High-throughput sequencing of the chicken gut microbiome

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Abstract

The chicken (*Gallus gallus domesticus*) is the most abundant and widely distributed livestock animal with a global population of over 21 billion. A commercially raised broiler chick takes five-weeks to reach market weight and this can be attributed to the selection of genetic traits, better feed formulation (in addition to enzyme supplementation) and an increased understanding of health and husbandry. The symbiotic, complex and variable community of the microbiome forms an important part of the gastrointestinal tract (gut) and is involved in gut-development and non-specific resistance to infection.

This study investigated the chicken gut microbiota using high-throughput 16S rRNA sequencing on an Illumina MiSeq and culture-based techniques. There was specific interest in the proventriculus of which there is limited research currently in the literature and the caecum because it contains the highest density of bacterial cells in the gut at 10^{11} cells per gram.

The results showed no significant difference in the first stages of the GIT which shared a low-diversity microbiota dominated by a few *Lactobacillus* species. The microbiota becomes more diverse in the latter parts of the small intestine where *Clostridiales* and *Enterobacteriaceae* were present in higher numbers. The caecum was the most diverse organ with the majority of species belonging to *Ruminococcaceae*, *Lachnospiraceae* and *Alistipes*. A number of novel species were isolated from the chicken gut and six of these were whole-genome sequenced.

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Non-author contributions

In Chapters Three and Four, the custom primers and java programs were designed by Martin Sergeant. In Chapter Six, the custom-script to determine the presence of spore-formation genes was written by Richard Brown.

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ANI	Average nucleotide identity
APEC	Avian pathogenic *Escherichia coli*
CFU	Colony Forming Unit
CMC	Carboxymethylcellulose
DDH	DNA-DNA hybridization
DNA	Deoxyribonucleic Acid
EGTs	Environmental Gene Tags
FAA	Fastidious anaerobe agar
FCR	Food-Conversion-Ratio
FISH	Fluorescent *in situ* Hybridization
GUT	Gastrointestinal tract
IMG	Integrated Microbial Genomes
LB	Lysogeny broth
LBA	Lysogeny agar
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time-Of-Flight
MDR	Multi Drug Resistant
MHA	Mueller-Hinton agar
NSP	Non-Starch Polysaccharides

OTU	Operational Taxonomic Unit
PCR	Polymerase Chain reaction
QIIME	Quantitative Insights Into Microbial Ecology
rRNA	Ribosomal Ribonucleic Acid
RT	Room Temperature
S.E.M	Standard error of the mean
SINA	SILVA incremental aligner
TAE	Tris-acetate

CHAPTER ONE

1. Introduction

1.1 The chicken

The chicken (*Gallus gallus domesticus*) is the most abundant and widely distributed livestock animal with a global population of over 21 billion [1, 2]. This equates to production of over 40 billion chickens per year to produce 61 million tons of meat and 55 million tons of eggs [3]. The poultry industry has succeeded in providing an affordable source of protein worldwide, taking a conventional broiler just 35-41 days and an organic broiler just 81 days to reach market weight [4, 5]. Due to intensive research, modern broilers required a third of the food to reach market weight than broilers did 60 years ago [6].

The chicken dominates UK poultry production, accounting for 93% of British poultry and through intensive animal husbandry, our country produces over 850 million broiler chickens annually, with a further 29 million egg-laying chickens [4, 7, 8]. It is therefore unsurprising that the UK chicken industry is worth over £3 billion to the UK economy [7, 8].

These figures indicate how important the chicken is as a protein source from both meat and eggs. Recently, there has been a rise in the demand for chicken from China and Brazil as their wealth increases. This, coupled with an ever-rising global population, is putting pressure on the livestock industry to meet demand; thus food security is an issue that needs to be addressed.

1.2 The gastrointestinal tract of the chicken

The primary function of the gastrointestinal tract (gut) is to convert food into components that can be utilised by the host [9]. The gut of the chicken comprises the crop, proventriculus, gizzard, duodenum, jejunum, ileum, caeca, colon, rectum and cloaca (Figure 1.1).

After swallowing, food first reaches the crop, which is covered in a thick, non-secretory epithelial layer. Food may remain here for up to six hours, undergoing fermentation by *Lactobacillus* species [10]. The contents of the crop empty into the proventriculus, from where they move quickly into the gizzard. Proton secretion from the mucosa of the proventriculus results in a low pH in both the proventriculus and the gizzard. In both sites, they are exposed to enzymes derived from bacteria, diet and saliva [11, 12]. The gizzard is made of thick muscle that grinds the food and chickens often purposely swallow small stones or grit, which sit in the gizzard to aid mechanical digestion [11].

The digesta then proceed to the small intestine, formed of the duodenum, jejunum and ileum. Here they encounter:

- bile salts released from the bile duct
- enzymes secreted by the pancreas
- mucus and digestive enzymes secreted by the intestinal mucosa

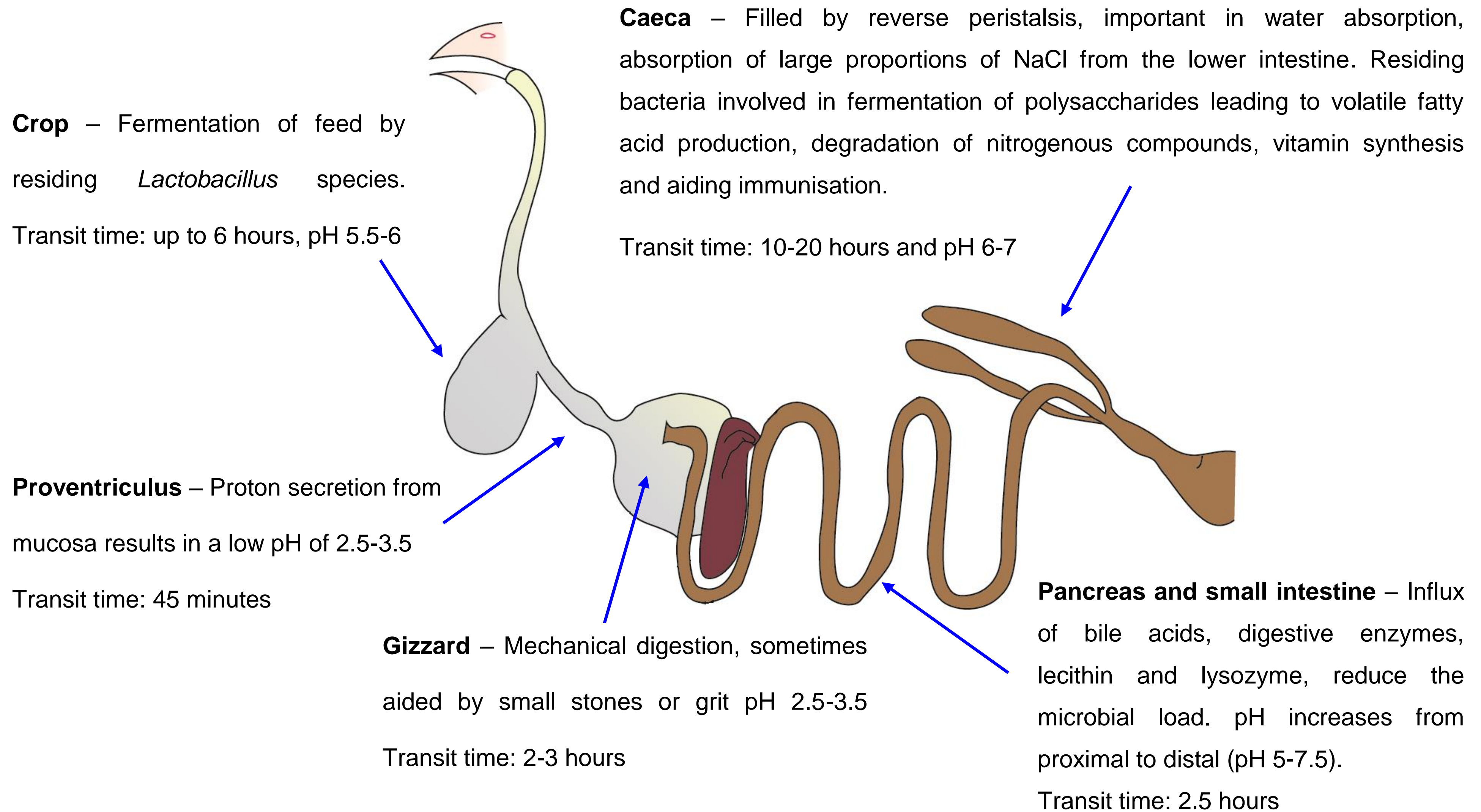


Figure 1.1 - Map of chicken gastrointestinal track showing structures, pH and transit times.

Edited from Poultry CRC [13]

Transit through the small intestine takes around 2.5 hours [2]. Once the digesta reach the ileocaecal junction at the beginning of the large intestine, they fill two caeca by reverse peristalsis. Here, the digesta can remain for as long as 10-20 hours, before being emptied back into the small intestine [2, 14, 15]. The role of the caeca has not been fully established. However, there are six probable functions:

- Water absorption [16]
- Absorption of large proportions of NaCl from the lower intestine [17]
- Fermentation of polysaccharides leading to volatile fatty acid production [18]
- Degradation of nitrogenous compounds [19]
- Vitamin synthesis [20]
- Aiding immunisation [20]

The latter four are all due to bacteria present in the caeca. The materials that enter the caeca from the small intestine are fine-particles and soluble, low molecular weight non-viscous molecules [21]. Up to 18% of excreted dry matter and 17% of excreted water enters the caeca, with a proportion entering birds that have been starved [22]. The faecal pellet is formed in the short, large intestine before passing into the cloaca, mixed with uric acid and exits through the vent [14, 15]. Caecal contents are voided several times a day and produce distinctive foul-smelling droppings.

1.3 The intestinal microbiota

Three terms are used when studying the gut as a habitat:

- Microbiota – the microorganisms present in a defined habitat
- Metagenome – the collection of genes and genomes of the microbiota
- Microbiome – the entire habitat, including the microbiota, their genes and genomes in addition to their interactions with each other and the host

A census of the microbiota is established through culturing or molecular methods such as analysis of 16S rRNA genes or other marker genes, which are amplified and sequenced from biological samples [23-25]. The metagenome was first described by Handelsman *et al.* (1998) in reference to the cloning and functional analysis of the collective genomes of soil microbiota [26]. Shotgun sequencing of DNA extracted from a biological sample, followed by assembly and annotation is now the most common way of completing functional analysis of the metagenome [23]. The term 'microbiome' was first used by Lederberg in 2001 to describe the "ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" [27]. This definition was similar to that of the microbiota; however, microbiome has taken on a slightly different meaning. It now encompasses the microbiota, the metagenome in addition to their products and the host environment [23, 28].

The gut microbiota plays an important role in host animal health and production. It can positively influence non-specific resistance to infection, immunology,

physiology, biochemistry and gut development. The gut of any animal forms a habitat for a complex and varied microbial community which is determined by the host, inter-species competition and diet [29]. The gut microbiomes of the human, wallaby, panda, cow and termite, amongst others, have been characterised through metagenomic and functional analyses [30-35].

There are two main approaches to characterising bacterial populations within animal guts: culture-based and culture-independent approaches. The traditional culture-based approach relies on growth (often on a selective medium) and biochemical tests to identify bacteria isolated under specific culture conditions; Henrich Kern initiated culture-based studies of the chicken gut microbiota in 1897 [36-38].

The culturing of an organism relies on many factors [39], including:

- Type of culture medium
- Presence of antibiotics
- Presence or absence of other organisms
- Temperature
- Atmosphere
- Incubation time
- Sample collection

Cultural approaches can be onerous and fail to provide a comprehensive picture, when the majority of microbiota have unknown growth requirements or cannot be cultured at all in the laboratory [38]. The first understanding of the ‘unculturable’ bacteria in the chicken came from microscopy in 1897, where the

bacteria observed by microscopy were in orders of magnitude greater than those that would grow on an agar plate using ordinary methods of cultivation; this was later referred to as ‘the great plate count anomaly’. [36, 40-42].

It was proposed that the observed bacteria that would not grow could be dead. However, it was later shown that cells can be metabolically active, even if unable to proliferate under laboratory conditions [43]. It has been estimated that only 20-40% of bacterial species from the gut can be cultured using currently available techniques which was confirmed by early metagenome and 16S rRNA gene sequencing studies [44-46]. This figure may be much lower for the caecal microbiota, given that one study found that only 10% of bacterial sequences obtained from this community represented known bacterial species [38].

Despite potential deficiencies, culture was the only available tool until three crucial developments:

- Sequencing technology invented by Fredrick Sanger in 1977 [47]
- Adoption of the 16S rRNA gene sequence to study phylogeny by Carl Woese in 1977 [48-50],
- The development of PCR by Kary Mullis in 1983 [51]

The 16S rRNA gene is 1542 bp in length and forms part of the 30S subunit with the 23S rRNA gene [52]. The sequences of some loops from the secondary structure are conserved across nearly all-bacterial species due to function. However, the structural parts are variable and only specific to one or more classes [53]. The 16S rRNA gene is universally present in prokaryotes allowing for comparisons of phylogenetic relationships and has highly variable regions to allow differentiation between species, while containing conserved regions that

enable the design of PCR primers (Figure 1.2) [54-56]. The conservative nature of the 16S rRNA gene sequence is a double-edged sword; it is very useful because it does not undergo significant lateral transfer and thus can be used for inference of deep phylogeny, however this extreme conservation limits the usefulness for discrimination of close relatives at strain or species level [57]. The “S” in 16S refers to Svedburg unit, which measures the particle size based on the rate of travel in a tube subjected to high g-force [23].

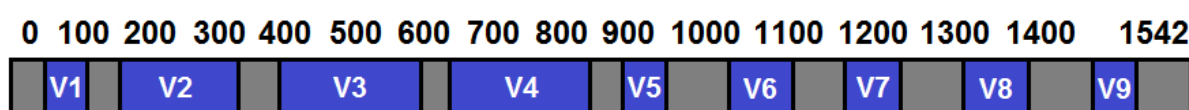


Figure 1.2 - 16S rRNA gene with conserved regions (grey) and variable regions (blue) with the length of the regions indicated in bp.

However, the use of 16S rRNA gene sequencing is not without issues such as: DNA extraction, PCR amplification and data analysis [55, 58-63]. A primary issue for determining species abundance with 16S rRNA gene sequencing data is gene copy number. As 16S rRNA gene operon copy numbers can vary from one to 15 in bacteria, observed relative abundances in 16S rRNA gene sequencing studies can differ from true abundance [56, 58, 64]. In response to an environmental change, the existence of multiple rRNA operons could have two potential functions: providing a multiplier effect on translation to allow the bacterium to grow rapidly and functional differentiation between rRNA operons would allow for differential expression of rRNA operons [65].

PCR amplification of 16S rRNA gene regions was used to detect food-borne pathogens in chicken products as long ago as 1992 [66]. However, 16S rRNA

gene sequencing was not applied to the microbiota of chickens until 2002 by Zhu *et al*. when they analysed the caecum of broilers [67].

The advent of high-throughput sequencing has enabled much deeper analyses of the chicken gut microbiota, generating copious 16S rRNA gene sequences and metagenome data [29, 60, 68].

Recent papers from Raoult's group [25, 69] have argued for a return to culture-based approaches, under the banner of "culturomics". His group claims using 212 different culture conditions, in addition to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass-spectrometry, provides more comprehensive results than DNA sequencing. The MALDI-TOF method involves bacterial identification based on peptidic spectra and comparison to a database [70]. They suggest that just 20 cultural conditions will result in 73% total species isolation. From three human faecal samples, they obtained 32,500 colonies, which yielded 340 species. Of these, 174 have never been described previously in the human-gut and 31 were completely novel species. This was compared to sequencing on the Roche 454 FLX-Titanium platform. However, it took three PhD students three years to isolate the colonies and the sequencing technology used would now be considered under-powered and does not give a true reflection on the sequencing resources that are now available.

However, it remains the case that a multi-pronged approach would almost certainly provide the best census of the organisms present in the chicken gut microbiota. Such an approach would combine the high speed, accuracy and throughput generated with modern sequencing techniques with isolation of

organisms through culture to optimise discovery and combine genotypic and phenotypic characterisation.

1.3.1 Microbial ecology

The advent of sequenced-based approaches allowed researchers to analyse bacterial populations that were previously inaccessible due to their rarity or inability to be cultivated in a laboratory [71]. However, this posed a new problem of placing sequences (such as 16S rRNA gene sequences) within a bacterial taxonomy. This resulted in two widely used methods of grouping sequences: into operational taxonomic units (OTU) or phylotypes [71].

