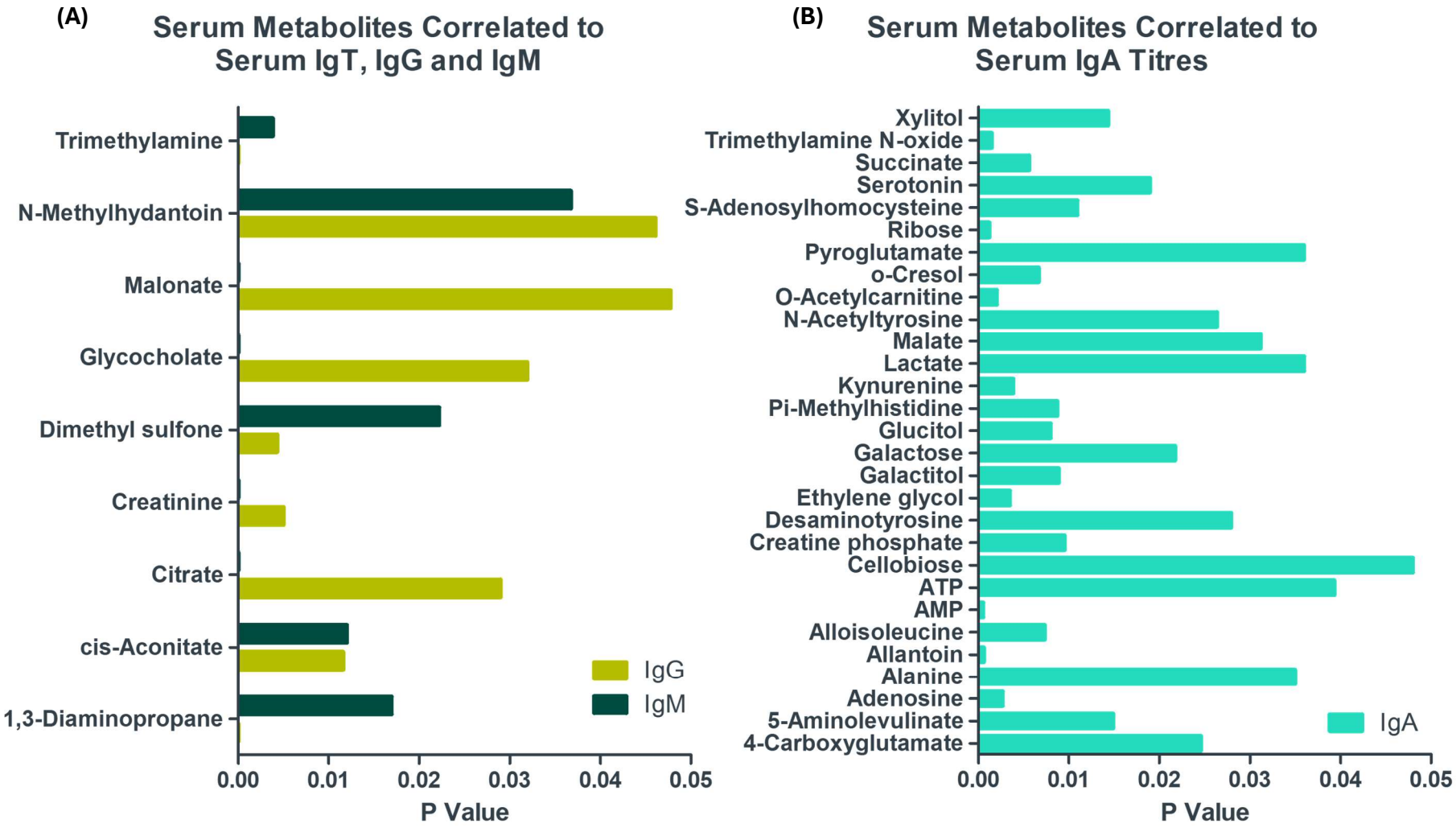


Figure 7.35 The P values for significant positive relationships between serum metabolites and serum NAb titres IgG and IgM (A) and IgA (B).

7.4 DISCUSSION

Utilising body weight and BWG as metrics for evaluating performance is a common practice (Lopes et al., 2013, Barbosa et al., 2017). While body weight is the basis for calculating BWG, it alone does not provide insights into feed efficiency. While maintaining consistently high body weight in broilers is advantageous, prioritising broilers capable of achieving a growth rate of 94% over those achieving 91% is more beneficial. Admittedly, the differences in performance observed are not substantial; however, they remain a valuable metric for assessing biological efficiency in differentiating broiler chickens. Even minor improvements in biological efficiency can have significant economic and environmental benefits when scaled across the entire poultry industry. The absence of data on feed efficiency complicates this evaluation. It is plausible that broilers exhibiting superior growth rates may incur higher costs attributable to increased feed consumption than the average bird within the flock.

Water retention (Ruiz-Palacios et al., 1981, Sanyal et al., 1984) and SI morphological structures, such as villus height and crypt depth (De Verdal et al., 2010), provide an insights to broiler gut health. Inadequate water reabsorption in the colon may signal poor gut health when considered with other factors such as microbiome community structure. Assessing morphological structures is a standard method to evaluate gut development and physiology (Zhang et al., 2015). A high villus-to-crypt ratio indicates longer, healthier villi and shorter, less active crypts, implying efficient nutrient absorption and a well-maintained intestinal lining (De Verdal et al., 2010). Longer villi provide greater surface area for nutrient absorption, while shorter crypts suggest balanced cell turnover, minimising disruptions to the intestinal lining. Conversely, a low villus-to-crypt ratio suggests shorter villi and deeper or elongated crypts, potentially indicating intestinal damage or disease. Shorter villi reduce nutrient absorption surface area, while deeper crypts imply increased cell turnover, often associated with inflammation and less effective nutrient absorption, leading to potential deficiencies (Kaspers and Schat, 2012). In this study, there were no differences in the morphological structures between genotypes at 40 dph or in relation to the faecal microbiome or metabolome.

7.4.1 Genotypic Disparities in Performance and Gut Health

Genotype A demonstrated slightly improved body weight and weight gain performance, whereas genotype B exhibited slightly better gut morphology, as evidenced by significantly higher villus-to-crypt depth ratio. Vacuoles in the SI epithelial wall were predominantly observed in genotype A, with only one bird from genotype B displaying a vacuole. Vacuoles in the SI lining, especially below the crypts, indicate pathological conditions or physiological processes such as infections or inflammation (Yamauchi and Tarachai, 2000, YAMAUCHI, 2007).

This underscores the independent nature of performance metrics, gut health parameters and the complexity of the microbiome. A limitation of this study was a lack of broilers with considerably inferior performance or gut health therefore optimal performance did not necessarily correlate with favourable gut health outcomes, which is the opposite of many reviews on the subject (Diaz Carrasco et al., 2019, Bindari and Gerber, 2022, Ducatelle et al., 2018b, Ducatelle et al., 2018a). Only three broilers concurrently demonstrated growth rates exceeding 94% and the highest villus-to-crypt ratio, indicating a limited overlap between exceptional performance and robust intestinal development. These findings emphasise the importance of considering multiple physiological parameters when assessing broiler health and performance.

The slightly improved biological efficiency observed in genotype A may be attributed to various factors beyond what was investigated in this chapter, including genetic characteristics related to growth and metabolic processes (Barbosa et al., 2017, De Verdal et al., 2010). Genotype B exhibited higher α diversity at 18 dph and increased β diversity at 7 and 40 dph. This diversity at the earlier time point may play a pivotal role in shaping morphological structures within the gut (Neveling et al., 2017).

The elevated α-diversity observed in genotype B at 18 dph could potentially contribute to enhanced gut health by fostering a robust and resilient microbial community (Kogut, 2013, Kogut, 2019). This enhancement may promote improved gut barrier function, competitive exclusion of pathogens, and immune modulation (Stanley et al., 2014).

The differences in NAb titres between genotypes observed at 18 dph stabilise by 27 dph, coinciding with the differentiation of IgM and IgA titres between the genotypes. By 40 dph, significant variations in IgG and IgM titres become apparent, suggesting the activation

of the immune system. The microbiome plays a crucial role in supporting immunological development (Broom and Kogut, 2018), with successional changes influencing immune exposure and antibody production (Oakley and Kogut, 2016). Genotype B displayed heightened levels of IgA at 18 dph, underscoring further immunological differences between the genotypes.

7.4.2 Age-Dependent Dynamics: Impact on Performance and Gut Health

In addition to observing a significant relationship between the microbiome and mycobiome faecal diversity at 7 and 18 dph, similar trends were identified at 7 and 13 dph in both CHAPTER 4 and CHAPTER 5. This implies that during the early stages of broiler development, a close interplay exists between the microbial and fungal communities within the GI tract. An important contribution of this research is the identification of a core mycobiome, a novel discovery not previously documented in the literature (Robinson et al., 2022a, Robinson et al., 2020c, Davies et al., 2022). This core mycobiome implies that fungal communities within the GI tract are not only transient but consist of resident populations undergoing successional changes over time. For example, *Aspergillus*, *Filobasidium* and *Melanodothis* significantly increased over time and were abundant at all ages whereas *Barnettazyma* was not in any faecal samples before 27 dph.