OTUs are defined as clusters of small subunits of the rRNA gene defined by sequence similarity that can be used to provide estimates of microbial taxa, while remaining theory-agnostic as to the definition of bacterial species [72]. Since Stackebrandt and Goebal's influential paper in 1994, OTUs have most often been defined with a cut-off of ≥97% nucleotide identity [73]. In that paper, the authors compared the relatedness of 16S rRNA gene sequences to standard DNA-DNA reannealing and stated that 16S rRNA sequences with <97% identity are most likely a different species. However, they claimed that if there is ≥97% identity then they can fall into the same species or different species by previously accepted criteria [73, 74]. Therefore, whilst an OTU might be considered a proxy for a species, there are various caveats to consider, including:

- Some named species have rRNA genes that share ≥97% identity resulting in OTUs representing multiple species
- Artefacts from read errors or chimeras may result in spurious OTUs
- Single species can have multiple copies of the gene that are below the 97% threshold thus causing the species to have more than one representative OTU [75]

These factors can lead to the number of OTUs far exceeding the number of expected species, although some errors can be corrected with the use of quality filtering tools [76, 77].

Phylotyping involves defining reference taxonomic outlines to classify the sequences to taxonomic bins. This often involves classifying a sequence according to its relationship with cultured and characterised organisms. However, this is problematic when organisms with the same phenotype belong to different lineages and organisms that belong to the same species have different phenotypes [71]. Another limitation of phylotyping is because it is based upon previously cultured species, therefore there is often a lack of well-defined taxonomy at genus and species level [71].

However, using an OTU-based method can overcome some of the limitations of phylotyping. As a taxonomy outline is not used, the assignment of OTUs to bins is not restricted by these outlines. Also, as methods for binning of OTUs are based on clustering and not classification, the differentiation between two sequences in the same OTU is dependent on other sequences in the dataset thus remaining theory-agnostic [71].

There are a variety of statistical approaches to analysing sequencing data representing species from an environment. Alpha-diversity is used to determine the diversity within the sample and beta-diversity is used to determine the difference in species composition between samples [74, 78, 79]. Rarefaction curves are used to plot alpha-diversity found within a sample: for example, the number of OTUs found in a set number of sequences. These are widely used to establish whether a sample has been sequenced to the required depth to observe all taxa and thus infer the total diversity of a sampled community [79].

Examples of alpha-diversity metrics commonly used are Chao1, Shannon and Simpson [80]. These metrics have advantages and disadvantages:

- Chao1 estimates species richness through the number of rare classes present in a dataset which means it will overestimate the species richness if there are lots of singletons [80, 81]
- Simpson's diversity index is a measure of the probability that two individuals randomly selected from a sample will belong to the same species (Gini-Simpson index is the probability they belong to different species), taking into account richness and evenness. However, it is weighted towards more abundant species [82-84]
- The Shannon index (or Shannon entropy) quantifies the uncertainty in the species identity of a randomly picked OTU from a dataset [85]

Beta-diversity is used to determine the distance or dissimilarity between samples [86, 87]. Here, the Bray-Curtis method quantifies the compositional dissimilarity between two samples, based on the counts at each site [86, 88]. Another method, UniFrac, measures the distance between samples based on

the lineages they contain. As it uses phylogeny, it is claimed to be more powerful because it can exploit different degrees of similarity between sequences [87].

Ecological theory has been applied to host metagenomes in an attempt to explain and predict compositional variability between and within hosts [89]. The late nineteenth century hypothesis of selection by the environment alone is now considered too naïve due to the increased understanding of dispersal limitation and diversification [89]. Metacommunity theory (an example of community assemblage theory) looks at the world as distinct areas of suitable habitat surrounded by unsuitable habitat. The theory is based upon predictions on the traits of individual organisms, the rate and extent of dispersal and the difference in conditions between the distinct areas. Using these predictions it enables the user to calculate how much the host metagenome is influenced by local adaptation or outside immigration [89].

1.4 Chicken gut microbiota

The microbiota of the chicken has been studied using both culture-dependent techniques [90-93] and culture-independent techniques [2, 29, 68, 94]. Bacterial colonisation of the gut of a chick is thought to occur soon after hatching when the chick ingests food. However, other studies have shown that bacteria can penetrate the eggshell prior to hatching and therefore colonisation may occur earlier than thought [95, 96]. In 18-19-day embryos *Bacillus* was isolated from the liver and large intestine and *Enterococcus* and *Micrococcus* from the gut [93, 97, 98].

Ingested feed is exposed to acidic environments and pepsins in the proventriculus and gizzard that results in a mostly sterile digesta. Furthermore, as the digesta enters the duodenum, the rapid shift in pH produces additional stress to any microbial survivors of gastric transit [99]. This is followed by the introduction of bile acids, digestive enzymes, lecithin and lysozyme, which provides an additional test to the remaining microbes. The result of these stresses often means that the upper regions of the chicken-gut have a lower bacterial load as the concentrations of pancreatic enzymes and highly active enterocytes are most abundant there. However, as the digesta pass through the gut, the concentration of enzymes and bile acids drop significantly due to catabolisation and absorption. This results in a more hospitable environment for any surviving bacteria to colonise and proliferate further down the gut [99].

1.4.1 Crop

The crop microbiota consists mainly of facultative anaerobic bacteria, primarily *Lactobacillus* species, at 10^8 – 10^9 cfu/g (Figure 1.3) [12, 100]. *Enterobacteria*, Gram-positive cocci and lactobacilli have been isolated from the digesta and mucosa of the crop [100]. Guan *et al.* (2003) determined that the population of lactobacilli changes readily during days one to seven. However, it stabilises after 14-days post-hatch [101].

Lactobacillus species detected in the crop include*: L. acidophilus, L. reuteri, L crispatus, L. salivarius, L. fermentum, L. amylovorus, L. aviarius, L. johnsonii* and *L. gallinarum* [100, 101]. Other bacterial taxa isolated from the chicken crop include *Bifidobacterium*, *Micrococcus luteus, Staphylococcus lentus, Enterobacter aerogenes, Pseudomonas aeruginosa, E. coli, E. fergunsonii,*

Bacteroides, *Eubacteriaceae*, *S. enterica* and *C. jejuni* [11, 100, 102]. The crop microbiota was found to be more similar to that of the duodenum rather than the ileum and caecum after 40 days [103]. However, in another study that included the gizzard, ileum, caecum and colon, the gizzard was found to have the most similar microbiota to the crop (Figure 1.3) [104, 105].

Lactobacilli form an almost complete layer, 2-3 cells thick on the superficial epithelium layers of the crop, irrespective of diet [12, 106]. Adherence occurs through the carbohydrate components of the bacterial cell wall, with a ~7 nm gap between bacteria and host cells, with no indication of migration through the crop wall [12].

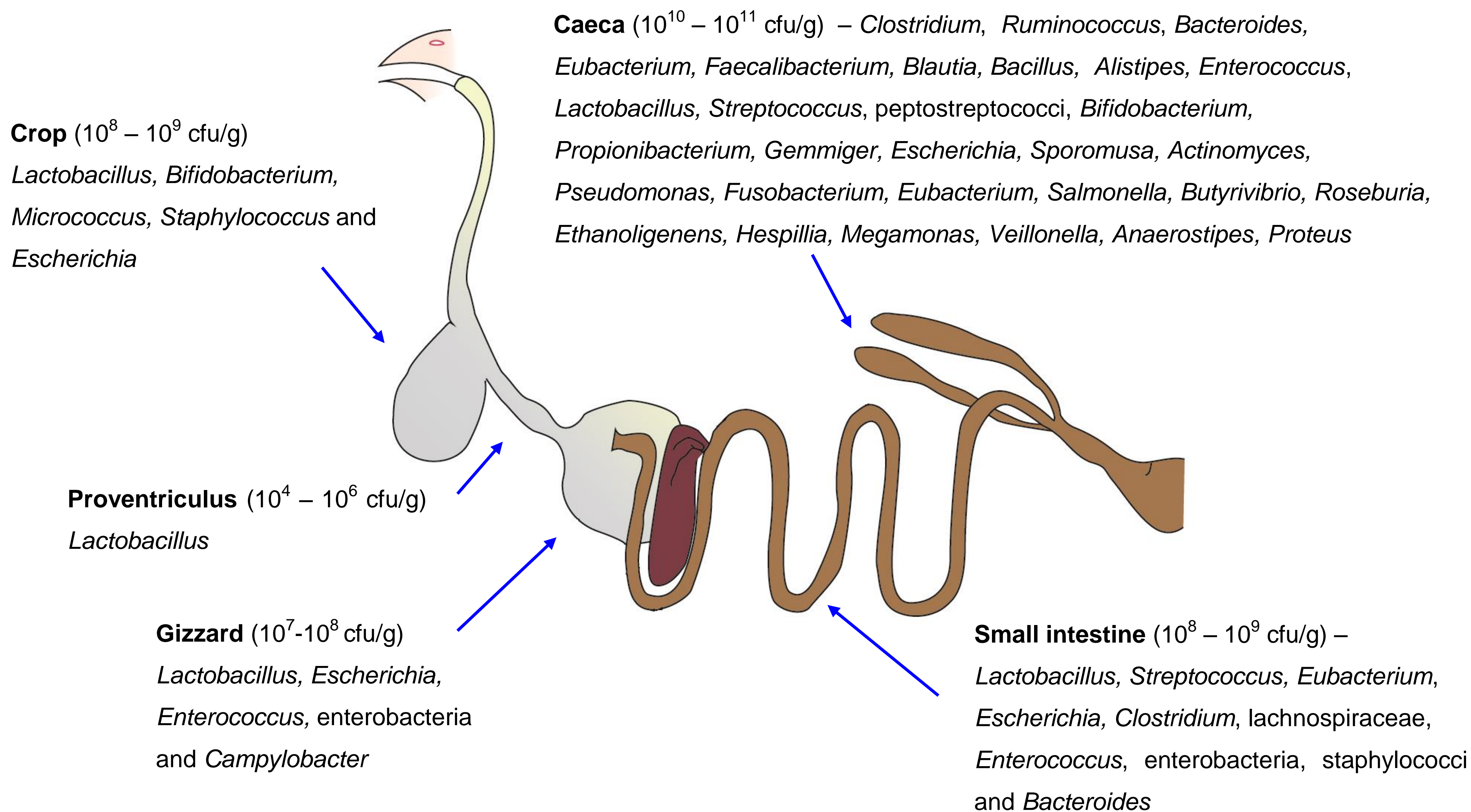


Figure 1.3 - Map of chicken gastrointestinal track showing structures with most common and abundant taxa.

Edited from Poultry CRC [13]

Colonisation of the crop by lactobacilli occurs within hours of the chick hatching and remains throughout the life of the chicken [12]. New epithelial cells are quickly re-colonised by bacteria from the lumen unless the chicken is starved, which happens often prior to slaughter [12]. Fluctuations in bacterial population size between meals are common due to the withdrawal of fermentable carbohydrates that lactic acid-producing bacteria require to proliferate. However, up to 10^6 lactobacilli can remain to inoculate and ferment the next meal [12, 102].

The probiotic nature of lactobacilli is illustrated when they are eliminated from the crop using high levels of penicillin; as a result coliforms increase from 10^5 cfu/g to 10^8 cfu/g[12]. Dominance by lactobacilli also confers a lower pH of 4.5, which causes a bacteriostatic effect on *E. coli* and *S. typhimurium* and a bactericidal effect for *Enterococcus faecalis*, *Micrococci* and vegetative cells of *Bacillus cereus* [12].

1.4.2 Proventriculus

The microbiota of the chicken proventriculus is thought to be dominated by lactobacilli, similar to that of the crop and gizzard (Figure 1.3) [44]. Whilst there have been numerous culture-dependent and culture-independent studies on the other organs of the chicken gut [2, 12, 19, 29, 60, 90-93, 101, 104, 107-112], only a single study has been published on the proventriculus [106], which reported 10^4 – 10^6 cfu/g of bacteria. The majority of these were lactobacilli, but unlike in the crop, the lactobacilli appeared not to adhere to the epithelium of the proventriculus.

1.4.3 Gizzard

The microbiota of the gizzard is closely related to that of the crop and is dominated by lactobacilli and *Clostridiaceae* (Figure 1.3) [104, 105]. Multiple studies by Engberg *et al.* found *L. salivarius* in considerable numbers, in addition to non-lactose fermenting, coliform bacteria, enterococci and other *Lactobacillus* species [109-111]. Total cfu/g counts in the gizzard have been recorded as 10^3 for aerobic bacteria, 10^2 for coliforms, 10^2-10^4 for *E. coli*, 10^2 for *Campylobacter*, 10^6-10^7 for lactobacilli, 10^5 for *Enterococcus* with a sum total of 10^7-10^8 [11, 109-111, 113]; *Clostridium perfringens* levels were found to be below 10^3 cfu/g in the gizzard [109]. Influx of acid from the proventriculus lowers bacterial counts and pathogen levels in the gizzard, compared to the crop, with significantly lower levels of total aerobic bacteria, *Campylobacter, E. coli* and coliform bacteria [11].

The type of feed ingested by the bird can affect the numbers of bacteria present in the gizzard. Broilers fed whole-wheat diets had a significant reduction in anaerobic bacteria, lactose-negative enterobacteria and enterococci, whereas pellet fed broilers experienced a shift from *L. salivarius* dominance to a mixture of lactobacilli species [109, 110].

1.4.4 Small intestine

Lactobacilli also dominate the microbiota of the small intestine, which increase in abundance from proximal to distal (Table 1.1) [114]. Low pH, short transit time and pancreatic and bile secretions all account for the reduced microbial abundance in the duodenum [100]. Deconjugation of bile acids and reduced

efficiency of digestive enzymes in the distal small intestine result in a more favourable environment for bacterial growth [100]. Furthermore, bacteria in the small intestine use 10-20% of carbohydrates and amino acids that would otherwise be used by the host [38].

Study	Section of small intestine	Method	Most dominant organisms
Salanitro *et al.* 1978 [93]	Duodenum	Culturing	*Streptococcus, Eubacterium, Lactobacillus* and *E. coli*
	Ileum		
Amit-Romach *et al.* 2004 [94]	Duodenum	16S rDNA primers and DNA gel band density	Lactobacilli, *E.coli and Clostridium*
	Jejunum		
	Ileum		
Gong *et al.* 2007 [115, 116]	Duodenum	16S rRNA clone library	*L. aviaries* and *L. salivarius*
	Jejunum		*L. aviaries*, *L. salivarius* and Lachnospiraceae
Stanley *et al.* 2012 [117]	Jejunum	16S rRNA pyrosequencing	*L. salivarius*, *L. crispatus*, *L. johnsonii*, *L. reuteri* and uncultured lactobacilli
Lu *et al.* 2003 [118]	Ileum	16S rRNA clone library	*L. salivarius, L. delbrueckii, L. acidophilus, L. crispatus*, *Streptococcus, Enterococcus,* Proteobacteria and *Clostridium*
Bjerrum *et al.* 2006 [5]	Ileum	Culturing and 16S rRNA clone library	Enterobacteria, enterococci, lactobacilli and staphylococci
Choi *et al.* 2014 [119]	Ileum	16S rRNA pyrosequencing	*Lactobacillus, Enterococcus, Streptococcus* and *Bacteroides*

Table 1.1 – Summary of the most abundant bacterial organisms throughout the small intestine.

Anaerobes, coliforms, lactic acid bacteria, enterococci and lactobacilli (especially *L. salivarius*) all increase in abundance from the duodenum to the ileum, regardless of pelleting or mashing of feed [110]. The diet of the broiler

has been shown to affect the microbiota of the ileum, with pellet-fed broilers containing more coliform bacteria, enterococci and *C. perfringens* compared to whole wheat fed broilers [109, 110]. Supplementation with xylanase in the broiler diet significantly increased anaerobic counts and lactic acid bacteria in the jejunum and increased the abundance of lactic acid in the ileum [109]. No *Lactobacillus* species have been found to be significantly abundant between high and low food-conversion-ratio (FCR) chickens [117].

1.4.5 Caeca

The caecal microbiota is the best-documented microbial community within the chicken gut. It is also the most abundant: at two weeks post-hatch the microbiota reaches 10^{11} cfu/g and is maintained at this level until at least six and a half weeks of age [90, 120]. Isolation of organisms from the caecal microbiota is difficult because 90% of them are facultative or obligate anaerobes, which often require exacting culture conditions [91, 94].

In a pioneering study by Barnes (1972), it was estimated that only a quarter of the microbiota could be isolated. They found that the majority were strict anaerobes, with counts of lactobacilli, streptococci, and coli-aerogenes fluctuating between 10^5 and 10^8 cfu/g [90]. At two weeks, peptostreptococci formed approximately 30% of the population. However, as the broilers matured the levels dropped to 9% and at four weeks, bifidobacteria and *Bacteroides* began to proliferate [90]. Since then, there have been several other reports documenting the constituents of the chicken caecal microbiota (Table 1.2).

Study	Method	Most abundant organisms
Barnes *et al.* (1972) [90]	Culturing	Lactobacilli, streptococci, coli-aerogenes, peptostreptococci, bifidobacteria, *Bacteroides*
Salanitro *et al.* (1974) [92]	Culturing	Gram-negative cocci, *Bacteroides*, *Clostridium, Eubacterium, Propionibacterium*, peptostreptococci, streptococci and facultative anaerobic cocci
Salanitro *et al.* (1974 &1978) [91, 107]	Culturing	*Gemmiger formicilis*, *P. acnes*, eubacteria, *C. clostridiiformis, Bacteroides,* Peptostreptococcus, *E. coli*
Coloe *et al.* (1984) [121]	Culturing	*Proteus*, Clostridia, *Bacteroides*, lactobacilli, streptococci, coliforms
Gong *et al.* (2002) [120]	16S rRNA gene clone library	*Faecalibacterium prausnitzii*-like, *Bacillus*, eubacteria, *Clostridium*, *E. coli*, *Enterococcus,* lactobacilli, ruminococci,
Zhu *et al.* (2002) [67]	16S rRNA gene clone library	*Clostridium coccoides, C. leptum, Sporomusa*, enterics, bacilli, *Bacteroides, Actinomyces,* P*seudomonas*
Lu *et al.* (2003) [118]	16S rRNA gene clone library	Bacteroidaceae*, Fusobacterium, Clostridium, Eubacterium,* ruminococci
Amit-Romach *et al.* (2004) [94]	Targeted rDNA primer and gel band density	Lactobacilli, *Clostridium, E.coli, Salmonella*
Bjerrum *et al.* (2006) [5]	Culturing and 16S rRNA gene clone library	*Bacteroides*, lactobacilli, *Ruminococcus, Clostridium, Eubacterium*
Wei *et al.* (2013) [24]	16S rRNA gene sequences in public domain census	*Ruminococcus, Clostridium*, *Eubacterium, Faecalibacterium, Blautia, Butyrivibrio, Lactobacillus, Roseburia, Ethanoligenens, Hespillia, Megamonas, Veillonella* and *Anaerostipes*
Sergeant *et al.* (2014) [2]	High-throughput 16S rRNA gene-fragment sequencing	*Megamonas, Veillonellaceae*, *Bacteroides*, *Alistipes*, Ruminococcaceae, lactobacilli

Table 1.2 – Summary of the most abundant bacterial organisms from selected studies of the caecal microbiota.

In the first culture-independent studies of the caecal microbiota, 25-72% of 16S rRNA gene sequences showed <95% homology to 16S rRNA gene sequences in BLASTN, compared to only 4% in the ileum [67, 116, 120]. A study into the diversity of succession of the caecal microbiota found significant differences in the population between three to seven days, 14 to 28 days and at 49 days. A series of successions from a transient community occurred in the young chickens and slowly moved to a more complex population with age. The caecal population was a subset of the ileum population until 14 days, when they became significantly different [118].

In a study of organic and conventional broiler chickens using both culture-dependent and culture-independent techniques in 2006, it was found there were significantly more lactose-negative enterobacteria and enterococci in the conventional broiler chickens. Using the Shannon index, they determined the organic broiler caecal samples were more diverse than the conventional broilers [5].

In an overview study of chicken caecum microbiota sequences in the public domain, Wei *et al.* (2013) estimated there could be 530-903 OTUs within the caecal microbiota and over 4,500 16S rRNA gene sequences would be required to achieve 99% of the diversity [24].

Recent advances in high-throughput DNA sequencing technology have allowed for deeper sequencing of the caecal microbiota. Sergeant *et al.* (2014) generated 414,070 16S rRNA gene-fragment sequences that were represented by 699 OTUs [2]. Using saturated rarefaction curves they established there were 200-350 OTUs in each chicken. Furthermore, 232 of the OTUs identified

showed <97% 16S rRNA gene sequence similarity to 16S rRNA gene sequences in GenBank and therefore could be novel species. This study found *Megamonas* was the most abundant genus, having over five times the number of 16S rRNA gene sequences as the next most abundant genus. Unnamed species from the *Veillonellaceae*, *Bacteroides*, *Alistipes* and *Ruminococcaceae* were in the top five most abundant organisms and two *Lactobacillus* OTUs were in the top ten. This has been the only study to report *Megamonas* as the most abundant genus. This genus belongs to an unusual class, the *Negativicutes*, which sit within the Firmicutes, yet possess a Gram-negative cell envelope. However, Zhu *et al.* (2002) did report high levels of *Sporomusa*, which also sits within the *Negativicutes* [2, 67].

The diet of a broiler is the strongest determinant of caecal microbiota in modern farming [122]. It has been suggested that the treatment and conditioning of the raw material affects the characteristics of the substrates for use by the microbiota [122]. The type of grain fed to broilers also has an effect on the microbiota, with corn-based diets selecting for low G+C bacteria (clostridia, enterococci and lactobacilli) and wheat based diets selecting for high G+C bacteria (bifidobacteria) [38]. In another study into the influence of xylanase and whole wheat fed to broilers, *Clostridium perfringens* and enterococci levels dropped in those fed whole wheat. However, levels of anaerobic bacteria, coliform bacteria, lactose-negative enterobacteria and lactobacilli showed no significant changes in abundance after feeding with whole-wheat or xylanase [109].

The presence of lactobacilli in the chicken caeca is antagonistic against *Campylobacter jejuni* and *C. coli* [123]. Of 150 lactic acid bacteria that were isolated from the chicken caeca, three *L. salivarius* strains were shown to have bacteriocins effective against *C. jejuni* and *C. coli*. This shows the potential of these strains to be used as probiotics to combat the growth of pathogens [123].

In a study of three chicken trials, 30.3% of OTUs were differentially abundant between the trials [124]. The chickens were sourced from the same hatchery, fed the same diet and reared in very similar conditions; however there was a high degree of variability in the microbiota between the trial flocks. They found that *Lactobacillus*, *Clostridium* and *Bacteroides* generated the largest differences between the flocks [124]. This level of variability between chickens kept in very similar conditions could explain why both culture-dependent and culture-independent studies find differences in the absolute and relative abundances of organisms, even before methodological differences in analysis are considered a source of variance [105, 124].

Although harvesting of caecal contents post-mortem is considered the gold standard in sampling this microbiota, this requires sacrificing of the bird and so prevents prolonged monitoring of temporal changes within the bird. To see if this problem would be avoided by use of faecal samples, the caecal and faecal microbiota were compared and quantitative differences were found [125]. Of the OTUs sequenced, 88.55% were shared and these represented 99.25% of all 16S rRNA gene sequences. There were more lactobacilli in the faeces, which was probably a result of small intestine origin. The study also found no statistically significant differences between the microbiota of caecal pairs, as

previously reported by Sergeant *et al.* (2014) [2, 125]. It was concluded that faecal samples could not be used as a substitute for caecal microbiota sampling, at least not without much greater sequencing depth [125]. And even though the same species might be detected in both samples, the communities had different alpha and beta-diversity values, which is unlikely to change with increased sequencing depth [125].

1.4.6 Non-bacterial organisms within the chicken gut

Bacteria are the most diverse and abundant form of life in the chicken gut. However, the chicken gut is host to microorganisms other than bacteria, albeit in low abundance [113]. Methanogenic archaea have been identified in the chicken caeca at levels of 10^4-10^8/g [29, 126, 127]. The abundance of archaea has found to increase with age, starting at three days; litter and flies were identified as a potential source of colonisation [126]. In a metagenomic study of two chicken caecal samples, 0.12-0.16% of the sequences were assigned to viruses, 0.8-1.1% were archaea (mainly methanogenic) and 0.1-0.2% were fungi; similar ratios were reported by Sergeant *et al.* (2014) [2, 29]. Archaea are more abundant in chickens that have been fed growth-promoter antibiotics with one study finding up to 2.2% of reads were assigned to archaea [68].

The levels of fungi detected in the chicken gut increase with age and are more common in the small intestine than the caeca [29, 128]. There were 50 fungal isolates that were assigned to *Aspergillus fumigatus, A. niger, Chrysonilia crassa, Mucor circinelloides, Rhizopus oligosporus* and *R. oryzae* [128].

Protozoan parasites within the *Eimeria* genus are often present in the environment where the chickens are raised and these disease causing organisms are often found in chickens [129].

1.5 The caecal metagenome

The first study of the chicken caecal metagenome in 2008, utilised 454 pyrosequencing to generate over 530,000 sequences and almost 200,000 environmental gene tags (EGTs) [29]. The majority of these were linked to carbohydrate metabolism with few respiratory genes present, representing the anaerobic environment of the caecum. In addition, virulence genes were also abundant; the majority of these (55-57%) were antibiotic resistant genes, with tetracycline and fluoroquinolones being most common. These classes of antibiotics are most common due to their routine use as antibiotic growth promoters until they were phased out in the EU between 2000 and 2005 [29]. In comparison to the human gut, mouse caecum and bovine rumen microbiomes, chicken and bovine microbiomes had lower abundances of invasion and intracellular resistance, prophage transposons and adhesion EGTs. However, there was no difference in the abundance of toxins and super-antigens, resistance to toxins and antibiotics and iron scavenging subsystems [29].

In a study of the caecal microbiome in response to anticoccidial and growth promoter treatment, they found enrichments of type IV secretion system genes, transport system genes and type I fimbrial genes, however no significant differences in antibiotic resistance gene counts [68]. The most prevalent functional groups were assigned to protein metabolism, amino acid synthesis

and carbohydrate utilisation. The significant enrichment of type I pili was probably due to the proliferation of *E. coli* in the monensin/tylosin and monensin/virginiamycin treatments, as the majority of the pili reads were similar to those found in *E. coli* [68].

Similarly to previous studies, Sergeant *et al.* (2014) discovered a large proportion of genes involved in carbohydrate metabolism [2]. As the caecum villi exclude less soluble, large polymers the number of cellulases and endohemicellulases accounted for 4% of the total reads, in comparison to 38% of reads assigned to oligosaccharide degrading enzymes. Bacitracin and tetracycline were the most common predicted antibiotic resistance genes [2].

1.6 Role of the microbiota in health and disease

There are many interactions between host, microbes and digesta within the gut [9]. The microbiota of the intestine form a protective barrier against pathogenic bacteria, preventing them from attaching to the host cells through competitive exclusion [130]. Enteric pathogens are a concern to the poultry industry due to production losses, reduced welfare of birds, increased mortality of birds and risk of contamination to products for human consumption (zoonosis) [131]. Zoonosis is the transmission of infectious diseases between animals and zoonotic bacterial pathogens can have detrimental effects on food safety, animal production and most importantly public health [132].

Using normal, germ-free and gnotobiotic mice, it was determined that the commensal gut bacteria and enteric viruses stimulate normal development of

the humoral and cellular mucosal immune systems [133]. Interactions between the mucosal immune system and gut microbiota maintain a normal level of inflammation throughout the life of a host [133]. Germ-free animals have a series of defects in gut-associated lymphoid tissues and antibody production than their colonised counterparts [134]. Furthermore, germ-free animals have a much slower turnover of intestinal epithelial cells (which line the gut and form a protective layer to the digesta) and an altered microvilli formation. This leads to decreased immune resistance and increased mortality when challenged with an enteric pathogen, something that is not observed in colonised animals [134].

A mechanism of pathogen-load control is the secretion of compounds that make the environment unfavourable for colonisation such as volatile fatty acids or bacteriocins [12, 123, 135]. The use of probiotics such as lactobacilli can have stimulatory effects of butyrate producing species and also re-establish balance in the gut [136].

As broilers are harvested whilst juvenile and immature there have been studies to develop and administer either single species or complex mixtures of bacterial species to newly hatched chicks from mature chickens [105]. It was found by Zhu *et al.* (2002) that only a complex mix of bacteria could supress *Salmonella*. However, they were unable to identify which species conferred the inhibition [67]. It has been demonstrated that colonisation of a pathogenic species can lead to preferable conditions for other pathogens. However, the presence of a pathogen in a healthy chicken does not always result in colonisation, significant changes in diversity or community structure [137, 138].

Necrotic enteritis was first described and attributed to *Clostridium welchii* (now *C. perfringens*) by Parish in 1961, when a large group of cockerels died after moving to a new location. Parish noted general signs of malaise and loss of appetite for three days, culminating in no food ingestion for 24 hours before death [139]. Necrotic enteritis levels dramatically increased after the banning of antibiotic growth promoters, showing they had a prophylactic effect in controlling the disease [140]. The spores of *C. perfringens* are ubiquitous in the environment and are therefore ingested via poultry feed regularly. However, it is thought that other predisposing factors such as high non-starch polysaccharides content and coccidiosis are required to cause disease [110, 131, 140, 141]. The cost of necrotic enteritis was estimated at $2 billion per year to the poultry industry in control measures and productivity losses [137, 142]. In a study of caecal microbiota in the birds that were challenged with *C. perfringens*, it was found that butyrate producers (*Eubacterium* species) were reduced in abundance in addition to *Weisella* species. Unclassified species in Mollicutes experienced a 3.7 fold increase in abundance and necrotic enteritis lesions were witnessed [137].

Coccidiosis has been studied in domesticated animals for over a century and the cost of the disease was estimated to be over £38 million in the UK alone in 1995 [143, 144]. Mortality, interruption of digestive processes and nutrient absorption, increased susceptibility to other diseases (such as necrotic enteritis) and reduced weight gain is caused by the multiplication of protozoan parasites within the *Eimeria* genus. The severity of the lesions caused by the disease are dependent on the number of oocysts ingested [141, 143].

C. jejuni is the most common cause of food-borne disease in the developed world [145, 146]. The transmission of *Campylobacter* is primarily through broiler flocks due to faecal shedding and coprophagia. However, it can also occur through feed and water [145, 147]. Broiler breed shows no significant impact on *C. jejuni* levels in the caeca [145]. In chickens, *C. jejuni* colonises the mucus of the epithelial cells primarily in the small intestine and caeca and is thought to be a commensal of the microbiota [145, 147]. However, it has been shown to cause intestinal inflammation and diarrhoea, leading to health issues with the feet and legs of the chicken [145].

The microbiota of high feed-conversion ratio (FCR) and low FCR birds have been compared in an attempt to identify the species that are more abundant in the high FCR birds [105, 117]. In a study of faeces from high and low FCR birds, it was found that eight families, including Fusobacteriaceae and Clostridiales family Incertae Sedis XIII (uncertain placement), were linked with high FCR and six were linked with low FCR birds, including Rikenellaceae, Enterobacteriaceae and Ruminococcaceae [148]. Butyrate producers and cellulose degraders in the caecum have been associated with high FCR chickens on multiple occasions [117, 124, 149].

1.7 Antibiotic growth promoters and antibiotic resistance

In 1946, Moore *et al.* fed chickens low levels of streptomycin and witnessed increased growth, noting decreased levels of coliform bacteria in the caeca

[150]. However, they failed to make the connection between increased growth with its significance for food animal production [151]. It was not until the inadvertent discovery in 1950 that feeding chickens fermentation waste from cyclotetracycline production as a source of vitamin B_{12}, led to improved weight gain and reduction in amount of feed required to bring the broilers to market weight [151, 152]. Antibiotics were shown to have similar effects in other livestock such as cattle and swine [151, 153, 154].

The suggested mechanisms of antimicrobial growth promoters include suppressing unrecognised infections, decreasing the microbial production of metabolites such as toxins, reducing the microbial destruction of essential nutrients, increased absorption of nutrients by the host because of a thinner intestinal wall and reducing the microbial-load of the chicken, thus reducing the competition for nutrients [155, 156].

The Netherthorpe report (1962) and later the Swann report (1969) into the use of antibiotics in animal husbandry and veterinary medicine in the UK highlighted the hazards posed by administration of antibiotics to livestock to human and animal health through the emergence of antibiotic resistant bacteria. It suggested the classification of certain antibiotics should be either “feed” or “therapeutic” to reduce the hazards to human health of resistance to clinically important antibiotics [157, 158].