The microbiome undergoes dynamic successional changes over time (Robinson et al., 2022b), with distinct shifts in taxa composition observed throughout the developmental stages (Jurburg et al., 2019). Initially, lactic acid-producing bacteria predominate (Yeoman et al., 2012), likely due to their early colonisation capabilities and rapid growth rate (Adhikari and Kwon, 2017). Similarly, *E. coli* exhibits a significant decrease over time, which is characteristic of its role as an initial coloniser that becomes less abundant in later stages (Yeoman et al., 2012). In this study, the microbiome composition at 40 dph is characterised by an increased presence of less abundant taxa, indicative of a more diverse microbial community. Of particular interest is the significant elevation of *Akkermansia muciniphila*, notably higher in genotype B, associated with improved gut health in mice (Zhao et al., 2019). Recent research has underscored the potential of *Akkermansia* as a probiotic agent with anti-inflammatory properties after being found to associate with increased *Lactobacilli* in high-fibre diets (Hou et al., 2020, Such et al., 2019), however, it must be used cautiously

as it has been demonstrated to promote necrotic enteritis (Yang et al., 2022). *A. muciniphila* protects against viral infections in mice (Hu et al., 2021). Further exploration of *Akkermansia muciniphila*'s functional role within the microbiome could unveil novel insights into its therapeutic potential in poultry health management.

At 7 dph, broilers displayed high GI metabolic activity, characterised by higher levels of specific faecal metabolites compared to later timepoints, such as N-acetylglucosamine and allantoin. Analysis of broiler samples across various developmental stages unveiled significant fluctuations in serum Ig and metabolites at 18 dph. Total immunoglobin, IgG and IgA had the highest titres at 18 dph and the highest serum metabolite concentrations, suggesting considerable metabolic activity on the grower diet. A surge in metabolic activity, observed through both faecal and serum metabolites noted at 18 dph. In the literature, a relatively stable metabolome would be expected by 20 dph (Yeoman et al., 2012, Oakley et al., 2014). Additionally, elevated IgA levels at this time point underscored its significance in the context of the microbiome, as some bacteria possess receptors for IgA, influencing mucosal integrity (Lillehoj and Lee, 2012, Pabst and Slack, 2019).

At 27 dph, genotype A broilers exhibited heightened IgM and IgA levels, indicating ongoing immunological development. By 40 dph, while immune markers continued to evolve, metabolite levels remained relatively stable, suggesting a potential equilibrium in metabolic processes. The immune response activation was evidenced by the progressive increase in serum NAb titres over time, with notable elevations observed in total Ig, IgG, and IgM levels. However, IgA levels peaked at 18 dph and declined by 27 dph, indicating a distinct pattern compared to other Ig. Contrary to expectations based on existing literature but in line with CHAPTER 6 findings, the serum had higher IgM titres than IgG at 40 dph.

Anomalies were observed in the sequencing process at 27 dph, where a considerable number of samples failed QC during shotgun metagenomic sequencing. These samples underwent processing and sequencing procedures identical to those of the remaining samples, and there were no discernible differences in genomic DNA yield. This occurrence coincided closely with the transition from the grower to the finisher diet, suggesting a potential association with dietary composition. For instance, the presence of excessive acidity in faecal samples may have adversely impacted downstream analytical procedures (Pankoke et al., 2019, Rothrock Jr et al., 2014). Further investigation into the

specific dietary factors influencing sample integrity is warranted to mitigate such discrepancies in future analyses.

7.4.3 Performance Metrics: Correlations and Influences

Biological efficiency metrics yielded significant insights for this study. The analysis revealed no positive correlation between NAb levels and body weight or morphological structures. These results are in align with CHAPTER 6, which also failed to establish any significant correlation or relationship between NAb titres and improved performance metrics, such as low FCR and high body weight. These findings collectively suggest that NAb titres may not serve as reliable indicators of broiler performance, with other factors exerting greater influence on performance outcomes.

Alistipes has been suggested as a marker of a mature microbiome (Richards et al., 2019). The presence of multiple *Alistipes* species demonstrated positive relationships with body weight. However, its abundance increased over time, indicating that this correlation may be influenced more by age than by weight alone. This supports the literature proposing *Alistipes* as a microbiome marker for GI microbiome maturity. Conversely, *L. aviarius* exhibited a notable increase in abundance over time and was significantly more prevalent in low-performing broilers. This suggests a potential link between the proliferation of *L. aviarius* and reduced performance in broilers. This finding aligns with the known polarising effects of *Lactobacilli* (Zou et al., 2018) and may be due to competition among *Lactobacillus* spp., indicating that *L. aviarius* might be competing with more beneficial bacteria. Understanding these microbial interactions could provide insights into optimizing the gut microbiota for improved broiler performance. In contrast, the yeast *Diutina* displayed a significantly higher relative abundance in the faeces of high-performing broilers. This highlights the potential beneficial role of *Diutina* in promoting improved performance outcomes. Furthermore, high faecal arabinitol concentrations at the intermediate time point of 18 dph in high-performing birds, coupled with elevated faecal and serum metabolic activity, suggests that arabinitol could serve as a potential biomarker for performance. Arabinitol is produced by yeasts, such as *Debaryomyces* and *Saccharomyces*, as a by-product of sugars, such as glucose (Kordowska-Wiater, 2015). High-performing broilers may

have a mycobiome that is more capable of sugar metabolism resulting in more faecal arabinitol to be detected.

TMAO may also serves as an indicator of microbiome maturity. Reduced serum TMAO levels over time suggest decreased conversion of TMA to TMAO, possibly due to diminished TMA production (Shehata, 2022) or altered liver function (Gatarek and Kaluzna-Czaplinska, 2021). Lower faecal TMA levels indicate reduced activity or abundance of TMA-producing gut bacteria (Rath et al., 2017). Elevated faecal choline and betaine levels suggest an underutilisation of these substrates by the microbiota, potentially due to decreased activity of metabolising bacteria (Barrett and Kwan, 1985). This indicates a microbiome shift reducing TMA production from choline and betaine (Shehata, 2022). A potential faecal marker is high faecal choline and betaine with low TMA, indicating a healthier microbiome. Establishing baselines for serum and faecal TMA/TMAO levels is essential. TMAO was consistently detected in the serum of all 200 broilers in CHAPTER 6 and in the faeces and serum of 10 broilers at 13 dph in **CHAPTER 5** and was detectable as early as 7 dph in CHAPTER 4. Consistently measuring TMAO in serum and faeces, driven by the GI microbiome, is valuable for biomarker use. Serum TMAO levels could track microbiome development, decreasing as the microbiome stabilises. Simultaneously, serum TMA levels could gauge optimal performance, as they negatively correlate with body weight. Manipulation of TMA and TMAO through microbiome management has been demonstrated in humans (Li et al., 2021b) (independent of diet (Ferrell et al., 2021)) and in rats (Jaworska et al., 2017).

7.4.4 Gut Health Markers: Insights and Implications

Microbial diversity has been widely recognised as an indicator of gut health (Díaz-Sánchez et al., 2019, Ducatelle et al., 2018b). However, these investigations have highlighted the need to broaden our perspective beyond bacterial diversity alone. Emerging evidence suggests a positive relationship between the microbiome and mycobiome's diversity in soil samples (de Menezes et al., 2017, Jiao et al., 2021b). This finding challenges the notion that focusing solely on bacterial diversity provides a comprehensive understanding of gut health. Instead, it underscores the interconnected nature of the microbial ecosystem, where the bacterial and fungal components interact (Jiao et al.,

2021a). By considering the diversity of both bacterial and fungal communities, researchers may gain deeper insights into the complex dynamics of the gut microbiota and its impact on broiler health and performance. These findings enhance our understanding of the gut microbiome and offer novel avenues for developing targeted strategies to optimise broiler health and productivity.

More virulence factors in the faeces of genotype B compared to genotype A suggest a potential disparity in gut health between the two genotypes. However, the reduction to only one virulence factor by 40 days post-hatch indicates a temporal decline in virulence factor abundance, possibly reflecting a natural progression towards a more balanced gut microbiota composition over time (Clavijo and Flórez, 2018). Alternatively, the presence of more virulence factors in genotype B could indicate a more robust immune response and better intestinal morphology in response to microbial challenges (Clavijo and Flórez, 2018). Genotype B may have developed a more robust immune defence mechanism, resulting in the clearance or containment of these factors more effectively (Koju et al., 2022). Additionally, genotype B exhibited better intestinal morphology, characterised by significantly greater villus-to-crypt ratio and less vacuoles present, further supporting a more resilient gut environment in this genotype.