The addition of growth-promoting antimicrobials to feed of livestock has been common practice worldwide, until recently. The use of antibiotics as growth-promoters in agriculture was banned in the EU in a two part process in 2005 and has also been restricted in North America [4, 159] due to fears of increased

antibiotic resistance in human pathogens. It was presumed that the removal of antibiotic growth-promoters would reduce exposure of bacteria to the antibiotic, thus decreasing the chance of resistance and the spread to humans via the food chain. This was demonstrated in Denmark where 105 tonnes were used for growth promotion in 1996 and was reduced to none in 2000 [160]. This resulted in a marked reduction of resistance to avoparcin, macrolides and virginiamycin in enterococci [160, 161].

However, the removal of the growth promoters, which acted as a prophylaxis, has resulted in an increase of some therapeutic drug treatments in livestock [161]. The use of total therapeutic antibiotics for poultry in the UK has gone up from 15 tonnes in 2005 (before the ban) to 60 tonnes in 2010. However, this remains much lower than the total amount used as growth promoters previously [4, 161, 162].

Infections caused by multi-drug resistant bacteria are a global health problem and is becoming increasingly more difficult with the emergence of multi-drug resistant organisms [163, 164]. A route by which pathogens can acquire antibiotic resistance genes is through the resistance reservoir present in the microbiota of healthy hosts [163, 165]. However, a study into 30,000-year-old permafrost sediments identified multiple antibiotic resistance genes, therefore suggesting resistance is a naturally occurring event and not only due to overuse [166].

Characterisation of the antibiotic resistance reservoir in the human microbiota uncovered 95 unique functional resistance genes to 13 antibiotics, suggesting the microbiota could contribute to the further emergence of multi-drug resistant

pathogens [163]. A similar study investigated the chicken gut microbiome of two free-range chickens and two chickens raised with feed containing antibiotics. 13 antibiotic resistance genes were identified; 11 from the chickens fed antibiotics. This was a much smaller study than the human microbiota, testing against only six antibiotics and using a clone library orders of magnitude smaller [132].

Antibiotic resistance has been found in common human and animal microbiota organisms. These include *E. coli* and enterococci [167-169] and disease causing organisms such as *Campylobacter* and *Salmonella* [170, 171]. They have been found within the chicken gut on multiple occasions and antibiotic resistance is common within caecal microbiome studies [2, 29, 68].

1.8 Enzymes in the chicken diet

1.8.1 Anti-nutritional effects of dietary non-starch polysaccharides in chickens

The diet of a chicken often consists of wheat, rye, barley, corn and sorghum cereals and the cell wall of these grains and cereals primarily consist of complex carbohydrates that are referred to as non-starch polysaccharides (NSPs) [172]. These NSPs were originally thought to provide the chicken with small nutritional contributions; however, it has been shown that the water-soluble NSPs have an anti-nutritional effect even when in minute quantities [173, 174]. This is because chickens lack the endogenous enzymes to effectively cleave and digest NSPs. The glycosidic bonds in dietary NSPs such as arabinoxylans, β-glucans, cellulose or pectic polysaccharides can only be

cleaved by enzymes derived from microbes [175]. β-glucans have been isolated from barley and fed to broilers, which led to a decrease in the rate of growth in addition to an increase in the viscosity of the digesta. Furthermore, it was demonstrated that the β-glucan polymer passes through the chicken-gut unchanged [173]. The anti-nutritional effects of the NSPs fall within three main areas; increase in intestinal viscosity, nutrient encapsulation and interaction with the intestinal microbiota.

1.8.2 Increase in intestinal viscosity

The β-glucans from barley and the arabinoxylans from the cell wall in cereals such as wheat and rye form viscous solutions when dissolved in water. The ability of these polysaccharides to form gels when mixed with fluid results in increased viscosity in the gut of the chicken [176]. The level of viscosity conferred by the NSP is determined by factors such as water solubility, how they are bound to other cell wall constituents, the presence of a charged group, the size of the molecule, the concentration and whether it is branched or linear. The high viscosity in the small intestine caused by the NSPs results in reduced digestion, absorption of protein, fat and starch [175]. The reduction in the nutrient absorption efficiency that causes depressed growth performance has been reported in diets containing barley, wheat or rye [177-179].

The presence of viscous polysaccharides has been shown to diminish glucose and salt diffusion [178]. It has been speculated that this is because digestion is dependent on the diffusion of enzymes and substrates and any hindrance to movement of these molecules will lead to a decrease in the efficiency of the

process [180]. It has been shown in rats that the addition of NSPs inhibits absorption of nutrients by increasing the relative thickness of the unstirred water layer, making it more difficult for the nutrients to diffuse through and reach the epithelium [181]. Viscous polysaccharides have been shown to depress lipid digestibility more than protein or starch. This is thought to be a consequence of bacterial overgrowth in the small intestine and the subsequent deconjugation of bile acids, thus resulting in reduced efficacy in solubilising lipids. Furthermore, it has been confirmed that the addition of carboxymethylcellulose (CMC), a non-fermentable gelling fibre, resulted in a decline in the digestion of lipids by decreasing the concentration of bile acids present in the chyme [182, 183]. Mathlouthi (2002) demonstrated that NSPs caused increased viscosity in the small intestine, which resulted in reduction of sodium and glucose transport, release of bile acids and pancreatic enzymes [174].

1.8.3 Nutrient encapsulation

Wheat consists of a bran layer (including aleurone), the endosperm and the germ (Figure 1.4). The aleurone layer contains tightly packed proteins, vitamins and minerals; but is protected by a tough pericarp and seed coat layer. The aleurone layer consists of cells with thick walls that protect the endosperm, which contains starch and protein. Therefore, for the chicken to release the nutrients within the layers, it has to have an effective enzyme suite [99, 184]. The grinding of the grains in the gizzard can cause rupture of the endosperm cell walls and hence aid digestion. However, some of the grains remain untouched after being consumed. This means the grains will escape digestion

and undergo fermentation in the hindgut, which leads to poor nutrient recovery [185].

1.8.4 Impact of dietary NSP on intestinal microbiota

Since NSPs are known to increase intestinal viscosity, this results in a slower feed passage rate and decreases the rate of digestion. This can facilitate the ability of the bacteria to colonise the higher areas of the gut that were not possible previously [141]. This ability to propagate in the small intestine due to the addition of dietary NSP has been demonstrated [99, 141, 180, 186]. In addition, bacterial species within the gut can also be altered with an increase in viscosity with the abundance of *Bacteroides*, *Clostridium* and *Lactobacillus* in the duodenum and jejunum greatly increasing (the ileum counts remain stable) [183].

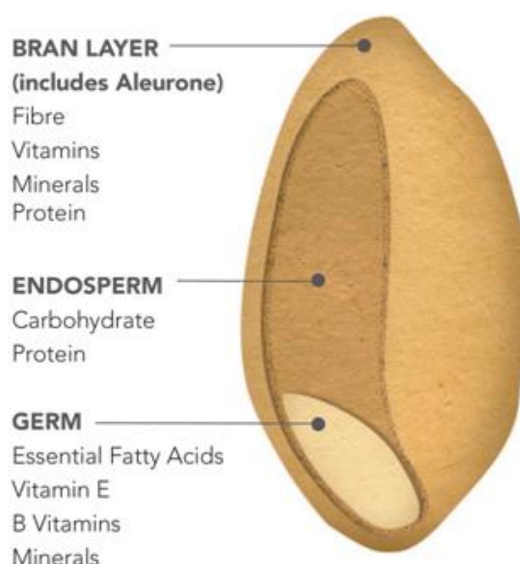


Figure 1.4 – Cross-section of a wheat grain showing different layers and their constituents.

Edited from: http://www.glnc.org.au/grains/attachment/grain-cross-section/

1.8.5 Adverse effects of intestinal bacteria on nutrient utilisation

Bacteria within the gut can have positive effects on the host such as the production of volatile fatty acids, polyamines and other nutrients [18, 183]. This has stimulatory effects on the intestinal mucosa by increasing the rate of secretion [18, 183, 187]. However, the presence of bacteria in the gut also leads to competition with the host for nutrients and the stimulation of rapid epithelial cell turnover, which leads to great energy costs for the host [141].

The ability of inhabiting bacterial species such as *Lactobacillus*, *Bifidobacterium*, *Bacteroides, Enterococcus* and *Clostridium* to cause the deconjugation of bile salts by hydrolysing the amide bond allows other species of bacteria to proliferate that would otherwise be unable to [141, 188]. In addition, chickens that have been kept germ-free show a greater FCR, less endogenous nitrogen loss and better absorption of lipids [189, 190].

1.9 Dietary supplementation with NSP-degrading enzymes

1.9.1 Mode of action of NSP-degrading enzymes

The multiple large polymers within NSPs form a viscous, mesh-like structure [184]. The addition of NSP-degrading enzymes aids the digestion of large polymers into shorter, unentangled fragments, thereby drastically reducing the intestinal viscosity. Enzymatic depolymerisation yields multiple products,

including simple sugars, oligosaccharides and low molecular weight polysaccharides [184]. The addition of a β-glucanase to hydrolyse β-glucans has been shown to increase the digestibility of nutrients in chicks (Figure 1.5a) [174, 191]. This has also been demonstrated with the addition of xylanase to hydrolyse xylan in a broiler diet (Figure 1.5b) [174].

1.9.2 Modification of intestinal microbiota and the prebiotic effects of enzyme hydrolysis products

The depolymerisation of NSPs by enzymes not only increases nutrient utilisation but has also been shown to reduce the bacterial load in the chicken gut. The addition of xylanase to a wheat-based diet reduced the total bacterial count by 60% due to decreased viscosity and increased transit times through the gut [192]. Furthermore, the formation of mannan-oligomers by hydrolysis can result in competitive exclusion of the intestinal binding sites.

This leads to a reduction in the colonisation and disease prevalence, thus allowing the intestinal mucosa to absorb more nutrients [193]. It has also been reported that the addition of xylanase significantly increases the amount of lactic acid producing bacteria in the small intestine [109]. These lactic acid bacteria form an integral part of the bacterial population in the crop, intestine and caeca. They have been attributed to maintaining the equilibrium between bacterial species in the gut and are available commercially as a prebiotic as they reduce the presence of *S. enterica* and the food-borne human pathogen *Campylobacter* [123].

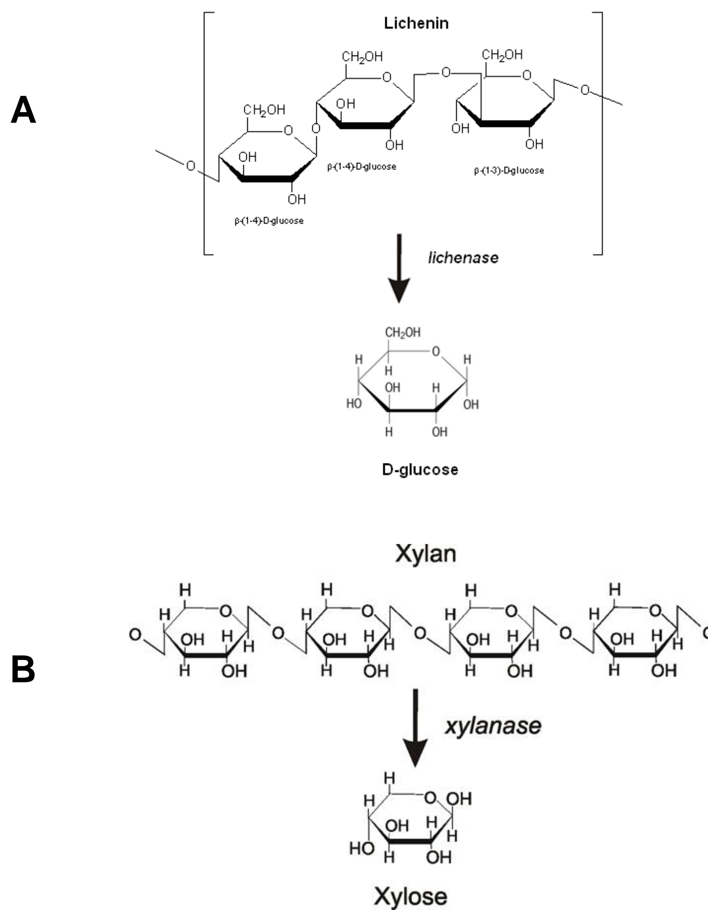


Figure 1.5 – **a)** Section of Lichenin (a β-glucan) and the product after the addition of lichenase **b)** Section of xylan and the product after the addition of xylanase.

Edited from: Sigma Aldrich

1.9.3 The addition of phytase

Unlike NSPs, phytate (the salt form of phytic acid, Figure 1.6) is a fixed chemical entity that is the storage form of phosphate in plants. It is also known to have anti-nutritive effects for most animals and does not provide a sufficient source of phosphorus, particularly in younger animals [99]. Phytic acid is a reactive anion that can form a variety of insoluble salts with minerals such as

calcium, copper, magnesium and phosphorus. As the chicken lacks endogenous phytase enzymes to hydrolyse the phytic acid, nutrients bound to it are poorly available to the host [194].

Phytate forms complexes with proteins when free in the gut causing them to be less vulnerable to proteolysis and to bind to digestive enzymes [99]. Furthermore, as the chicken gut cannot readily extract the organic phosphorus from the phytic acid found in cereal grains and oilseed meals, inorganic phosphorus has been frequently added to chicken feeds [195]. However, because the chicken is unable to utilise all of the added inorganic phosphorus, this leads to excess phosphorus excretion and potential eutrophication from agricultural waste seepage into waterways, thus financial penalties are incurred when waste is disposed.

A particular focus of industrial companies is on the enzyme phytase, which can be added to the diet to aid in phytic acid digestion [196, 197]. Phytase is effective in improving the retention of dietary phosphorus and it has been suggested that it could aid the retention of amino acids and energy [194, 198]. The amount of phytase required by the chicken decreases with age because the feed passage rate drops significantly in older birds and so the opportunity for phytase activity is increased [99]. However, the greater the presence of calcium in the diet, the less efficient the phytase becomes. This is because calcium precipitates the phytate and interacts with the soluble substrate, reducing its susceptibility to enzyme attack [99, 199].

The effect of phytase on the microbial ecology of the gut was studied in 2015 using *in situ* fluorescent hybridization (FISH) of eight targets. It was found

phytase increased the pH of the ileum in addition to total bacterial abundance in the ileum; particularly the abundance of lactobacilli and enterococci [112]. However, only the ileum microbial contents were analysed, therefore it is unknown what the effects are to other parts of the chicken gut. Furthermore, the use of targeted probes could result in missed changes to other members of the microbiota that could be important.

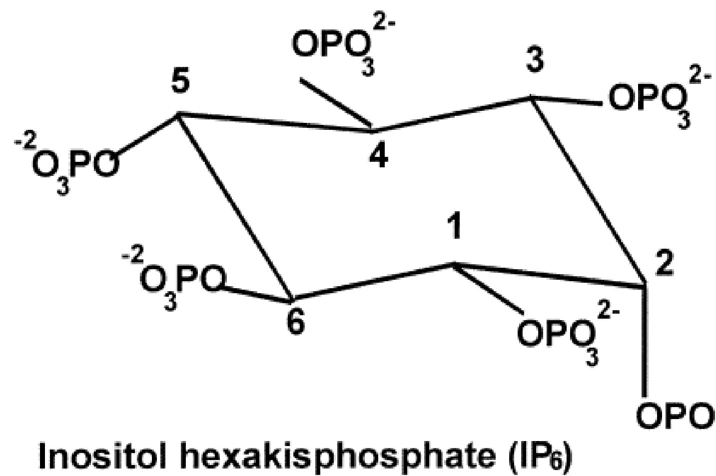


Figure 1.6 – Schematic diagram of phytic acid [99].

1.10 Aims and objectives of this study

Extensive culture-dependent analyses have been performed on the chicken gut, with the exception of the proventriculus. However, there has been limited high-throughput sequencing of the microbial communities colonising the organs that form the proximal chicken gut— almost all previous studies have focussed on the small intestine and caecum. The overall aim of the study was to complete high-throughput sequencing of the gut microbiota to analyse the spatial heterogeneity of the microbiota in chickens fed a standard or phytase-supplemented diet (Chapter Three and Four). Efforts were made to culture isolates from each gut section (Chapter Five) and novel isolates were genome-sequenced (Chapter Six). The microbiotas from the crop, proventriculus, gizzard, duodenum, ileum and caecum were studied in six chickens: three fed a standard diet and three fed a standard diet supplemented with phytase.

The objectives were to: -

- Provide a taxonomic census of the chicken gut
- Map the spatial heterogeneity of the chicken gut
- Identify the effect of phytase on the microbiota
- Isolate bacterial species from the microbiota with a particular focus on novel bacterial species
- Perform whole genome sequence analysis of novel isolates

CHAPTER TWO

2. Materials and methods

2.1 Sample collection

Gut samples were collected from Ross broilers on two diets at 35 days, housed indoors under standard commercial conditions at ADAS UK Ltd. They were fed on a wheat-based diet with 5% maize and one of the groups had a phytase enzyme supplement at 2,500 FTU/kg. There was no feed withdrawal before the sacrificing of the chickens. The broilers were randomly selected from a dietary group before being euthanized by cervical dislocation and the gut organs removed. The samples (whole organ, including contents) were weighed and labelled according to the diet, organ and number of the broiler in the order they were sacrificed (Table 2.1). The samples were plunged in liquid nitrogen and transported to the laboratory on dry ice and stored at the University of Birmingham or University of Warwick until processing.

2.2 Suppliers

All media, chemicals and reagents used in this study were obtained from Lab M, Life Technologies, Oxoid or Sigma-Aldrich unless otherwise stated.

2.3 Media

All media were prepared as specified in the manufacturer's instructions and autoclaved at 121 ^{o}C for 15 minutes at 15 psi (Table 2.2). All antibiotics or supplements to be added to the media were sterilised by filtration through a 0.22 μm filter, except horse blood.

Sample ID	Weight (kg)
Control 1 crop	1.95
Control 1 proventriculus	
Control 1 gizzard	
Control 1 duodenum	
Control 1 ileum	
Control 1 caecum	
Control 2 crop	2.28
Control 2 proventriculus	
Control 2 gizzard	
Control 2 duodenum	
Control 2 ileum	
Control 2 caecum	
Control 3 crop	2.21
Control 3 proventriculus	
Control 3 gizzard	
Control 3 duodenum	
Control 3 ileum	
Control 3 caecum	
Phytase 1 crop	2.59
Phytase 1 proventriculus	
Phytase 1 gizzard	
Phytase 1 duodenum	
Phytase 1 ileum	
Phytase 1 caecum	
Phytase 2 crop	2.93
Phytase 2 proventriculus	
Phytase 2 gizzard	
Phytase 2 duodenum	
Phytase 2 ileum	
Phytase 2 caecum	
Phytase 3 crop	2.91
Phytase 3 proventriculus	
Phytase 3 gizzard	
Phytase 3 duodenum	
Phytase 3 ileum	
Phytase 3 caecum	

Table 2.1 – Chicken gut samples for DNA extraction and microbial culturing

Medium	Atmosphere	Temperature (°C)	Antibiotic	Pre-condition
Blood culture bottle	Anaerobic	37	n/a	Serial dilution
Blood culture bottle	Aerobic	37	n/a	Serial dilution
Brain-heart infusion agar	Anaerobic	37	n/a	Serial dilution
Fastidious anaerobe agar	Anaerobic	37	n/a	Serial dilution
Fastidious anaerobe agar	Anaerobic	37	Rifampicin	Serial dilution
Fastidious anaerobe agar	Anaerobic	37	Colistin	Serial dilution
Fastidious anaerobe agar	Anaerobic	37	Gentamicin	Serial dilution
Fastidious anaerobe agar	Anaerobic	37	n/a	Ethanol
Fastidious anaerobe agar	Anaerobic	37	n/a	Heating sample
Fastidious anaerobe agar + 5% horse blood	Anaerobic	37	n/a	Serial dilution
Fastidious anaerobe agar + 5% horse blood	Anaerobic	30	n/a	Serial dilution
Fastidious anaerobe agar + 5% horse blood	Microaerophilic	37	n/a	Serial dilution
Lysogeny agar	Aerobic	37	n/a	Serial dilution
Lysogeny agar	Anaerobic	37	n/a	Serial dilution
M9 minimal agar	Anaerobic	37	n/a	Serial dilution
Marine agar	Aerobic	37	n/a	Serial dilution
Marine agar	Anaerobic	37	n/a	Serial dilution
McConkey agar	Aerobic	37	n/a	Serial dilution
McConkey agar	Anaerobe	37	n/a	Serial dilution
Mueller-Hinton agar	Anaerobic	37	n/a	Serial dilution
Mueller-Hinton broth	Anaerobic	37	n/a	Serial dilution
Mueller-Hinton agar with sodium thioglycolate	Anaerobic	37	n/a	Ethanol

Medium	Atmosphere	Temperature (^oC)	Antibiotic	Pre-condition
Mueller-Hinton agar with sodium thioglycolate	Anaerobic	37	Vancomycin	Ethanol
Orange serum agar	Aerobic	37	n/a	Serial dilution
Orange serum agar	Anaerobic	37	n/a	Serial dilution
Schaedler agar	Anaerobic	37	n/a	Serial dilution
Schaedler broth	Anaerobic	37	n/a	Serial dilution
Schaedler broth with sodium thioglycolate	Anaerobic	37	n/a	heating sample

Table 2.2 – Media used for the cultivation and isolation of bacteria from the gut samples

2.3.1 Bacterial media

Fastidious anaerobe agar (FAA), consisting of a peptone mix, sodium chloride, starch, agar, sodium bicarbonate, glucose, sodium pyruvate and l-cysteine was routinely used for the culturing of anaerobic or facultative anaerobic bacteria on solid media. The medium was made as per the manufacturer's instructions (46 g per litre of dH_2O) and autoclaved. This medium was made fresh when required, as it could not be stored due to the reducing reaction of l-cysteine with oxygen in air. For blood agar plates 2-5% defibrinated horse blood was added once the agar cooled to 50-60 ^oC. Schaedler anaerobic broth and Mueller-Hinton broth supplemented with l-cysteine and sodium thioglycolate were used when culturing in liquid. For Lysogeny-broth (LB), 20 g of LB powder was added per litre of dH_2O and autoclaved. For making Lysogeny agar (LBA), 15 g of bacto-agar was added per litre of LB.

2.3.2 Bacterial growth conditions

Anaerobic or facultative anaerobic bacteria were grown on FAA plates in an anaerobic cabinet (Don Whitley) for two-five days at 37 ^{o}C. All LBA plates were incubated for one to three days at 37 ^{o}C or three days at room temperature (RT) in an aerobic or anaerobic atmosphere.

2.3.3 Bacterial glycerol stocks

For anaerobic or facultative anaerobic bacteria a 10 µl inoculation loop was used to scrape colonies from a fresh agar plate. The colonies were resuspended in Mueller-Hinton broth with 40% glycerol and supplemented with l-cysteine. All other bacteria were grown overnight in LB and a 500 µl aliquot was mixed with glycerol to a final concentration of 30%. All glycerol stocks were stored at -80 ^{o}C.

2.4 Buffers and solutions

2.4.1 Buffers and solutions for agarose gel electrophoresis

Tris-acetate (TAE) buffer was prepared as a 50 x stock solution and diluted to 1 x with dH_2O for the working solution. The stock solution consisted of 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) for one litre. Fermentas 6 x loading dye (#R0611) was added to the samples as required.

2.5 Isolation of DNA

2.5.1 Isolation of DNA from gut samples using Qiagen Stool kit

Gut samples were removed from -80 ^{o}C storage and 200 mg of sample was removed through scalpel excision of the organ and placed into a 2 ml screw cap tube. To help the lysis of cells, 1.4 ml of buffer ASL and 0.2 g of 100–300 μM acid washed glass beads (Sigma-Aldrich, Poole, UK) were added followed by disruption with 2 × 30 sec pulses at 6.2 m/s in a FastPrep FP120 machine. The tube was put into a heat-block preheated to 95 ^{o}C for 10 minutes. Each sample was vortexed for 15 seconds and centrifuged for one minute at 13,000 rpm before 1.2 ml of the centrifuged sample was pipetted into a new 2 ml microfuge tube.

To each sample, one InhibitEX tablet, which had been crushed into a coarse powder with a scalpel blade, was added and vortexed for one minute until completely dissolved. The purpose of the InhibitEX tablet is to bind to potential PCR inhibitors in the sample. The sample was subsequently centrifuged for three minutes at 13,000 rpm before pipetting all the supernatant into a new 2 ml microfuge tube and discarding the pellet. As carry over pellet would have had detrimental effects to future steps, the sample was centrifuged again at 13,000 rpm for three minutes and the supernatant processed.

In a new 1.5 ml microfuge tube, 15 μl of Proteinase K and 200 μl of supernatant from the second centrifugation was added followed by 200 μl buffer AL and incubated at 70 ^{o}C for 10 minutes. Proteinase K degrades and digests proteins

in the sample and buffer AL is a lysis buffer. To this, 200 µl of 100% ethanol was added and vortexed for 15 seconds to form a lysate. The purpose of the DNA is to allow for more efficient binding of the lysate to the column. The lysate was added to a QIAmp spin column and centrifuged at 13,000 rpm for one minute. The collection tube was discarded and replaced with a new collection tube before 500 µl buffer AW1 was added and centrifuged for one minute at 13,000 rpm. The collection tube was discarded again and 500 µl of buffer AW2 was added, centrifuged for three minutes at 13,000 rpm and the flow through was discarded prior to centrifuging again for a further minute to remove residual buffer AW2. AW1 buffer contains a higher proportion of ethanol to remove excess salt and improve the pH conditions. AW2 is a longer spin to remove digested proteins or other impurities. To elute the DNA 200 µl of buffer AE was pipetted onto the membrane and incubated for two-five minutes at RT then centrifuged for one minute at 13,000 rpm. Eluted DNA was stored at -20 ^{o}C until required.

2.5.1.1 RNAase step

Due to the high levels of RNA extracted using this kit, an RNase step was occasionally added after the InhibitEX tablet step. To the supernatant, 2 µl of RNase A (100 mg/ml) was added and incubated for 30 minutes at 37 ^{o}C. The protocol then continued from the Proteinase K step (detailed in Section 2.5.1).

2.5.2 Isolation of DNA from caecal sample using Powermax soil extraction kit

To a Powermax bead solution tube 200 mg of caecal sample was added and placed in a FastPrep FP120 machine for 2 × 30 sec pulses at 6.2 m/s. To the homogenised solution, 1.2 ml of solution C1 was added and vortexed for 30 seconds. Solution C1 is a lysis buffer and contains SDS which breaks down lipids and fatty acids in the cell membrane. The tubes were then fixed to the vortexer with tape and vortexed for 10 minutes, before centrifuging for three minutes at 13,000 rpm. The supernatant was transferred to a clean 2 ml microfuge tube and 500 µl of solution C2 was added prior to inverting the tube twice and incubating on ice for 10 minutes. Solution C2 removes inhibitors from the sample. The sample was centrifuged at 13,000 rpm for four minutes and the supernatant added to a new 2 ml microfuge tube. To the supernatant, 400 µl of solution C3 was added, inverted twice and incubated on ice for a further 10 minutes followed by a four minute centrifugation at 13,000 rpm. Solution C3 is another inhibitor removal reagent. The supernatant was added to a 5 ml tube containing 3 ml of solution C4 (after shaking to mix) and the tube was inverted twice. Solution C4 contains a high concentration of salt which allows the DNA to bind to the column. To a spin filter membrane, 1 ml of the solution was applied and centrifuged for two minutes at 13,000 rpm. This step was repeated until the full 3 ml and the supernatant had passed through the column. Subsequently, 1 ml of solution C5 was applied to the membrane and centrifuged for three minutes at 13,000 rpm. Solution C5 is an ethanol wash that reduces the salt concentration and removes other contaminants. The flow-through was discarded before centrifuging for a further five minutes at 13,000 rpm to ensure

all residual solution C5 was removed. The collection tube was replaced with a sterile 1.5 ml microfuge tube before 100 µl of solution C6 was pipetted onto the centre of the spin filter and incubated for two-five minutes at RT followed by centrifugation for one minute at 13,000 rpm. Solution C6 is a low salt elution buffer and therefore the DNA is removed from the membrane. Eluted DNA was stored at -20 ^{o}C until required.

2.5.3 Isolation of genomic DNA from solid medium cultures

Colonies were taken from a fresh agar plate using a 10 µl inoculation loop and resuspended in 500 µl of resuspension buffer. The absorbance was taken at $_{OD}$600 and more cells were added to increase the absorbance to four as required. Tubes were subsequently centrifuged for five minutes at 13,000 rpm to form a compact cell pellet. The cell pellet was resuspended in 180 µl of lysis buffer supplemented with 1 mg/ml lysozyme and RNAse A followed by incubation at 37 ^{o}C for 30 minutes. After incubation 200 µl of buffer AL and 15 µl of Proteinase K was added. Samples were vortexed before 200 µl of 100% ethanol was added and incubated at 70 ^{o}C for 10 minutes. The sample was then applied to the Qiagen stool extraction kit column and centrifuged at 13,000 rpm for one minute. The flow-through was discarded before the column was washed with 500 µl of AW1 buffer with centrifugation at 13,000 rpm for one minute followed by 500 µl of AW2 buffer with centrifugation at 13,000 rpm for one minute. DNA was eluted with 50 µl of buffer AE and centrifuged at 13,000 rpm for one minute. Eluted DNA was stored at -20 ^{o}C until required.

2.5.4 DNA extraction from agarose gels

Following DNA gel electrophoresis (detailed in Section 2.7.2), DNA fragments of interest were excised from an agarose gel with a clean scalpel blade whilst using a UV-illuminator to visualise the DNA bands. Excess agarose around the DNA band was removed prior to placing it into a 1.5 ml microfuge tube and weighing. If the gel slice weighed 400 mg the DNA band was cut in half. After weighing, buffer QG (from Qiagen gel extraction kit) was added in correspondence to the weight of the gel slice (600 µl of buffer QG for a 200 mg gel slice). After incubation at 50 ^{o}C for 10 minutes the tube was vortexed every two minutes for 15 seconds. Buffer QG enables the solubilisation of agarose and provides the best conditions for binding of the DNA to the column. After the gel slice had completely dissolved, the solution was applied to a QIAquick column and centrifuged at 13,000 rpm for one minute and the flow-through was discarded. The reservoir could only hold 800 µl; therefore any samples over 800 µl were loaded in two parts. To remove any traces of leftover agarose from the membrane, 500 µl of buffer QG was applied to the column and centrifuged at 13,000 rpm for one minute and the flow-through discarded. To wash and remove salts from the sample 750 µl of buffer PE was added to the column and incubated at RT for two-five minutes. Buffer PE removes salt and other impurities from the column. The column was subsequently centrifuged at 13,000 rpm for one minute and the flow-through discarded before being centrifuged again for a further one minute at 13,000 rpm to remove residual buffer PE. To elute the DNA, 30 µl of buffer EB was added directly to the centre of the membrane in the column, incubated at RT for two-five minutes and centrifuged for one minute at 13,000 rpm. Buffer EB is basic and contains low salt

concentrations and thus is used to elute DNA from the column. Eluted DNA was stored at -20 ^{o}C until required.

2.6 DNA quantification

2.6.1 Nanodrop 1000

The sample pedestal of a Nanodrop 1000 (Thermo Scientific, USA) was cleaned by pipetting 2 µl of dH_20 onto it and wiping with Whatman filter paper. The Nanodrop was blanked with 1 µl of appropriate solution before pipetting 1 µl of sample and taking a reading. The concentration, 260/280 nm and 260/230 nm ratios were recorded before washing the pedestal with 2 µl dH_20 after use.

2.6.2 Qubit 2.0

A working solution was made by diluting Qubit dsDNA reagent (Life technologies, USA) 1:200 with dsDNA buffer. To 0.5 ml tubes, 190 µl of working solution was added for standards and 198 µl was added for samples. To the standard tubes, 10 µl of Qubit standard was added and to the sample tubes, 2 µl of sample was added. The tubes were mixed by vortexing for three seconds ensuring bubbles were not formed. The tubes were incubated in the dark at RT for two minutes prior to quantification on the fluorometer and the concentrations recorded. All samples under 10 ng/µl were quantified using a high-sensitivity kit and for all other samples a broad-range kit was used.

2.7 Genetic manipulations

2.7.1 Polymerase chain reaction

The polymerase chain reaction (PCR) was used for amplifying genes for cloning and sequencing in addition to checking plasmid inserts. All designed primer pairs had similar melting temperatures (Tm) and were synthesised by Life Technologies. Table 2.3 shows the reaction conditions used throughout this study and Table 2.4 details the primer sequences.

2.7.2 Analysis of DNA by agarose gel electrophoresis

DNA was analysed by electrophoresis on 0.8-1.2% agarose gels depending on fragment length. For target fragments under 750 bp a 1.2% concentration was used, up to 1.5 kb 1% and anything over 1.5 kb a 0.8% concentration was used. Agarose was dissolved in the working stock of TAE buffer and melted in a microwave.