7.4.5 Conclusive Insights: Integrating Genotypic Variations, Age Dynamics, Performance, and Gut Health

Objective 1 aimed to investigate bacterial successional changes as described in the literature, analyse their correlation with faecal and serum metabolism, and explore their relationship with the faecal mycobiome throughout the broiler life cycle. While the microbiome findings aligned with existing literature, the mycobiome, contrary to expectations of being transient and inconsistent across ages, showed more complexity. The hypothesis was confirmed: bacterial successional changes in broilers aligned with established patterns, were accompanied by alterations in faecal and serum metabolism, and correlated with variations in the faecal mycobiome. Additionally, the diversity of the faecal bacterial microbiome (shotgun metagenomics) correlated with the diversity of the fungal mycobiome (ITS1 rRNA metabarcoding), further validating the proposed hypothesis.

Objective 2 focused on identifying performance markers through weight measurements and BWG assessments. It was hypothesised that broilers with good performance would exhibit favourable gut health characteristics, such as increased body weight, BWG, a high villus-to-crypt ratio, and low water retention. Positive trends were identified, with notable potential markers including *Akkermansia* and *Alistipes*, as highlighted in the literature however limited correlations to biological efficiency and gut health were observed.

For objective 3, gut health markers were examined by evaluating intestinal structure and morphology, as well as faecal water retention. While significant relationships were found, they contradicted expectations based on findings from other chapters as outlined in objective 5. Performance markers identified in previous chapters were assessed for their applicability, but none were validated in this new cohort of broilers. However, metabolic processes such as the TCA cycle and trimethylamine metabolism showed significant differences between performance and gut health parameters across all chapters. High performance did not correlate with significantly higher microbial richness and diversity, but there was a notable presence of *Lactobacillus aviarius* and *Alistipes*. Thus, the hypothesis that performance markers from previous chapters would be applicable and consistent was disproven.

The attainment of the 4th objective culminates at 18 dph, marking a pivotal juncture in the study. Positioned as an intermediate time point, it presents a strategic window for potential interventions. Notably, this age witnesses the peak of metabolic activity, both in faecal and serum samples.

CHAPTER 8

GENERAL DISCUSSION

8.1 GENERAL DISCUSSION

Identifying biomarkers of intestinal health in commercial poultry, particularly broilers, is a research area of immense promise. This work provides insights into the microbiome, mycobiome, metabolome and immune system. Given the substantial population of chickens, particularly broilers, the potential impact of this research on the poultry industry is significant. (Nations, 2019) One of the key aspects of this thesis was the emphasis on non-invasive methodologies, which are designed to minimise animal stress (Videvall et al., 2018). This approach is not only ethically sound but also strategically important. Another promising avenue is routine blood collection in veterinary practices, potentially leading to identifying detectable biomarkers within serum samples, offering significant advantages for the poultry industry.

The first objective of this research was to validate and refine methodologies for assessing broiler gut health. This involved selecting appropriate sample types, optimising genomic DNA extraction techniques, and evaluating various sequencing methodologies. The goal was to develop a robust suite of tools that provide comprehensive insights into broiler gut health, enhancing our understanding of their overall gut health and performance. The methodologies established and validated in CHAPTER 3 achieved this aim.

Shotgun metagenomic sequencing offered a broader perspective on the faecal microbiome and provided more profound insights into the intestinal ecosystem than 16S rRNA gene amplicon sequencing (Gilroy et al., 2021). When applied to broiler faeces, shotgun metagenomic sequencing retrieved more information on low-abundance taxa and achieved a higher taxonomic resolution than 16S sequencing, consistent with the literature expectation that 16S rRNA analysis recovers less than 31% of the genera compared to shotgun metagenomic sequencing (Durazzi et al., 2021, Campanaro et al., 2018, Laudadio et al., 2018).

The observed similarity in microbiome composition from shotgun metagenomic data across different collection dates and ages throughout this thesis affirms what has been published on the microbiome using 16S rRNA barcoding (Yeoman et al., 2012, Jurburg et al., 2019, Schokker et al., 2021). Utilising both shotgun metagenomic and ITS1 rRNA amplicon sequencing enabled comprehensive profiling of bacteria and fungi and facilitated

the exploration of functional genes such as those associated with AMR and virulence. However, the study also highlighted certain limitations and areas for improvement. Specifically, the analysis of viral populations faced challenges due to insufficient reads, indicating that current shotgun metagenomic sequencing techniques are inadequate for a comprehensive description of the viral component of the microbiome. These methods struggled to distinguish between free and integrated bacteriophages, pointing to the need for more specialised methodologies to enrich and separately capture the bacteriophage population. Despite these limitations, the study provided valuable insights into the viromes of different GI locations, as discussed in CHAPTER 5.

Faecal sampling is non-invasive and allows longitudinal studies of the same broilers despite this sampling method being described as variable (Schokker et al., 2021). This thesis found that the faecal microbiome diversity and major taxa resembled the successional changes of the GI microbiome composition described in the literature (Yeoman et al., 2012, Jurburg et al., 2019, Schokker et al., 2021) and there were still small but significant differences in abundance that could be detected. Admittedly the faeces do not capture the density and diversity of bacteria to the same capacity as the caecal content, which is most commonly researched (McNab, 1973, Ocejo et al., 2019b, Di Marcantonio et al., 2022, Kairmi et al., 2022), but there was overlap between faeces and caecum as well as faeces and SI, although there was no overlap between the caecum and the jejunum. All these GI locations have different functions and different microbiomes (Glendinning et al., 2019, Gong et al., 2007, Yeoman et al., 2012, Shang et al., 2018), no single GI location can fully represent the entire tract.

Including untargeted metabolomics allowed for identifying and quantifying various metabolites within different sample types. The serum metabolome was reproducible. The faecal metabolome was expected to be reproducible (Beauclercq et al., 2019b) but instead varied across collection dates in CHAPTER 4. However, this could also be because the faecal metabolome at 7 dph is too variable between different flocks. Serum metabolome analysis proved effective in distinguishing between high performing and lower performing broilers based on their body weight at 13 dph, highlighting its potential as a valuable indicator for assessing early-stage performance. No relationship to performance and gut health with lactate was observed, suggested in the literature as a marker (Lei et al., 2013), which may be detected at higher concentrations in serum if the gut barrier function is suboptimal but

requires knowledge of the initial host concentration. Lactate and glucose had the highest concentration and were most easily detectable at all ages across all sample types. There were higher faecal lactate concentrations in chick faeces compared to adults, which is consistent with the high presence of lactic-acid-producing bacteria.

The second objective of this research was to investigate the microbial landscape of broilers beyond bacteria and the functional metabolome to elucidate the interrelationships among different microbial components across various life stages of broilers. The exploration of fungal communities proved to be valuable and insightful. Fungal species richness and evenness might play a role in determining microbiome stability (Robinson et al., 2022b), yet current literature predominantly focuses on bacteria, overlooking contributions from fungi and viruses to GI microbiome stability. A positive trend was observed between fungal and bacterial diversity in faecal samples collected at 7 and 18 dph in CHAPTER 7 and in SI and caecal samples, as well as faecal samples at 13 dph in CHAPTER 6. This finding underscores the importance of considering fungi as a key microbiome component, alongside bacteria, mainly as chicks, as this trend was not observed in 27 and 40 dph faecal samples. Fungi are a source of antibiotics (Karwehl and Stadler, 2016) and may explain the presence of AMR on this farm where antibiotics have never been used. It was notable that the ITS sequencing provided much more information than shotgun metagenomics, but future improvement of sequencing depth and fungal databases may reduce this gap.

The third objective of this research was to identify and establish suitable markers indicative of broiler gut health and performance. The goal was to facilitate the adoption of these markers within the poultry industry to improve gut health and productivity levels. The research successfully identified several key microbial and metabolic markers that correlate with broiler gut health and performance in CHAPTER 5, CHAPTER 6, and CHAPTER 7. Still, these results were not reproducible, as the same faecal and serum metabolites or taxa did not consistently associate with biological efficiency in all chapters.

The presence of *Akkermansia municipia* and *Alistipes* spp. was found to correlate positively with weight and overall performance in CHAPTER 7, warranting further exploration of their potential as a valuable biomarker for assessing broiler health. It was found in faeces from 27 dph and showed a positive trend in weight. Recent research has underscored the potential of *Akkermansia* as a probiotic agent with anti-inflammatory properties after being found to associate with increased *Lactobacilli* in high-fibre diets (Hou

et al., 2020, Such et al., 2019), however, it must be used cautiously as it has been demonstrated to promote necrotic enteritis (Yang et al., 2022). *Akkermansia muciniphila* is associated with improved gut health in mice (Zhao et al., 2019) and protects against viral infections in mice (Hu et al., 2021). Further exploration of *Akkermansia muciniphila*'s functional role within the microbiome could unveil novel insights into its therapeutic potential in poultry health management. *Alistipes* is considered an indicator of the mature microbiome in chickens (Richards et al., 2019). In CHAPTER 7, the presence of multiple *Alistipes* species demonstrated positive relationships with body weight. However, the abundance increased over time, indicating that this correlation may be influenced more by age than by weight alone.