PCR/Polymerase	Initial denaturation		Denaturation		Annealing		Extension		Number of cycles	Final Extension	
	Time (secs)	Temp (°C)	Time (secs)	Temp (°C)	Time (secs)	Temp (°C)	Time (secs)	Temp (°C)		Time (secs)	Temp (°C)
Velocity	60	98	30	98	30	55	45	72	35	300	72
Phusion	30	98	10	98	30	55	45	72	30	300	72
Pfu	60	95	30	95	30	55	180	72	30	300	72
Myfi	60	95	15	95	15	57	45	72	30	-	-
16S rRNA Sanger	180	93	15	93	30	62	90	68	35	-	-
16S rRNA Illumina	180	94	30	94	30	55	60	68	30	300	68

Table 2.3 – PCR thermocycling conditions for all PCRs used in the project.

References to this table will be made when referring to specific PCRs.

Primer name	Sequence (5'-3')	Which study
27F	AGAGTTTGATCMTGGCTCAG	Sanger sequencing
1492R	CGGTTACCTTGTTACGACTT	Sanger sequencing
V3 Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	V3-V4
V4 Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC	V3-V4
V4 Forward	TCGTCGGCAGCGTCAGAGTGTATAAGAGACAGCMGGATTAGATACCCKGG	V4-V6
V6 Reverse	TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGGTTGCGCTCGTTRYGG	V4-V6

Table 2.4 – Primer sequences that were used in this study.

Once the melted agarose had cooled sufficiently SYBR safe (Life Technologies) was added to a final concentration of 1:10000 for DNA visualisation. The molten agarose was poured into a plastic cast and a plastic comb was used to produce wells before being removed once the gel had set. The gel was then placed into a horizontal electrophoresis tank with the TAE buffer level sufficiently high enough to cover the gel. If required, loading dye was added to the sample at a ratio of 6:1 and run at 80-100 volts. Hyperladder 1 kb (Bioline, UK) was used as a ladder on all gels. To visualise the DNA, the gel was transferred to a freestanding UV transilluminator or to a BioRad gel-doc system.

2.7.3 Post-PCR DNA purification with Qiagen PCR purification kit

Amplicons were purified by adding a 5:1 ratio of buffer PI to the post-PCR mix (if the PCR volume was 100 µl, 500 µl of buffer PI was added). When using Reddy mix (ThermoScientific) or Go-Taq G2 (Promega), 5 µl of 3 M sodium acetate was added to ensure optimum pH of ≤7.5 for binding DNA to the membrane. Buffer PB binds to DNA >100 bp and therefore any primers should not bind to the column. The solution was pipetted onto the membrane of a QIAquick column and centrifuged at 13,000 rpm for one minute. The flow-through was discarded and the column placed back into the collection tube. The column was washed with 750 µl of PE buffer by centrifugation at 13,000 rpm for one minute. To aid the removal of salts, buffer PE was left on the column for two-five minutes at RT. The flow-through was discarded and the column placed back into the tube and centrifuged for a further one minute at 13,000 rpm to

remove residual buffer PE, before the column was placed into a 1.5 ml microfuge tube. To elute the DNA, 30 µl of buffer EB was added directly to the centre of the membrane in the column, incubated at RT for two-five minutes and centrifuged for one minute at 13,000 rpm. Purified amplicon DNA was stored at -20 ^{o}C until required.

2.7.4 Post-PCR DNA purification with AMPure beads

After PCR, 22.5 µl of dH_20 was mixed with 22.5 µl of post-PCR mix. To this, 72 µl of AMPure beads were added and incubated for 10 minutes at RT. Using a magnetic particle concentrator the beads were pelleted against the side of the low-binding microfuge tube and the supernatant was removed. The beads were then washed twice with 200 µl of 80% ethanol, with vortexing between washes. The beads were dried at 37 ^{o}C prior to the addition of 10 µl of buffer TE to elute DNA. Purified DNA was stored at -20 ^{o}C until required.

2.8 DNA sequencing

2.8.1 Sanger DNA sequencing

Plasmids and amplicons were Sanger sequenced using a BigDye terminator kit and ABI Prism 3700/3730xl (Applied Biosystems) by Functional Genomics Laboratory, School of Biosciences, University of Birmingham or GATC- Biotech. These single primer-sequencing reactions required 3.2 pmol or 2.5 pmol final concentrations of primers respectively. For plasmid DNA 100-300 ng was

required and 20-80 ng for amplicons. Each reaction required a final volume of 10 µl (Table 2.5). A combination of Geneious and DNA baser software was used to analyse the sequencing data.

Solution	GATC – amplicon	Functional Genomics facility UoB - amplicon	Functional Genomics facility UoB - plasmid
Template (ng)	20-80	20-80	100-300
Primer (pmol)	2.5	3.2	3.2
Water (µl)	Up to 10	Up to 10 µl	Up to 10 µl

Table 2.5 – Sanger sequencing set up for amplicon or plasmid DNA.

2.8.2 Illumina 16S rRNA gene sequencing library preparation

The 16S rRNA gene was amplified from isolated metagenomic DNA using custom V3-V4 and V4-V6 primers (Table 2.4) with extensor ready mix (Thermo scientific) using the conditions shown in Table 2.3. These primers contained a barcode and two indexes to enable unique identification of each sample after sequencing. The post-PCR mix was cleaned up using AMPure beads (detailed in Section 2.7.4) and quantified using the Qubit 2.0 broad-range kit (detailed in Section 2.6.2). The fragment size was determined using an Agilent bioanalyzer (detailed in Section 2.8.4), which enabled library quantification. Samples were

diluted accordingly to 4 nM using resuspension buffer. From each library 5 µl was taken and added to a separate low-binding microfuge tube to pool the 4 nM libraries. To denature the DNA, 5 µl was taken from the pool and added to 5 µl of 0.2 N NaOH, vortexed and centrifuged at 280 rpm for one minute at RT. The denatured pool was incubated at RT for five minutes before 10 µl was removed and added to 990 µl of chilled HT1 buffer and placed on ice until required. The pool was diluted to a final concentration of 6-12 pM using chilled HT1 buffer. The final denatured DNA pool was then sequenced on a MiSeq using either the Illumina MiSeq V2 2x250 bp paired end protocol or MiSeq V3 2 x 300 bp paired end protocol.

2.8.3 Illumina Nextera XT library preparation

The buffers required for the protocol were removed from -20 ^{o}C storage and thawed on ice or on the bench. After the input DNA was diluted to 0.2 ng/µl, 5 µl was added to 10 µl of TD buffer in a 0.2 ml tube. ATM buffer (5 µl) was added to the tube and mixed by pipetting up and down five times prior to centrifugation at 280 rpm for one minute at RT. The tubes were subsequently placed in a thermocycler for five minutes at 55 ^{o}C and then held at 10 ^{o}C. To each tube, 5 µl of NT buffer was added and mixed by pipetting up and down five times before briefly centrifuging and incubating at RT for five minutes. After the incubation, 15 µl of NPM was added to each tube in addition to 5 µl of each index primer and mixed up and down by pipetting five times and briefly centrifuged. The samples were placed in a thermocycler and the Illumina program in Table 2.3 was used. Post-PCR samples were cleaned with AMPure

beads (detailed in Section 2.7.4) and the bioanalyzer (detailed in Section 2.8.4) was used to determine fragment length and the libraries were diluted accordingly. The same method was used to denature and pool the libraries as outlined in 2.8.2 Illumina 16S rRNA gene sequencing subsection.

2.8.4 Agilent bioanalyzer high sensitivity DNA kit

The high sensitivity DNA dye concentrate and gel matrix were removed from 4 ^{o}C storage and equilibrated to RT in 30 minutes. Then, 15 µl of high sensitivity DNA concentrate was added to the gel matrix in a microfuge tube before vortexing the solution. The mix was centrifuged at 2240 rpm for 10 minutes and stored until needed away from light at 4 ^{o}C. Once required, the gel-dye mix was allowed to equilibrate at RT for 30 minutes prior to use. A high-sensitivity chip was placed on the chip priming station and 9 µl of gel-dye mix pipetted into the appropriate well. The plunger was depressed until held by the priming station clip for exactly one minute before releasing. After the plunger had rebounded for five seconds it was pulled back to the starting position. A further 9 µl of gel-dye mix was added to other wells as specified. In all remaining wells, 5 µl of marker was pipetted before 1 µl of ladder was added to the specified ladder well. As the quantitative range of the High sensitivity DNA kit was 5-500 pg/µl, samples $\leq$50 µg/µl were diluted 1:10 and samples $\leq$100 µg/µl were diluted 1:20 with dH_2O. In the 11 sample wells, 1 µl of sample was added, if there were less than 11 samples, 1 µl of marker was added to each empty well. The chip was vortexed at 2400 rpm for one minute before running on an Agilent 2100

Bioanalyzer instrument for 45 minutes. The data was processed using an Agilent 2100 computer program.

2.9 Bioinformatic analysis

2.9.1 Processing of 16S rRNA gene-fragment sequences

Fastq files were de-multiplexed (including trimming of barcodes and primers and the removal of low-quality reads) using default Illumina software. Sequences were split further according to the barcode at the beginning of read 2 and assigned to a sample. Forward and reverse reads were joined using a custom java program to produce contigs. A quality-filtering step to discard reads with more than three mismatches was included. If any mismatches were identified, the base with the highest quality value was inserted into the contig. Each contig was subsequently assigned to OTUs using the UPARSE pipeline.

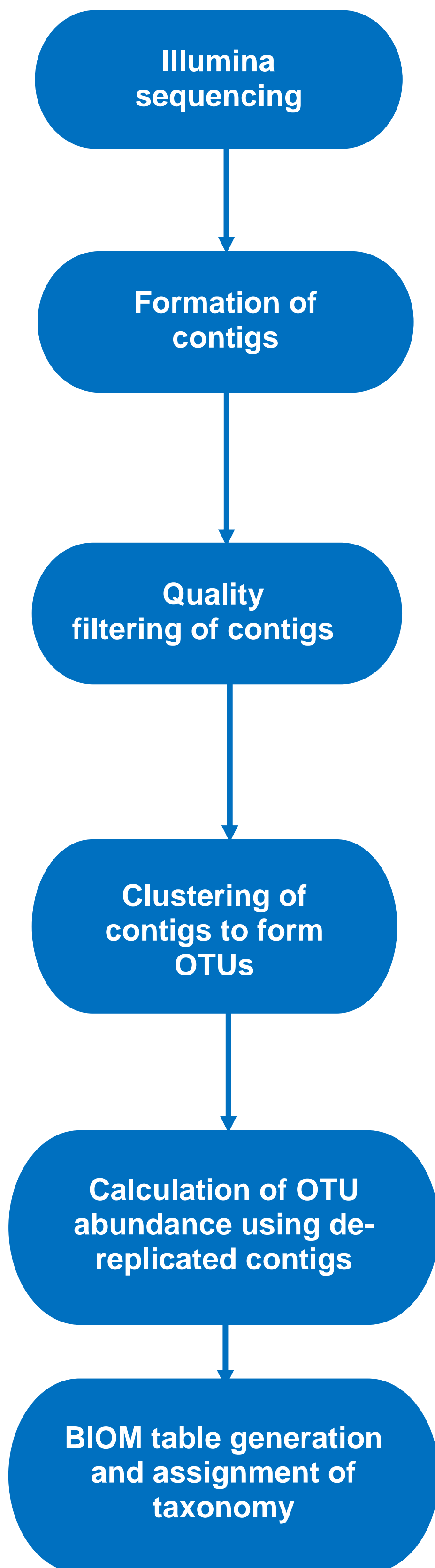
Initially, 16S rRNA gene-fragment sequences were de-replicated using a custom java program before sorting by size and singletons were excluded using *usearch –sortbysize –minsize 2*. Sequences were clustered at the default 97% identity level and chimeras were removed using *usearch –cluster_otus*. An extra chimera checking step was applied to OTUs when they were compared to the default GOLD database using the command *usearch –uchime_ref*. De-replicated reads were mapped against the OTU sequences with *usearch –usearch_global –strand plus –id 0.97*, which calculated the abundance of each OTU. A custom java program assigned the original reads (prior to de-replication) to each OTU and correlated them to each sample.

The OTU sequences were assigned taxonomic classification using assign_taxonomy.py in QIIME, which used the RDP classifier. An OTU consisting of the OTU counts for each sample and taxonomic classification was constructed using *make_otu_table.py* (Figure 2.1).

2.9.2 Alpha rarefaction and beta diversity analysis of 16S rRNA gene-fragment sequences

QIIME was used to perform alpha rarefaction analyses on the OTU tables generated from section 2.9.1 using the *alpha_rarefaction.py* command. The metrics utilised to determine diversity within the samples were: number of observed species, Chao1, Simpson diversity and Shannon diversity. The depth of rarefaction was determined by either the median number of sequences or the lowest number of sequences assigned to a sample within a group that was analysed.

To determine the diversity between the samples, *jackknifed_beta_diversity.py* was used. The depth of the rarefaction was determined by lowest number of sequences assigned to a sample within a group that was analysed. Bootstrap trees were produced using the unweighted UniFrac output from jackknife beta diversity with colour-coded bootstrap support.



Sequences trimmed to remove barcodes, primers and low quality sequences by a default program on the MiSeq.

Forward and reverse reads joined together to form a contig with a quality filtering step to remove contigs with more than three mismatches using a custom java program.

Using a custom java program the contigs were de-replicated then using the UPARSE pipeline, were sorted by size and contigs that appeared only once in the dataset were removed.

Using the UPARSE pipeline the contigs were clustered at 97% identity to form OTUs and chimeras were removed. Chimera check was completed again after comparing the OTUs to the GOLD database

The de-replicated contigs were mapped against the OTUs sequences which calculated the abundance of each OTU using the UPARSE pipeline.

Custom java program matches original reads to OTUs and then assigns them to each sample in a BIOM table. Taxonomy is assigned to each OTU using the RDP classifier and QIIME.

Figure 2.1 – Flow chart of steps used to generate analysable data in QIIME from raw sequence data

2.9.3 Statistical analysis of 16S rRNA gene-fragment sequences

To determine statistical significance between samples or organs, the OTU table was rarefied ten times and concatenated into a single OTU table using *multiple_rarefactions.py* in QIIME. The tests of significance were performed using a two-sided Student's two-sample t-test within *make_distance_boxplots.py* and the Bonferroni p-value was recorded. OTU significance was determined using *group_significance.py* and either a parametric T-test or ANOVA depending on the number of samples being compared.

2.9.4 Phylogenetic analysis of 16S rRNA gene sequences

To refine the taxonomic resolution of the most abundant OTUs in both diets, the sequences were imported into ARB and aligned using the SINA (SILVA incremental aligner) tool. The aligned OTU sequences were then added to the existing LTPs 115 SSU tree using the ARB parsimony (quick add marked) function. Further trees were built using the ARB neighbour joining distance matrix function to assess the confidence of each OTU position within the tree. To confirm the taxonomy of these sequences, 1,000 bootstrap trees were built to provide a consensus tree with the ARB estimation bootstrap values. The percentage on each branch point provided bootstrap support of each placement.

2.9.5 Analysis of Sanger sequenced isolates

The AB1 file from the sequencing of the isolate was loaded into Geneious, which calculated the sequence length, number of ambiguities, percentage of high quality bases and also provided a chromatogram trace. Sequences were trimmed manually according to quality before loading into BLASTN. Isolates were aligned against each other using BLASTN and if the comparison yielded ≤97% the sequences were retained for further analysis. For forward and reverse 16S rRNA gene sequences, DNABaser was utilised; both sequences were imported before the ends were automatically trimmed (optimising for high-quality samples) and assembled to form a contig. As a general rule, only isolates that gave ≤97% identity to the nearest named species via BLASTN were sequenced in both directions.

CHAPTER THREE

3. Optimisation of 16S rRNA gene-fragment sequencing on the chicken gut microbiota

3.1 Introduction

The organs of the chicken gastrointestinal tract consist of the crop, proventriculus, gizzard, small intestine (duodenum, jejunum and ileum), caecum and large intestine [113]. Each of the organs are differentiated functionally and morphologically and this can affect the microbiota that inhabit these environments [119]. The gut microbiota plays an important role in animal health; inhibiting the proliferation of pathogenic bacteria, synthesising vitamins for the host, aiding feed digestion and driving the development of the immune system [9, 29]. Due to these multifunctional roles, it is important to identify and understand the complex microbiota of the chicken gut.

The aim of this study was to map the spatial heterogeneity of bacteria from the chicken gut at a greater depth, identify any differences in the diets of six chickens (three fed a control-diet and three fed a phytase supplemented diet) using alpha rarefaction and beta diversity analyses and identify the microbiota of the proventriculus. The digesta from six organs of the chicken gut (crop, proventriculus, gizzard, small intestine first, small intestine last and caecum) were studied using 16S rRNA gene sequencing of the V3-V4 hypervariable region (full details of methods are detailed in Chapter Two).

There has previously only been one published study that sequenced multiple gut organs from a single chicken [119]. This previous study used a Roche 454 FLX Titanium sequencer to sequence the V1-V3 region of the 16S rRNA gene and generated 111,970 sequences assigned to 2,803 OTUs from three chickens. The

proventriculus was not sequenced in this previous study and there has been no published data regarding the proventriculus using culture-independent techniques and a single culture-dependent study that only referred to lactobacilli [106].

3.2 Methods

Methods for DNA extraction, PCR amplification set up, DNA purification, DNA quantification, DNA sequencing using an Illumina MiSeq and subsequent bioinformatic analysis is described in Chapter Two (Materials and Methods).

3.2.1 Normalisation of OTU coverage

As 16S rRNA gene operons can be present in multiple copies (one to 15 copies per cell), the reads representing an OTU may not provide an accurate representation of cellular abundance for that OTU [58]. PICRUSt was therefore used to normalise OTU reads using a 16S rRNA gene copy number predictor. The reads of 16S rRNA gene operons per genome were taken from *Integrated Microbial Genomes* (*IMG*). These series of scripts were used on a galaxy interface at http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=PICRUSt_normalize.

The output from PICRUSt was applied to the OTU table using a custom python script that divided reads by the number of 16S rRNA gene operons (e.g. *Escherichia* have seven gene operons so each *Escherichia* read would be divided by seven).

3.3 Results

3.3.1 Exclusion of organellar sequences

The processing of sequences resulted in a dataset of 2,482,715 reads from all samples. An initial phylum-to-genus-level taxonomic representation generated from OTU tables showed that most reads from the organs of the proximal gut originated from chloroplasts (Figure 3.1a). The OTU table was then searched for chloroplast OTUs, which confirmed that >85% of reads from four samples (control 2 proventriculus, control 3 proventriculus, phytase 1 proventriculus and phytase 1 gizzard) were assigned to chloroplast OTUs and therefore should be excluded from the OTU table (Table 3.1).

Further investigation of OTUs 6 and 561 (present in high abundance, but classified only to domain level) using BLASTN revealed that they belonged to the mitochondria of wheat (*Aegilops speltoides*) and a legume (*Lotus japonicus*). The chloroplast and mitochondrial OTU reads were removed from the OTU table before the command for summarise taxa was run again leading to an increase in the relative proportion of clostridia and bacilli in the proventriculus and gizzard (Figure 3.1b).

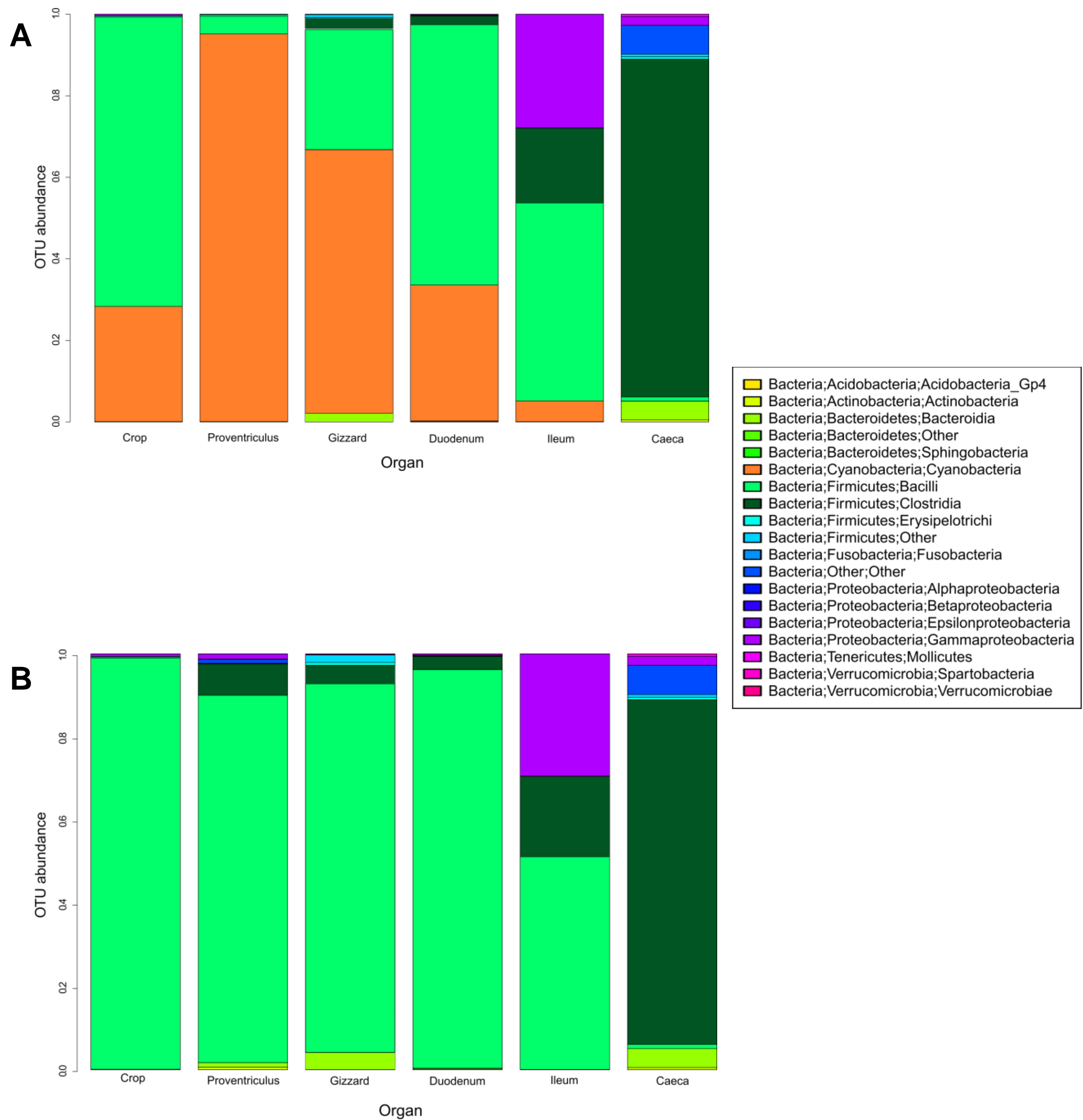


Figure 3.1 – a) Summary of taxonomic composition of combined diets including organellar OTUs from V3-V4 16S rRNA gene-fragment sequencing.

b) Summary of taxonomic composition of combined diets excluding organellar OTUs from V3-V4 16S rRNA gene-fragment sequencing.

Organ	Number of reads	Number of non-bacterial reads	Reads after deletions	% of reads deleted
Crop	194,420	55,671	138,749	28.7
Crop	287,587	17,262	270,325	6.0
Proventriculus	178,112	167,058	73,889	93.8
Proventriculus	249,729	216,106	11,054	86.5
Gizzard	172,620	71,768	100,852	41.6
Gizzard	215,628	121,285	94,343	56.2
Duodenum	46,520	29,666	16,854	63.7
Duodenum	191,274	15,330	175,944	8.0
Ileum	130,661	4,034	460,971	3.1
Ileum	338,040	3,878	126,627	1.1
Caecum	191,620	66	191,554	0.034
Caecum	286,884	75	286,809	0.026

Table 3.1 - Number of reads for organs (control and phytase), the number of organellar reads and the remaining reads after organellar reads were excluded.

3.3.2 Overview

In total, 862 OTUs were sequenced which were assigned to nine bacterial phyla, 18 classes, 31 orders, 64 families and 110 genera. The number of samples, reads, assigned OTUs and core OTUs per organ for each diet are summarised in Table 3.2. Figure 3.2 shows that only some samples were sequenced to a depth great enough to uncover all of the organisms present. Exclusion of organellar reads from

the V3-V4 16S rRNA gene-fragment sequencing dataset resulted in insufficient read numbers to perform a comparison of control and phytase diets in the crop, proventriculus, gizzard, duodenum and ileum (Figure 3.2). However, there were sufficient reads for analysis of the caecum.

Organ	Num. of samples	Total reads	Lowest num. of reads within organ	Num. of OTUs	Num. of core OTUs	Num. of core OTUs between diets
Crop	3	25,355	3,341	87	16	10
Crop	3	51,581	6,825	125	19	
Proventriculus	3	2,438	143	232	26	9
Proventriculus	3	6,786	371	115	13	
Gizzard	2	1,654	709	142	42	12
Gizzard	3	18,915	57	120	16	
Duodenum	2	3,562	928	243	58	17
Duodenum	3	35,122	4,560	225	23	
Ileum	3	17,937	4,655	174	127	6
Ileum	3	54,012	38	105	10	
Caecum	3	69,531	17,578	467	223	197
Caecum	3	111,263	22,401	696	319	

Table 3.2 – Summary of control (red) and phytase (blue) diet organs from V3-V4 16S rRNA gene-fragment sequencing after PICRUSt normalisation.

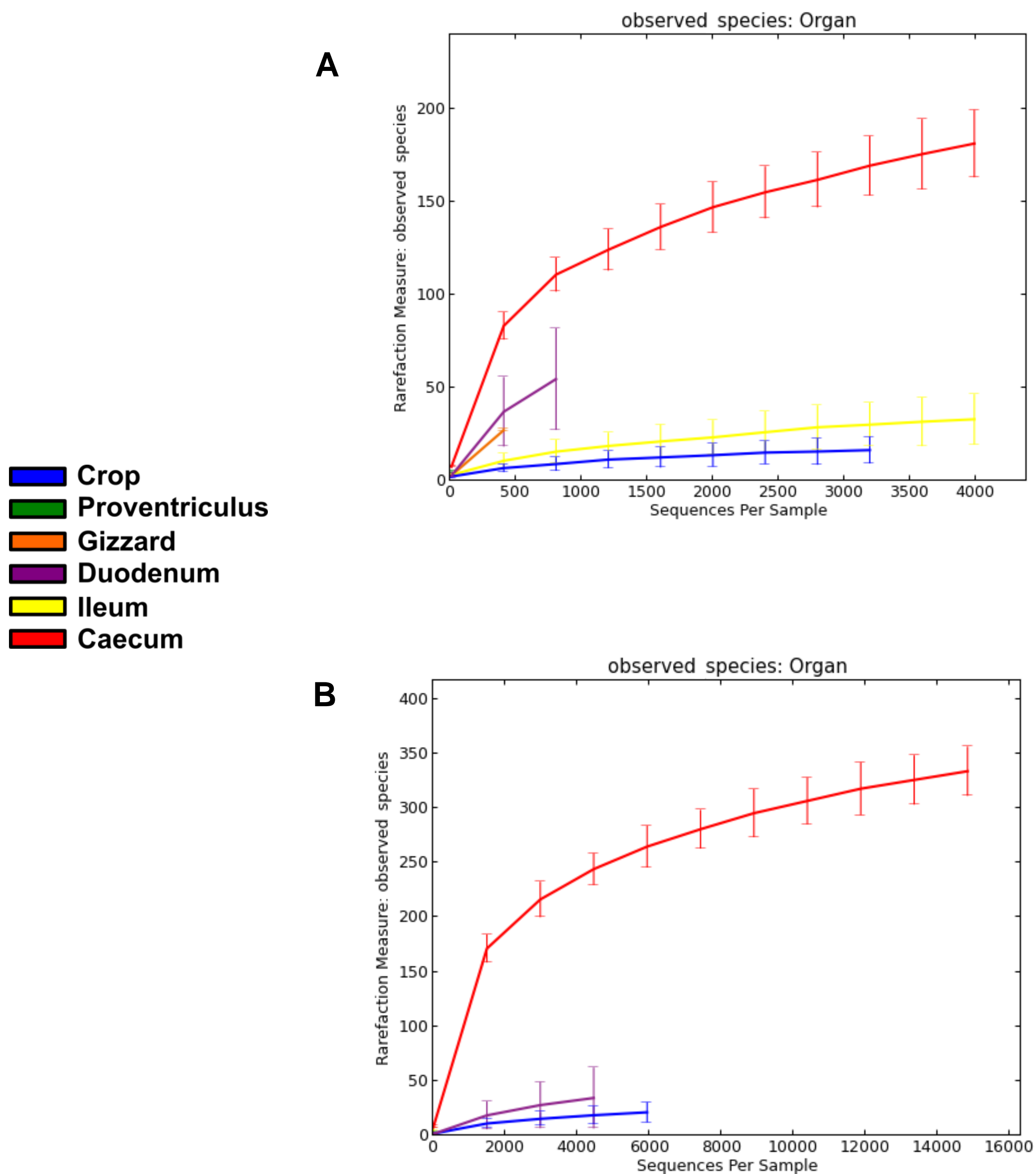


Figure 3.2 – a) Number of observed species against the number of sequences in control-diet organs.

b) Number of observed species against the number of sequences in phytase-diet organs.

*error bars are S.E.M.

3.3.3 Caecum

There were 69,531 reads represented by 467 OTUs from all caecum control-diet samples (n=3). OTUs were assigned to five named bacterial phyla and one unassigned bacterial phylum. At phylum level the majority of reads were assigned to Firmicutes (83.2%), followed by Bacteroidetes (7.6%) and Proteobacteria (4.8%),

At family level, Lachnospiraceae (35.2%) was the most abundant, followed by Ruminococcaceae (30.1%), Rikenellaceae (7.6%) and Enterobacteriaceae (4.8%). Within the Clostridiales family 15.4% of reads were unassigned. *Coprococcus* (19.1%) was the genus with the greatest number of reads. *Faecalibacterium* (13.2%), *Alistipes* (7.6%), *Escherichia/Shigella* (4.8%), *Butyricicoccus* (1.8%) and *Dorea* (1.1%) were the only other genera that accounted for ≥1% of total reads.

From 467 OTUs, 223 were identified in all caecum control-diet samples (n=3) of which 190 were Clostridiales. The most abundant OTU (OTU 20) represented 16% of total reads and was assigned to *Coprococcus*. There were four Lachnospiraceae (OTU 20, OTU 24, OTU 746 and OTU 393) OTUs in the top ten most abundant OTUs and three Ruminococcaceae (OTU 8, OTU 28 and OTU 55) (Table 3.3a).

There were 111,263 reads represented by 696 OTUs from all caecum phytase-diet samples (n=3). OTUs were assigned to five named bacterial phyla and one unassigned bacterial phylum. The most abundant phylum was Firmicutes (86.4%), followed by unassigned bacterial phylum reads (9.5%) and Bacteroidetes (2.6%).

There were two main assigned families; Lachnospiraceae (34.2%) and Ruminococcaceae (23.7%), with 20.2% of reads unassigned to family level. Bacteroidales reads were only attributed to Rikenellaceae in the caecum. Lactobacillaceae was the only family within Lactobacillales that had >0.1% of total reads. The most abundant named genus was *Coprococcus* (12.4%) with *Faecalibacterium* (8%) the second. Other genera with >1% of total reads were *Alistipes* (2.6%), *Blautia* (2.4%), *Dorea* (1.8%) and *Lactobacillus* (1.4%).

319 OTUs were present in all caecum phytase-diet samples (n=3) with Clostridiales OTUs the most common of the core OTUs. The most abundant OTU was assigned to *Coprococcus* (OTU 20), which accounted for 10.3% of total reads. Half of the most abundant OTUs were Lachnospiraceae (OTU 20, OTU 24, OTU 18, OTU 14 and OTU 216).

Alpha-rarefaction was completed on the caecum samples at a depth of 17,500 reads (Table 3.3b). Figure 3.3a showed the number of observed species against the number of reads and indicated the number of species began to level off at 17,500 reads, however more reads would be required to reach saturation (Figure 3.3a). The Simpson diversity figure (Figure 3.3b) showed that the microbiota shared a similar level of diversity between the diets, which is indicated in table 3.3b, where the control-diet is assigned 0.926 and the phytase-diet is assigned 0.939.