The composition of *Lactobacilli* at 7 dph emerged as a possible factor for performance, highlighting the significance of early-life gut microbiota in influencing long-term productivity. *Lactobacilli* can have a polarising effect on the microbiome (Zou et al., 2018). They are an important source of probiotics (Pokorná et al., 2019, Adhikari and Kwon, 2017), critical for immune development (Gonmei et al., 2019) and pathogen resistance (Okamoto et al., 2018), but the composition of *Lactobacilli* species is important (Angelakis and Raoult, 2010), which was seen in this thesis. Higher performers had higher abundances of *L. reuteri*, *L. crispatus*, and *L. gallinarum* in faeces at 7 dph, whilst lower performers had higher relative abundance of *L. vaginalis* and *L. johnsonii* at 7 dph. The longitudinal study revealed *L. aviarius* in low performers despite only being present in the faeces of 3 birds at 7 dph. *Lactobacillus aviarius* was also highly abundant in March 2020, 7 dph, the collection date, with the lowest body weight at 7 and 35 dph. *L. salivarius* is said to improve body growth (Yang et al., 2023). It was detected, but there were no significant differences or associations with performance or gut health.

Another significant finding was the consistent association of the yeast *Diutina* with better performance in the longitudinal study and in 7 dph faeces from the March 2020 collection. This implies that *Diutina* could positively affect performance or serve as an indicator of optimal performance in broilers. *Diutina* can remove phosphorus from an environment (Sun et al., 2021), relevant to the gut microbiome and poultry nutrition (Selle and Ravindran, 2007) and farm pollution. *Diutina rugosa SD-17* isolated from chicken faeces has been proposed as a potential chicken probiotic due to improved glucan production, transient colonisation of the intestinal tract, high adhesion to Caco-2 cells and

ability to resist a simulated intestinal environment (Wang et al., 2019b). *Diutina* has demonstrated significantly improved morphological structures, FCR, body weight and increased T lymphocytes (Wang et al., 2019a), suggesting improved resistance to pathogens.

Trimethylamine metabolism compounds TMA, TMAO, choline, and betaine were highlighted as potential markers in faecal and serum metabolomes and detected in faecal and serum metabolomes at all ages sampled. TMA appears to be particularly important in the early stages of the broiler life cycle, potentially indicating microbiome maturity. TMAO may also serve as an indicator of microbiome maturity. Reduced serum TMAO levels over time suggest decreased conversion of TMA to TMAO, possibly due to diminished TMA production (Shehata, 2022) or altered liver function (Gatarek and Kaluzna-Czaplinska, 2021). Lower faecal TMA levels may indicate reduced activity or abundance of TMA-producing gut bacteria (Rath et al., 2017). Elevated faecal choline and betaine levels suggest an under-utilisation of these substrates by the microbiota, potentially due to decreased activity of metabolising bacteria (Barrett and Kwan, 1985). This would imply a microbiome shift toward reducing TMA production from choline and betaine (Shehata, 2022). A potential faecal marker is high faecal choline and betaine with low TMA, indicating a healthier microbiome. Establishing baseline levels for serum and faecal metabolites involved in trimethylamine metabolism is necessary. However, high levels of TMA at later stages may be undesirable, warranting further investigation into its role and implications on broiler gut health and performance.

Generic loss of bacterial diversity is generally associated with poor gut health and performance (Hou et al., 2020, Rodrigues et al., 2020, Gilroy et al., 2021, Bhagat et al., 2023). It is suggested as a marker for poultry gut health (Ducatelle et al., 2018a). The observation of a positive trend between bacterial and fungal diversity in the faeces of broiler chicks in this study in notable. Fungal diversity was less variable than bacterial diversity, suggesting that changes in fungal diversity may be easier to detect and monitor. The relationship between fungal and bacterial diversity requires further research. Structural equation modelling and random forest analysis have shown that the balance between positive and negative bacterial-fungal associations is linked to soil microbial diversity and nutrient cycling across habitats (Jiao et al., 2021b). This understanding, fundamental to

terrestrial ecosystems, may also apply to the chicken microbiome, reflecting the foundational role of soil microbial ecology in microbiome research.

Broilers in the longitudinal study possessed a core faecal mycobiome during the broiler life cycle; *Wickerhamomyces* were found at a relative abundance of over 0.6% at all time points. *Metschnikowia* exhibited a relative abundance of 0.3-0.4% from 7 to 27 dph. *Wickerhamomyces* and *Metschnikowia* are genera of yeast-like fungi (Kurtzman et al., 2011). *Alternaria* was present within the core mycobiome from 18 dph onwards. This indicates that the broiler mycobiome is not merely transient (Robinson et al., 2022a) but includes resident fungi within the GI microbiome. *Aspergillus*, *Filobasidium* and *Melanodothis* exhibit a significant increase in abundance over time, which may indicate successional changes in the mycobiome or continued existence within the environment. The top genera in the literature saw *Microascus*, *Trichosporon*, and *Aspergillus* as over 80% of the genus (Davies et al., 2022), consistent with the findings that *Microascaceae* and *Aspergillaceae* were in the top five most abundant families for all collection dates in broiler faeces at 7 dph and within the top 20 across the broiler life cycle.

NAbs are a component of the host immune system that are present without external immune interventions, such as vaccination or prior exposure to pathogens (Berghof et al., 2015b). These antibodies exhibit broad specificity but low binding affinity. The levels of NAbs typically increase with age (Kaspers and Schat, 2012), which this thesis has affirmed in CHAPTER 7, possibly due to environmental exposure over time or GI microbiome maturation (Broom and Kogut, 2018). The average antibody titres fell below those reported by Berghof et al. (Berghof et al., 2018) but were detectable in broiler serum as early as 13 dph, which is valuable as previous research was limited to much older layers. There are no differences in NAb titres between genotypes or sex, and they do not seem to be related to biological efficiency in broilers. Modulating and managing NAb titres, such as through selective breeding, could hold promise for enhancing broiler stock immunity, thus providing increased protection against pathogens and diseases (Berghof et al., 2018, Berghof et al., 2019). Furthermore, lower levels of IgM in plasma have been linked to stress, while birds exhibiting high-feather pecking behaviour demonstrate significantly lower antibody titres compared to control and low-feather pecking counterparts (van der Eijk et al., 2019). IgM was unexpectedly higher in serum than IgG in broilers at 35 and 40 dph, which may be an inadvertent effect of breeding as NAb titres are inheritable (Berghof et al., 2015b).

The fourth objective of this research was to establish the optimal age within the broiler life cycle for implementing markers of gut health and performance. A limitation of this research was the lack of birds that were substantially inferior performers or exhibited defined poor gut health, which is why IQR ranges had to be used to categorise performance based on biological efficiency, e.g. body weight, FCR and growth. The selection of birds was random, and they all performed similarly, as indicated by the substantial overlap between groups. This limitation may have constrained the ability to detect more pronounced differences and establish definitive stages for biomarker application.

At 7 dph, faecal samples exhibited similarities in the presence of major taxa and faecal diversity. The overall findings at this age align with established knowledge (Glendinning et al., 2022, Yeoman et al., 2012, Jurburg et al., 2019) and emphasise the importance of early-life microbiota. The first-week post-hatch is critical for the development of the broiler chick microbiome (Ramírez et al., 2020), shaped by initial microbial exposures and subsequent successional changes (Wielen et al., 2002, Zhou et al., 2021). Faecal profiles were collected from birds reared under identical environmental conditions, dietary regimes, and genetic backgrounds over three years. There were some variations between individual birds and within collections, such as biological efficiency and the *Lactobacilli* composition. Taurine was investigated as a marker because elevated bile acid deconjugation may signify an increase in bacterial species with this capability, impacting microbial nutrient availability and thus influencing microbiome composition (Bailey, 2010). Taurine was measured in all metabolome studies and was detectable in faeces as early as 7 dph. It was associated with the body weight of broilers at 7 dph in March 2020. Still, taurine did not consistently correspond to productivity, gut health, or the faecal microbiome. A notable contribution of this research is investigating endogenous phytase activity in broilers, particularly as early as 7 dph. This early assessment is advantageous for the poultry industry, potentially allowing for more effective modulation and management of the microbiome. Understanding and leveraging endogenous phytase activity could offer a sustainable alternative to phytase feed additives (Selle and Ravindran, 2007, Sommerfeld et al., 2019), thereby potentially reducing production costs for poultry farmers.

By 18 dph, there was a notable increase in metabolic activity, and differences between lower and higher-performing broilers were more pronounced. For example, serum arabinitol levels appeared to differentiate performance at this stage, as measured by growth. Arabinitol is produced by yeasts, such as *Debaryomyces* and *Saccharomyces*, as a by-product of sugars, such as glucose (Kordowska-Wiater, 2015). High-performing broilers may have a mycobiome that is more capable of sugar metabolism, resulting in more faecal arabinitol being detected. This metabolic marker could potentially indicate superior performance, warranting further investigation into its reliability and applicability across different broiler populations. The data at 18 dph was limited to a single collection point, restricting the ability to draw robust conclusions about this age as an optimal intervention stage. These findings suggest that this age may be promising for biomarker discovery in broiler chicks due to the observed heightened faecal and serum metabolic activity. Investigations at 27 dph yielded unexpected challenges, as several samples failed during shotgun and ITS1 rRNA metagenomic sequencing. This setback suggests that this age may not be optimal for biomarker identification using current methodologies. Nevertheless, these findings underscore the need for continued research to refine sequencing techniques and explore alternative biomarkers that may be more stable and reliable across different developmental stages.

While many nutritional interventions may not directly influence performance metrics (Yang et al., 2018), they often induce significant changes in serum metabolic profiles, indicating substantial alterations in metabolism. The investigations at 35 dph demonstrate the potential of serum metabolome as a source of biomarkers, having consistently detected 20 compounds in the serum of all broiler chickens; the reproducibility challenge highlighted in the longitudinal study emphasises the need for methodological refinement in biomarker research within poultry science. The ability to reliably measure metabolites in serum at this stage is promising, indicating the feasibility of using metabolic profiling to monitor microbiome changes in broilers. However, the lack of reproducible serum markers demonstrating a relationship to performance or gut health suggests that further refinement and validation are necessary to establish robust biomarkers for this developmental stage.

The faecal microbiome at 40 dph was characterised by the sudden presence of numerous low-abundance taxa. This observation marks a distinct shift in microbial

composition at this developmental stage, which coincides with the endpoint of the broiler life cycle. Identifying these low-abundance taxa highlights the dynamic nature of the gut microbiome during the later stages of broiler development (Jurburg et al., 2019). While these taxa may hold significance for functionality, their sporadic presence complicates their utility as consistent biomarkers for production purposes. Given that 40 dph represents the endpoint of the broiler life cycle, identifying stable biomarkers at this stage is critical for informing breeding practices aimed at enhancing broiler traits relevant to production.

No significant correlations were found between the microbiome, mycobiome, metabolome, and morphological structures, highlighting another limitation of this thesis due to the absence of broilers with clearly defined poor gut health parameters. Consequently, even the purportedly reliable marker of gut health, measuring the villus-to-crypt ratio, was not sensitive enough to differentiate chickens based on performance. This suggests that factors beyond gut health alone influence productivity.

In mixed-sex broiler flocks, it is commonly observed that male broilers exhibit higher growth rates and improved FCR compared to female broilers (Kers et al., 2018). Although significant growth rate disparities between the sexes typically manifest around 21 dph, microbial variations discerned as early as 3 dph suggest that factors beyond growth may influence microbiome composition (Lumpkins et al., 2008). Despite the established literature, no significant differences in sex were observed across any chapters. This finding contrasts with previous reports (Zucker and Beery, 2010) meaning if any of the proposed performance biomarkers are validated, they should be applicable to mixed-sex flocks.

8.2 FUTURE WORK

One promising area for future research is the validation of potential markers with targeted metabolomics. This approach could provide more precise insights into the metabolic pathways and biomarkers relevant to broiler health and performance. Identifying arabinitol in serum, believed to have derived from the mycobiome as a potential biomarker at 18 dph, underscores the dynamic nature of metabolic activity during broiler development. These initial findings necessitate validation across more extensive and

diverse broiler populations to ensure robustness and generalisability. Although 18 dph shows promise as a critical age for biomarker discovery in broilers, our experiences at 27 dph highlight the ongoing challenges and complexities in biomarker discovery research.

Investigating trimethylamine metabolism in faeces and serum has the potential to identify markers of gut microbiome function and diversity. This line of research could yield significant findings that enhance our understanding of the metabolic activities within the broiler GI microbiome and its implications for health and performance.

The composition of *Lactobacilli* in the gut microbiome warrants further investigation. Administering specific strains of *Lactobacilli* to broiler chickens may improve their performance, provided that the correct strains are selected to avoid polarising effects. The synergistic relationship between *Akkermansia municipia* and *Lactobacilli* (Lu et al., 2021) should be explored in more detail better to understand its impact on gut health and overall performance. Another area of interest is the role of *Alistipes*, which indicates a mature microbiome. Research should aim to determine whether *Alistipes* can be implemented effectively in the commercial poultry industry as a marker for performance or gut health in broilers. This could lead to improved strategies for managing and optimising broiler health based on microbiome composition.

The connection between fungi and AMR is another possible area for future study. Reproducing the core mycobiome in longitudinal studies will provide deeper insights into the interactions between fungi and bacteria in broiler chicks in GI microbiome stability during the broiler life cycle. Particular attention should be given to *Diutina*, especially in phosphorus utilisation by the mycobiome, as it may serve as a valuable performance marker or probiotic.

8.3 SUMMARY

This thesis yielded several key findings that enhance our understanding of broiler microbiome dynamics and metabolic profiles. Methodologically, shotgun sequencing was effective for bacterial analysis but inadequate for fungal research, indicating a need for alternative approaches to study fungi. Variations in *Lactobacilli* species composition

highlighted their significant impact on broiler health and performance. Identifying a core mycobiome across different life stages provided valuable insights into the stability of fungal communities in broilers. Additionally, the reproducibility of both faecal and serum samples demonstrated their potential as reliable biomarkers for assessing broiler gut health and performance.

APPENDICES

CHAPTER 5: SPATIAL VARIATION OF THE BROILER GASTROINTESTINAL TRACT AT 13-DAYS POST HATCHING

Appendix 1: List of fungi with significantly different relative abundances across three GI tract locations in broilers at 13 dph

ORDER	P-value	COMPARISON
Cystofilobasidiales	6.09E-06	Jejunum vs. Caecum
Cystofilobasidiales	0.000127	Faeces vs. Caecum
Capnodiales	0.00854	Faeces vs. Caecum
Diaporthales	0.0221	Jejunum vs. Caecum
Dothideales	0.0479	Jejunum vs. Caecum
Dothideales	0.0182	Faeces vs. Caecum
Eurotiales	7.46E-05	Faeces vs. Caecum
Eurotiales	0.000554	Faeces vs. Jejunum
Filobasidiales	0.00719	Jejunum vs. Caecum
Filobasidiales	0.00327	Faeces vs. Caecum
Helotiales	0.0351	Jejunum vs. Caecum
Helotiales	0.00617	Faeces vs. Caecum
Hypocreales	0.000753	Jejunum vs. Caecum
Hypocreales	0.000255	Faeces vs. Caecum
Leucosporidiales	0.00159	Jejunum vs. Caecum
Leucosporidiales	0.00774	Faeces vs. Caecum
Malasseziales	0.0113	Jejunum vs. Caecum
Onygenales	0.0236	Faeces vs. Jejunum
Pleosporales	3.69E-05	Faeces vs. Caecum
Saccharomycetales	6.28E-05	Faeces vs. Caecum
Saccharomycetales	0.000454	Faeces vs. Jejunum
Tremellales	0.000548	Jejunum vs. Caecum
Tremellales	6.19E-05	Faeces vs. Caecum
Trichosporonales	0.000689	Jejunum vs. Caecum
Trichosporonales	0.0346	Faeces vs. Caecum
Wallemiales	0.0271	Jejunum vs. Caecum
Wallemiales	0.00235	Faeces vs. Caecum

Pleosporales	9.63E-05	Jejunum vs. Caecum
Xylariales	0.000175	Faeces vs. Caecum
Xylariales	4.38E-05	Jejunum vs. Caecum

Appendix 2: List of metabolites with significantly different concentrations in three GI tract locations in broilers at 13 dph

COMPOUND NAME	P VALUE	Metabolic Family
Trimethylamine	2.49E-06	Phenol
4-Guanidinobutanoate	3.98E-05	Amines, Organic Acids
Lactate	4.74E-05	Organic Acids
Isobutyrate	5.24E-05	Organic Acids
Glycine	5.45E-05	Amino Acids
Pyruvate	5.60E-05	Keto Acids
Succinate	6.07E-05	Organic Acids
Ethylene glycol	6.24E-05	Alcohols and Polyols
4-Aminobutyrate	6.46E-05	Amino Acids
2-Methylglutarate	8.47E-05	Organic Acids
Glutarate	8.47E-05	Organic Acids
Glycerol	8.47E-05	Alcohols and Polyols
Levulinate	8.47E-05	Keto Acids
Adipate	8.68E-05	Organic Acids
Ethylmalonate	9.96E-05	Organic Acids
Glucitol	0.000103	Alcohols and Polyols
5-Aminopentanoate	0.000107	Amines, Amino Acids
Guanidoacetate	0.000108	Amines, Organic Acids
N,N-Dimethylformamide	0.000111	Amines
Arabinitol	0.00013	Alcohols and Polyols
Glucarate	0.000133	Alcohols and Polyols, Organic Acids
Gluconate	0.000134	Alcohols and Polyols, Organic Acids
Xylitol	0.00014	Alcohols and Polyols
Sucrose	0.000147	Monosaccharides and Disaccharides
Putrescine	0.000148	Amines
Mannitol	0.000152	Alcohols and Polyols
Propionate	0.000153	Fatty Acids
Ribose	0.000155	Monosaccharides and Disaccharides
Ethanol	0.000156	Alcohols and Polyols
Glycerate	0.000157	Alcohols and Polyols, Organic Acids
Trehalose	0.000165	Monosaccharides and Disaccharides
3-Hydromuconate	0.000172	Organic Acids
Erythritol	0.000182	Alcohols and Polyols
Mannose	0.000187	Monosaccharides and Disaccharides
3-Methyladipate	0.000189	Organic Acids
Lactose	0.000189	Monosaccharides and Disaccharides
Methylsuccinate	0.000191	Organic Acids
1,6-Anhydro-D-glucose	0.000193	Monosaccharides and Disaccharides

Fructose	0.000193	Monosaccharides and Disaccharides
Arabinose	0.000193	Monosaccharides and Disaccharides
Lactulose	0.000193	Monosaccharides and Disaccharides
Maltose	0.000201	Monosaccharides and Disaccharides
N6-Acetyllysine	0.000201	Amines, Amino Acids
Butyrate	0.000203	Fatty Acids
Glucose	0.000203	Monosaccharides and Disaccharides
Fucose	0.000211	Monosaccharides and Disaccharides
Cadaverine	0.000217	Amines
Glucuronate	0.000223	Monosaccharides and Disaccharides
Serine	0.000226	Amino Acids, Amines, Aromatics
Galactose	0.000228	Monosaccharides and Disaccharides
Isocaproate	0.000231	Organic Acids
Tropate	0.000232	Aromatics, Organic Acids
Propylene glycol	0.000248	Alcohols and Polyols
Glucose-1-phosphate	0.000252	Monosaccharides and Disaccharides, Phosphates
Glutaric acid monomethyl ester	0.000259	Organic Acids
Galactitol	0.000265	Alcohols and Polyols
D-Threitol	0.00027	Alcohols and Polyols
Agmatine	0.000271	Amines
N-Acetylglucosamine	0.000271	Amines, Monosaccharides and Disaccharides
S-Sulfocysteine	0.000274	Amino Acids, Sulfur Compounds
2-Phosphoglycerate	0.000277	Alcohols and Polyols, Organic Acids
N-Nitrosodimethylamine	0.000277	Amines
Threonine	0.000279	Amino Acids, Organic Acids
2-Oxobutyrate	0.00028	Keto Acids
Cellobiose	0.000283	Monosaccharides and Disaccharides
myo-Inositol	0.000283	Alcohols and Polyols
Glucose-6-phosphate	0.000306	Monosaccharides and Disaccharides, Phosphates
2-Oxovalerate	0.000316	Keto Acids
N-Phenylacetylglycine	0.000322	Amino Acids
3-Methyl-2-oxovalerate	0.000323	Keto Acids
Xylose	0.000332	Monosaccharides and Disaccharides
Acetate	0.000344	Fatty Acids
Ethanolamine	0.000344	Alcohols and Polyols, Amines
Uridine	0.000346	Nucleotides and Nucleosides
Butanone	0.000357	Ketones
AMP	0.000363	Nucleotides and Nucleosides
IMP	0.000363	Nucleotides and Nucleosides
N-Methylhydantoin	0.000366	Amines
Alanine	0.000383	Amino Acids
Threonate	0.000392	Alcohols and Polyols, Organic Acids
Ascorbate	0.000394	Alcohols and Polyols
N-Acetylglycine	0.000394	Amino Acids

2-Oxoglutarate	0.000424	Amino Acids
2'-Deoxyguanosine	0.000461	Nucleotides and Nucleosides
3-Methylglutarate	0.000474	Organic Acids
Acetone	0.00048	Ketones
O-Phosphoserine	0.000483	Amino Acids, Phosphates
N-Acetylserotonin	0.000521	Alcohols and Polyols, Aromatics, Amines
Indole-3-acetate	0.000531	Aromatics, Organic Acids
Cytidine	0.000539	Nucleotides and Nucleosides
sn-Glycero-3-phosphocholine	0.000539	Alcohols and Polyols, Amines, Phosphates
Homovanillate	0.000543	Organic Acids, Phenols
1,3-Dihydroxyacetone	0.000545	Alcohols and Polyols
Guanosine	0.00055	Nucleotides and Nucleosides
Galactonate	0.000556	Alcohols and Polyols, Organic Acids
5-Hydroxyindole-3-acetate	0.000613	Phenols, Organic Acids
Betaine	0.000624	Amino Acids
Isovalerate	0.000634	Fatty Acids
Syringate	0.000634	Phenols, Organic Acids
UDP-glucose	0.000642	Monosaccharides and Disaccharides, Nucleotides and Nucleosides
Glycolate	0.000649	Alcohols and Polyols, Organic Acids
UDP-N-Acetylglucosamine	0.000649	Amines, Monosaccharides and Disaccharides, Nucleotides and Nucleosides
O-Phosphocholine	0.000657	Amines, Phosphates
UDP-galactose	0.000659	Monosaccharides and Disaccharides, Nucleotides and Nucleosides
2'-Deoxyadenosine	0.000754	Nucleotides and Nucleosides
Adenosine	0.00077	Nucleotides and Nucleosides
O-Phosphoethanolamine	0.000775	Amines, Phosphates
N,N-Dimethylglycine	0.000778	Amino Acids
Xanthosine	0.000778	Nucleotides and Nucleosides
UMP	0.000806	Purines and Pyrimidines
N-Acetyltyrosine	0.000837	Amino Acids, Phenols
2-Phenylpropionate	0.000899	Aromatics
Choline	0.000963	Alcohols and Polyols
Homogentisate	0.00097	Phenols, Organic Acids
Kynurenine	0.000997	Amines, Amino Acids, Aromatics, Keto Acids
UDP-glucuronate	0.001034	Monosaccharides and Disaccharides, Nucleotides and Nucleosides
Isopropanol	0.001061	Alcohols and Polyols
3-Hydroxykynurenine	0.00107	Amino Acids, Keto Acids, Phenols
Inosine	0.00107	Nucleotides and Nucleosides
Desaminotyrosine	0.001073	Phenols, Organic Acids
Pipecolate	0.001094	Amino Acids
trans-Aconitate	0.001124	Organic Acids

1,7-Dimethylxanthine	0.00124	Purines and Pyrimidines
Methylamine	0.001253	Amines
4-Hydroxyphenylacetate	0.001359	Phenols, Organic Acids
2'-Deoxyinosine	0.001485	Nucleotides and Nucleosides
Riboflavin	0.001485	Alcohols and Polyols, Amines, Aromatics
Thymine	0.001601	Purines and Pyrimidines
?-Alanine	0.001655	Amino Acids
Citrate	0.001947	Organic Acids
Phenylacetate	0.00195	Aromatics, Organic Acids
3,5-Dibromotyrosine	0.002074	Amino Acids, Phenols
Tiglylglycine	0.00223	Amino Acids
Cystine	0.002256	Amino Acids, Sulfur Compounds
3,4-Dihydroxybenzeneacetate	0.002345	Organic Acids
Acetoacetate	0.002573	Keto Acids
Tyramine	0.002634	Amines, Phenols
3-Phenylpropionate	0.00278	Aromatics, Organic Acids
Thymol	0.002824	Phenols
Cysteine	0.002868	Amino Acids, Sulfur Compounds
1,3-Diaminopropane	0.002958	Amines
Dimethyl sulfone	0.003004	Sulfur Compounds
2-Hydroxyisovalerate	0.003075	Organic Acids
?-Methylhistidine	0.003103	Amino Acids, Aromatics
Malate	0.003213	Organic Acids
Theophylline	0.003366	Purines and Pyrimidines
2-Hydroxyisobutyrate	0.003405	Organic Acids
Sarcosine	0.00359	Amino Acids
NADPH	0.003894	Amines, Monosaccharides and Disaccharides, Nucleotides and Nucleosides
N-Carbamoyl-?-alanine	0.003939	Amines, Organic Acids
2-Oxoisocaproate	0.004256	Keto Acids
3-Hydroxyisobutyrate	0.004447	Organic Acids
5,6-Dihydrouracil	0.004762	Purines and Pyrimidines
Trimethylamine N-oxide	0.004937	N-oxides
Malonate	0.004969	Organic Acids
Citraconate	0.004988	Organic Acids
NADH	0.005086	Amines, Monosaccharides and Disaccharides, Nucleotides and Nucleosides
Acetaminophen	0.005145	Amines, Phenols
1,3-Dimethylurate	0.005453	Purines and Pyrimidines
5-Methoxysalicylate	0.005668	Organic Acids, Phenols
Tryptophan	0.006101	Amino Acids, Aromatics
Creatinine	0.006773	Amines, Ketones
Asparagine	0.007413	Amino Acids
T-Methylhistidine	0.007461	Amino Acids, Aromatics
2-Aminoadipate	0.007726	Amino Acids, Organic Acids

Citrulline	0.008219	Amines, Amino Acids
Tyrosine	0.009745	Amino Acids, Penols
N-Acetylornithine	0.009897	Amines, Amino Acids
2-Hydroxyphenylacetate	0.010777	Organic Acids
p-Cresol	0.01103	Phenols
Hydroxyacetone	0.011261	Alcohols and Polyols, Ketones
4-Hydroxy-3-methoxymandelate	0.011555	Phenols, Organic Acids
2-Furolglycine	0.012073	Amino Acids
5,6-Dihydrothymine	0.012073	Purines and Pyrimidines
Pyridoxine	0.012262	Alcohols and Polyols, Amines, Aromatics
Carnitine	0.012262	Amines, Organic Acids
Leucine	0.012485	Amino Acids
o-Cresol	0.012647	Phenols
Vanillate	0.014095	Organic Acids, Phenols
Glutathione	0.014918	Amino Acids, Sulfur Compounds
Galactarate	0.01577	Alcohols and Polyols, Organic Acids
Pantothenate	0.016119	Alcohols and Polyols, Amino Acids
5-Hydroxytryptophan	0.017372	Phenols
Hippurate	0.017484	Amino Acids, Aromatics
3,5-Dibromotyrosine	0.017552	Amino Acids, Phenols
Taurine	0.017552	Amines, Sulfur Compounds
N-Isovaleroylglycine	0.017918	Amino Acids
4-Aminohippurate	0.018601	Amino Acids, Amines, Aromatics
Catechol	0.019261	Phenols
Valine	0.020021	Amino Acids
Phenylalanine	0.020021	Amino Acids, Aromatics
Creatine phosphate	0.020919	Amino Acids, Amines, Phosphates
Pyrocatechol	0.021082	Phenols
Aspartate	0.022749	Amino Acids, Organic Acids
Glutamylphenylalanine	0.022956	Amino Acids
Creatine	0.026973	Amino Acids, Amines
N-Acetylglutamine	0.027288	Amino Acids, Amines, Organic Acids
Saccharopine	0.028147	Amino Acids, Organic Acids
dTTP	0.028147	Nucleotides and Nucleosides
2-Hydroxy-3-methylvalerate	0.028256	Organic Acids
Methylmalonate	0.028256	Organic Acids
3,4-Dihydroxymandelate	0.028697	Organic Acids
Methylguanidine	0.029485	Putrefaction

Appendix 3: T-test (P<0.05) significant differences and respective relative abundances comparing genotypes at each time point.

			Relative Abundance	
Age	*Taxon*	P Value	Genotype A	Genotype B
7	*Candidatus Arthromitus sp FB turkey*	0.026	0.150	2.428
7	*Corynebacterium stationis*	0.014	0.281	0.879
7	*Enterococcus hirae*	0.015	0.253	2.842
7	*Escherichia coli*	0.012	0.053	5.427
7	*Jeotgalicoccus schoeneichii*	0.042	0.002	0.011
7	*Lactobacillus crispatus*	0.008	15.420	26.616
7	*Lactobacillus johnsonii*	0.037	26.454	23.769
7	*Limosilactobacillus vaginalis*	0.015	12.203	7.204
7	*Ligilactobacillus aviarius*	0.000	0.311	1.843
7	*Limosilactobacillus reuteri*	0.019	8.862	5.654
7	*Mammaliicoccus lentus*	0.047	0.026	0.135
7	*Staphylococcus arlettae*	0.044	0.000	0.012
7	*Staphylococcus cohnii*	0.041	0.003	0.123
7	*Staphylococcus nepalensis*	0.025	0.009	0.137
18	*Akkermansia muciniphila*	0.026	0.047	0.549
18	*Bacilli bacterium*	0.049	0.004	0.022
18	*Candidatus Arthromitus sp SFB turkey*	0.027	0.218	0.994
18	*Clostridium spiroforme*	0.038	0.032	0.178
18	*Corynebacterium freneyi*	0.033		0.000
18	*Enterococcus gallinarum*	0.025	0.000	0.008
18	*Faecalibacterium sp An122*	0.033	0.071	0.703
18	*Fournierella massiliensis*	0.018	0.000	0.012
18	*Gemmiger sp An50*	0.041	0.001	0.040
18	*Gordonibacter sp An232A*	0.050	0.003	0.020
18	*Limosilactobacillus vaginalis*	0.001	13.871	7.022
18	*Ligilactobacillus aviarius*	0.000	0.457	6.004
18	*Parasutterella SGB9260*	0.025	0.008	0.053
18	*Rothia koreensis*	0.002	0.000	0.011
18	*Ruminococcaceae bacterium*	0.033	0.018	0.155
18	*Salinicoccus sediminis*	0.044	0.000	0.001
18	*Staphylococcus equorum*	0.014	0.007	0.000
18	*Staphylococcus xylosus*	0.020	0.032	0.001
18	*Weissella jogaejeotgali*	0.036	0.021	0.000
27	*Akkermansia muciniphila*	0.032	0.050	2.282
27	*Brachybacterium faecium*	0.044		
27	*Candidatus Harrysmithimonas galli*	0.033	0.019	0.004

27	*Lachnospiraceae bacterium*	0.046	0.007	0.085
27	*Limosilactobacillus vaginalis*	0.033	0.182	0.827
27	*Ligilactobacillus aviarius*	0.042	8.633	5.287
27	*Limosilactobacillus reuteri*	0.013	1.130	8.506
27	*Staphylococcus cohnii*	0.042	3.803	1.876
40	*Akkermansia muciniphila*	0.006	0.084	0.002
40	*Alistipes inops*	0.016	0.049	3.869
40	*Alistipes onderdonkii*	0.032	0.296	0.843
40	*Alistipes putredinis*	0.029	0.285	1.167
40	*Anaerotruncus colihominis*	0.020	0.021	0.064
40	*Bilophila wadsworthia*	0.035	0.007	0.040
40	*Blautia sp An249*	0.014	0.051	0.144
40	*Candidatus Cryptoclostridium obscurum*	0.018	0.120	0.514
40	*Candidatus Heritagella intestinalis*	0.034	0.009	0.028
40	*Candidatus Heteroclostridium caecigallinarum*	0.037	0.017	0.052
40	*Candidatus Heteroscilispira lomanii*	0.029	0.007	0.001
40	*Candidatus Metaruminococcus gallistercoris*	0.024	0.005	0.001
40	*Candidatus Neoruminococcus faecicola*	0.028	0.000	0.005
40	*Clostridia bacterium 12CBH8*	0.002	0.003	0.009
40	*Clostridia bacterium UC5 1 1D1*	0.005	0.001	0.016
40	*Clostridiales bacterium CHKCI006*	0.020	0.000	0.012
40	*Enterococcus cecorum*	0.036	0.079	0.022
40	*Faecalibacterium SGB63299*	0.024	0.110	0.507
40	*Flavonifractor sp An306*	0.042	0.712	0.142
40	*Flavonifractor sp An82*	0.021	0.002	0.011
40	*Gordonibacter pamelaeae*	0.035	0.002	0.070
40	*Gordonibacter sp An232A*	0.047	0.002	0.019
40	*Gordonibacter urolithinfaciens*	0.045	0.052	0.104
40	*Lachnoclostridium sp An138*	0.027	0.001	0.011
40	*Lachnoclostridium sp An76*	0.008	0.098	0.012
40	*Lactobacillus gallinarum*	0.004	0.044	0.285
40	*Ligilactobacillus aviarius*	0.001	44.364	22.099
40	*Massilimicrobiota timonensis*	0.007	0.420	12.918
40	*Massilistercora timonensis*	0.025	0.144	0.014
40	*Subdoligranulum sp DSM 109015*	0.027	0.000	0.002

Appendix 4: Faecal Metabolites with a Significant Difference Between Time point, ANOVA, P<0.001, MetaboAnalyst 5.0

METABOLITE	P VALUE		FISHER'S LSD	
Asparagine	1.58E-11	18 - 40	18 - 7	7 - 40
Fructose	4.71E-14	40 - 18	7 - 18	40 - 7
3-Hydroxy-3-methylglutarate	9.65E-15	40 - 18	7 - 18	40 - 7
Glycylproline	4.95E-17	40 - 18	7 - 18	40 - 7
O-Phosphoethanolamine	7.89E-05	7 - 18	7 - 40	

N-Nitrosodimethylamine	8.35E-06	7 - 18	7 - 40
Sarcosine	5.88E-06	7 - 18	7 - 40
3,5-Dibromotyrosine	2.32E-06	7 - 18	7 - 40
5-Methoxysalicylate	2.27E-06	7 - 18	7 - 40
Acetoin	2.38E-07	7 - 18	7 - 40
Taurine	4.62E-18	7 - 18	7 - 40
Mannitol	3.54E-29	7 - 18	7 - 40
Phenylalanine	1.02E-31	7 - 18	7 - 40
Lysine	3.91E-34	7 - 18	7 - 40
Ferulate	0.00020137	40 - 18	7 - 18
Tryptophan	2.81E-05	40 - 18	7 - 18
Acetylsalicylate	1.34E-05	40 - 18	7 - 18
5-Hydroxylysine	9.31E-06	40 - 18	7 - 18
Kynurenate	5.04E-06	40 - 18	7 - 18
cis-Aconitate	9.92E-07	40 - 18	7 - 18
O-Phosphocholine	9.08E-10	40 - 18	7 - 18
Pantothenate	1.96E-10	40 - 18	7 - 18
N-Phenylacetylphenylalanine	9.06E-11	40 - 18	7 - 18
2-Hydroxyphenylacetate	1.78E-11	40 - 18	7 - 18
Butanone	5.70E-05	40 - 18	40 - 7
3-Methyl-2-oxovalerate	5.22E-05	40 - 18	40 - 7
2-Hydroxy-3-methylvalerate	4.14E-05	40 - 18	40 - 7
2-Oxoisocaproate	3.79E-05	40 - 18	40 - 7
Trehalose	3.62E-05	40 - 18	40 - 7
Glucuronate	5.29E-06	40 - 18	40 - 7
3-Chlorotyrosine	9.78E-07	40 - 18	40 - 7
3-Methylglutarate	4.91E-07	40 - 18	40 - 7
Methylsuccinate	1.66E-07	40 - 18	40 - 7
Agmatine	2.32E-08	40 - 18	40 - 7
Ribose	2.01E-09	40 - 18	40 - 7
Cellobiose	2.28E-10	40 - 18	40 - 7
Glutamate	4.23E-11	40 - 18	40 - 7
Glucitol	3.98E-18	40 - 18	40 - 7
3-Phenyllactate	2.62E-18	40 - 18	40 - 7
Gluconate	7.06E-20	40 - 18	40 - 7
Glucose-6-phosphate	2.42E-20	40 - 18	40 - 7
Ï€-Methylhistidine	0.00026235	18 - 40	18 - 7
5-Aminolevulinate	0.00019351	18 - 40	18 - 7
Galactonate	8.80E-05	18 - 40	18 - 7
Arabinose	4.22E-05	18 - 40	18 - 7
N-Acetyltyrosine	3.29E-05	18 - 40	18 - 7
Galactose	1.10E-05	18 - 40	18 - 7
O-Acetylcholine	8.42E-06	18 - 40	18 - 7
Trimethylamine N-oxide	5.17E-06	18 - 40	18 - 7
Phenylacetate	2.43E-06	18 - 40	18 - 7
Carnosine	1.53E-06	18 - 40	18 - 7
Tyrosine	8.33E-07	18 - 40	18 - 7
Creatine phosphate	8.16E-07	18 - 40	18 - 7

Pyrimidine	4.55E-07	18 - 40	18 - 7
N-Acetylcysteine	4.37E-07	18 - 40	18 - 7
5-Hydroxyindole-3-acetate	2.08E-07	18 - 40	18 - 7
Formate	1.56E-07	18 - 40	18 - 7
Î³-Glutamylphenylalanine	1.04E-07	18 - 40	18 - 7
N6-Acetyllysine	5.20E-08	18 - 40	18 - 7
Glucose	5.19E-08	18 - 40	18 - 7
Imidazole	4.56E-08	18 - 40	18 - 7
Riboflavin	2.93E-08	18 - 40	18 - 7
Betaine	1.25E-09	18 - 40	18 - 7
Succinate	9.90E-10	18 - 40	18 - 7
Sucrose	4.75E-10	18 - 40	18 - 7
Trigonelline	1.02E-10	18 - 40	18 - 7
Biotin	6.45E-11	18 - 40	18 - 7
Fucose	3.10E-12	18 - 40	18 - 7
Lactate	2.47E-12	18 - 40	18 - 7
Methanol	4.60E-13	18 - 40	18 - 7
Dimethylamine	3.87E-13	18 - 40	18 - 7
Glycine	6.61E-17	18 - 40	18 - 7
Ethanol	5.90E-20	18 - 40	18 - 7
Threonate	1.41E-23	18 - 40	18 - 7
Lactose	2.20E-25	18 - 40	18 - 7
Isoleucine	7.63E-30	18 - 40	18 - 7
1,3-Dimethylurate	4.72E-30	18 - 40	18 - 7
Leucine	3.25E-33	18 - 40	18 - 7
Valine	6.94E-35	18 - 40	18 - 7
Alanine	6.74E-07	7 - 40	
2-Hydroxyisobutyrate	0.00030732	7 - 18	
Vanillate	0.00026188	7 - 18	
Urocanate	0.00013529	7 - 18	
N-Acetylornithine	2.15E-05	7 - 18	
Ibuprofen	0.00017342	40 - 18	
Kynurenine	7.27E-05	40 - 18	
N-Acetylglucosamine	5.62E-06	40 - 18	
Xylitol	1.83E-07	40 - 18	
4-Hydroxy-3-methoxymandelate	5.79E-08	40 - 18	
Aspartate	3.52E-08	40 - 18	
Xanthine	0.00021545	18 - 7	
Acetate	9.47E-05	18 - 7	
N-Phenylacetylglycine	1.04E-05	18 - 7	
4-Pyridoxate	8.00E-05	18 - 40	
Lactulose	2.32E-13	18 - 40	

Appendix 5: T-test (P<0.05) significant differences in faecal metabolite concentrations comparing genotypes at each time point.

			AVERAGE CONCENTRATION (μM)	
AGE	FAECAL METABOLITE	P VALUE	GENOTYPE A	GENOTYPE B
7	Glutamate	0.048	329.2	160.9
7	Pimelate	0.049	80.6	49.7
7	Propylene glycol	0.024	45.0	86.3
7	Sucrose	0.024	91.0	122.6
18	Galactose	0.001	1247.2	3146.8
40	2-Hydroxyisocaproate	0.012	14.6	41.4
40	3-Methyl-2-oxovalerate	0.002	6.9	27.5
40	4-Hydroxy-3-methoxymandelate	0.003	9.7	17.8
40	Phenylacetate	0.011	56.4	181.5

Appendix 6: Serum Metabolites with a Significant Difference Between Time point, ANOVA, P<0.05, MetaboAnalyst 5.0

METABOLITE	P VALUE	FISHER'S LSD	FISHER'S LSD	FISHER'S LSD
N-Methylhydantoin	1.07E-09	18 - 27	40 - 18	40 - 27
Leucine	0.009805	27 - 18	27 - 40	
Allantoin	5.78E-06	18 - 27	18 - 40	
Glucitol	7.24E-06	18 - 27	18 - 40	
3,4-Dihydroxybenzeneacetate	1.55E-05	18 - 27	18 - 40	
Alloisoleucine	4.53E-05	18 - 27	18 - 40	
Desaminotyrosine	6.95E-05	18 - 27	18 - 40	
Trimethylamine N-oxide	0.000145	18 - 27	18 - 40	
T -Methylhistidine	0.000157	18 - 27	18 - 40	
2-Furoylglycine	0.000244	18 - 27	18 - 40	
Xylitol	0.001046	18 - 27	18 - 40	
Homovanillate	0.001669	18 - 27	18 - 40	
Lactate	0.002179	18 - 27	18 - 40	
3-Hydroxy-3-methylglutarate	0.008617	18 - 27	18 - 40	
2-Phosphoglycerate	0.009293	18 - 27	18 - 40	
Urea	0.011333	18 - 27	18 - 40	
Na-Acetyllysine	0.013132	18 - 27	18 - 40	
2-Hydroxyphenylacetate	0.015084	18 - 27	18 - 40	
Acetate	0.00156	27 - 18	40 - 18	
O-Acetylcholine	0.003621	27 - 18	40 - 18	
4-Carboxyglutamate	1.42E-08	18 - 27	40 - 27	
Acetoacetate	3.71E-06	18 - 27	40 - 27	
Sarcosine	9.89E-06	18 - 27	40 - 27	
S-Sulfocysteine	1.34E-05	18 - 27	40 - 27	
Galactitol	1.36E-05	18 - 27	40 - 27	

O-Phosphoserine	4.09E-05	18 - 27	40 - 27
2-Hydroxyvalerate	8.93E-05	18 - 27	40 - 27
5-Aminolevulinate	0.000204	18 - 27	40 - 27
2-Hydroxyisocaproate	0.000994	18 - 27	40 - 27
N-Acetylglucosamine	0.001239	18 - 27	40 - 27
2-Hydroxyglutarate	0.001329	18 - 27	40 - 27
Malate	0.003564	18 - 27	40 - 27
Melatonin	0.0047	18 - 27	40 - 27
Glycylproline	0.007654	18 - 27	40 - 27
2-Hydroxyisovalerate	0.008784	18 - 27	40 - 27
N-Carbamoylaspartate	0.009016	18 - 27	40 - 27
Xanthurenate	0.010744	18 - 27	40 - 27
Malonate	1.59E-13	40 - 18	40 - 27
cis-Aconitate	8.01E-05	40 - 18	40 - 27
trans-4-Hydroxy-L-proline	0.000137	40 - 18	40 - 27
Glycocholate	0.000661	40 - 18	40 - 27
Isoleucine	0.001022	40 - 18	40 - 27
Citrate	0.009745	40 - 18	40 - 27
Anserine	0.013432	40 - 18	40 - 27
2-Hydroxy-3-methylvalerate	0.016137	40 - 18	40 - 27
Oxypurinol	0.004046	18 - 27	
Glycerate	0.005817	18 - 27	
N-Acetyltyrosine	0.007452	18 - 27	
4-Pyridoxate	0.002024	40 - 27	
N-Acetylglutamine	0.010391	40 - 27	

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