A

OTU ID	Taxonomy	% of total OTU reads in control diet	% of total OTU reads in phytase diet
4	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia/Shigella	4.8	N/A
7	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; Alistipes	7.6	2.6
8	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Faecalibacterium	13	8.0
10	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	N/A	6.1
14	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	N/A	2.4
18	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	N/A	2.8
20	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus	16	10.3
21	Bacteria; Firmicutes; Clostridia	N/A	2.6
22	Bacteria	N/A	2.1
24	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	3.3	3.7
28	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillibacter	1.5	N/A
45	Bacteria; Firmicutes; Clostridia; Clostridiales	1.5	N/A
55	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Butyricicoccus	1.5	N/A
216	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	N/A	2.3

OTU ID	Taxonomy	% of total OTU reads in control diet	% of total OTU reads in phytase diet
393	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus	1.4	N/A
746	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	2.8	N/A

B

Diet	Seqs/Sample	Chao1 Ave.	Chao1 Err.	Observed species Ave.	Observed species Err.	Shannon Ave.	Shannon Err.	Simpson Ave.	Simpson Err
Control	17,500	307.662	31.747	257.9	28.281	5.229	0.482	0.926	0.034
Phytase	17,500	420.146	17.507	348.933	23.606	5.701	0.479	0.939	0.033

Table 3.3 – **a)** Top ten abundant OTUs after V3-V4 16S rRNA gene-fragment sequencing of control and phytase diet caecum samples.

b) Alpha rarefaction results of the caecum from control and phytase diet samples after V3-V4 16S rRNA gene-fragment sequencing.

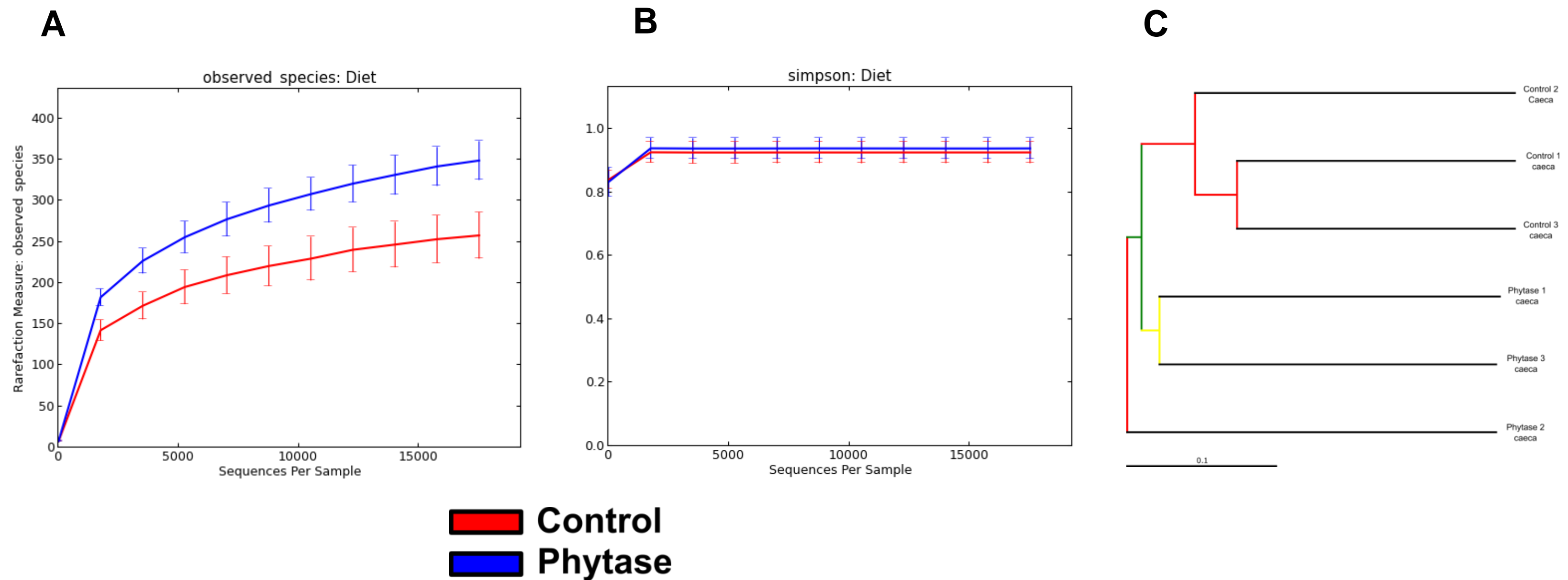


Figure 3.3 – a) Number of observed species against the number of sequences in control and phytase diets in the caecum after V3-V4 16S rRNA gene-fragment sequencing.

b) Simpson diversity index against number of sequences in control and phytase diets in the caecum after V3-V4 16S rRNA gene-fragment sequencing.

c) Jackknife beta diversity bootstrapped tree of caeca samples from control and phytase diets after V3-V4 16S rRNA gene-fragment sequencing.

Red=75-100%, yellow=50-75%, green=25-50%, and blue =< 25%

*error bars are S.E.M

There was no significant difference in diversity or significant OTUs between the control and phytase diets in the caecum with a p-value of >0.05.

Jackknifed beta diversity was completed on the caecum samples at a depth of 17,500 reads. This resulted in the control and phytase diets clustering separately, with two of the control-diet caecal samples clustering together with high jackknife support (75-100%). The support between the diets was 25-50% showing weak bootstrap support and one phytase-diet sample clustered further away from the other two (phytase 2 caecum).

3.3.4 Phylogenetic analysis of abundant OTUs from the caecum

Many trees failed to provide better resolution for OTUs that were assigned previously with the RDP classifier. In total, five of the most abundant OTUs from the caecal samples could be clustered at species level with their closest bacterial relative using the 16S rRNA gene V3-V4 hypervariable region (Table 3.4). Output from ARB indicates two OTUs (OTU 8 and OTU 28) were associated with *Faecalibacterium prausnitzii* and *Oscillibacter valericigenes* respectively with a bootstrap value of 99%, however, the branch length of OTU 28 indicates this is a different species.

OTU	Closest bacterial relative
7	*Alistipes putredinis*
8	*Faecalibacterium prausnitzii*
20	*Ruminococcus torques*
28	*Oscillibacter valericigenes*
55	*Eubacterium desmolans*

Table 3.4 – Five most abundant OTUs from V3-V4 16S rRNA gene-fragment sequencing of caecal samples, which were placed in an ARB tree with >50% confidence levels and their closest bacterial relative

3.4 Discussion

This study used V3-V4 16S rRNA gene-fragment sequencing to study bacteria in the crop, proventriculus, gizzard, duodenum, ileum and caecum from chickens fed either a normal diet or a diet supplemented with phytase. To the authors' knowledge, this is the first study to sequence the microbiota of the proventriculus and to use V3-V4 16S rRNA gene-fragment sequencing to study multiple regions of the chicken gut.

In this study, there were two major issues with using V3-V4 16S rRNA gene-fragment sequencing to survey the chicken gut microbiota. The first problem arose when trying to amplify the V3-V4 region from the DNA from the gizzard

and duodenum of the control-diet samples. Multiple attempts were made to amplify a product from two samples, using a range of PCR cycling conditions, primer concentrations, template DNA concentrations and indices. However, all failed. The second issue was the V3-V4 primers amplified organellar DNA present in the digesta from the feed consumed by the chicken. As this study was only interested in non-organellar bacteria, all chloroplast reads were removed and in some organs this resulted in the loss of over 600,000 reads. Due to the absence and/or low number of reads within samples it was not possible to generate reliable and accurate alpha rarefaction comparison data or beta diversity statistics for all organs.

The identification of 862 OTUs and 110 genera were similar to those found by Wei *et al.* (2013). However, the recovery of OTUs and genera were probably limited due to the abundance of chloroplast assigned reads in this study. The abundance of chloroplast reads was highest in the proventriculus and lowest in the caecum. This could be because the bacterial load in the proventriculus is among the lowest in the gut whereas the caeca has the highest bacterial load. Furthermore, the digesta has undergone relatively little degradation in the foregut, thus the relative abundance of organellar DNA is increased. The removal of sequences assigned to chloroplast OTUs made the analysis and comparison of organs difficult due to the low number of reads remaining in each sample.

A further limitation of 16S rRNA gene-fragment sequencing is the difficultly to determine OTU taxonomy beyond genus level. Therefore, the functions of the OTUs could be difficult to assign and predict. If the identified OTUs are

genotypically and phenotypically similar to their identified closest bacterial relatives, then it could be assumed they would have similar traits to those discussed.

Due to the caecum being the only organ to provide sufficient number of reads after sequencing, this is the only organ discussed in detail with regards OTU taxonomy. *Alistipes putredinis* was the closest bacterial relative to one of the most abundant OTUs in the caecum. Although the species *A. putredinis* has not been described within the chicken microbiota previously, *Alistipes* as a genus (reclassified from *Bacteroides* in 2003) is commonly found in the gut [2, 90, 92, 93, 121]. *A. putredinis* is bile-resistant and produces volatile-fatty acids [200]. Any benefits of *A. putredinis* to the chicken gut are unknown. However, organisms in the same genus are associated with increased FCR [149].

An OTU that *Ruminococcus torques* was the closest bacterial relative to, occurred in high abundance in multiple parts of the chicken gut but was the most abundant OTU in the caecum of both diets. It has previously been isolated from the chicken caecum and has been linked with improved performance in the caecum, perhaps because it can degrade mucin [5, 149, 201]. Mucin production can fluctuate in chickens in response to probiotics, enzymes, antibiotics and feed withdrawal [149, 202]. Furthermore, drops in nutrient availability within the gut can cause mucolytic bacteria to use mucus as a substrate, thus reducing the protective mucus layer [149]. The abundance of the *R. torques* in both diets could indicate high mucin production in the caecum and particularly in the control-diet allowing the organism to proliferate.

Eubacterium desmolans was the closest bacterial relative of OTU55, which was abundant in the control caecal samples and has previously been identified by 16S rRNA gene sequencing of the caecum. In that study it was observed to fall dramatically in chickens challenged with *C. perfringens [137]*. *Eubacterium* species have been isolated from the chicken on multiple occasions and is thought to be a dominant genus in the caecum in older chickens [91, 118, 121]. Inositol is the sole carbohydrate fermented by *E. desmolans*, which in environments enriched with inositol results in the production of volatile-fatty acids beneficial for chicken health [18, 183, 203].

This study found no significant difference in microbiota between control and phytase diets in the caecum. However, differences could have been masked by the low read numbers. The lack of usable data generated from 16S rRNA gene-fragment sequencing of the V3-V4 hypervariable region led to the design and use of primers that excluded chloroplast reads to enable greater depth of sequencing of the microbiota within organs with high chloroplast abundance (detailed in Chapter Four).

CHAPTER FOUR

4. Identification of the chicken gut microbiota using V4-V6 16S rRNA gene-fragment sequencing

4.1 Introduction

This chapter followed up on the 16S rRNA gene-fragment sequencing of the microbiota within the chicken gut. The V4-V6 hypervariable region of the 16S rRNA gene was sequenced in this study due to the amplification and sequencing of chloroplast DNA when using the V3-V4 16S rRNA gene-fragment which resulted in the exclusion of over 600,000 reads (as detailed in Chapter Three). Using the V4-V6 16S rRNA gene region almost mitigated this problem and allowed for deeper sequencing of samples to provide greater insight into the microbiota present in chloroplast dominant organs.

Chloroplasts are evolutionarily descended from bacteria and thus there is a homology between the 16S rRNA genes [204, 205]. There are limited regions of the 16S rRNA gene that allow almost universal bacterial 16S rRNA gene amplification without chloroplast amplification and the use of the 799F primer in this study was because chloroplast 16S rRNA genes have two base pair mismatches at 798 and 799 (*E. coli* numbering) [205].

The same samples that were sequenced with V3-V4 16S rRNA gene-fragment primers were sequenced with V4-V6 rRNA gene-fragment primers (Table 2.4).

4.2 Methods

Methods for DNA extraction, PCR amplification set up, DNA purification, DNA quantification, DNA sequencing using an Illumina MiSeq and subsequent bioinformatic analysis is described in Chapter Two (Materials and Methods).

4.3 Results

4.3.1 Overview

An alpha rarefaction plot of observed species showed that at a depth of approximately 10,000 sequences the curves began to level off and by 150,000 the majority of samples had levelled off completely indicating no more OTUs would be discovered with a higher number of sequences (Figure 4.1).

Application of the PICRUSt normalisation tool to the data reduced the relative OTU reads from ~10.5 million to ~2.5 million (Figure 4.2a/b). A total of 1160 OTUs were assigned to the reads; however 14 of these OTUs were excluded due to being assigned to organellar OTUs. The number of samples, reads, assigned OTUs and core OTUs per organ for each diet are shown in Table 4.1. This showed the minimum number of reads was 31,478, considerably higher than the number achieved through V3-V4 16S rRNA gene-fragment sequencing (38 reads). Furthermore, the V4-V6 primers were able to amplify a product using the digesta from each organ, therefore providing sequencing data for all three samples per organ, per diet, unlike the V3-V4 16S rRNA gene-fragment sequencing.

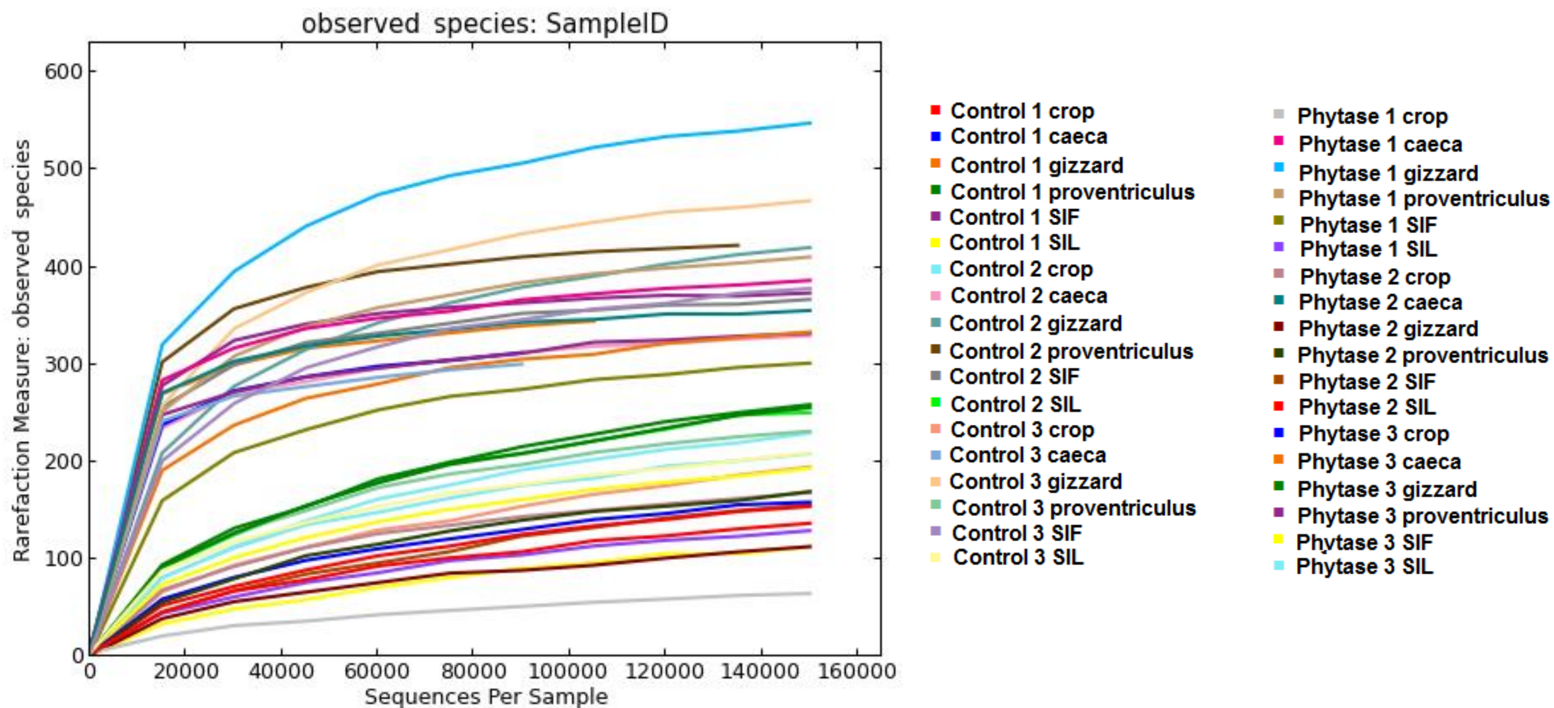


Figure 4.1 – Alpha rarefaction curve at a depth of 150,000 sequences for all samples after V4-V6 16S rRNA gene-fragment sequencing.

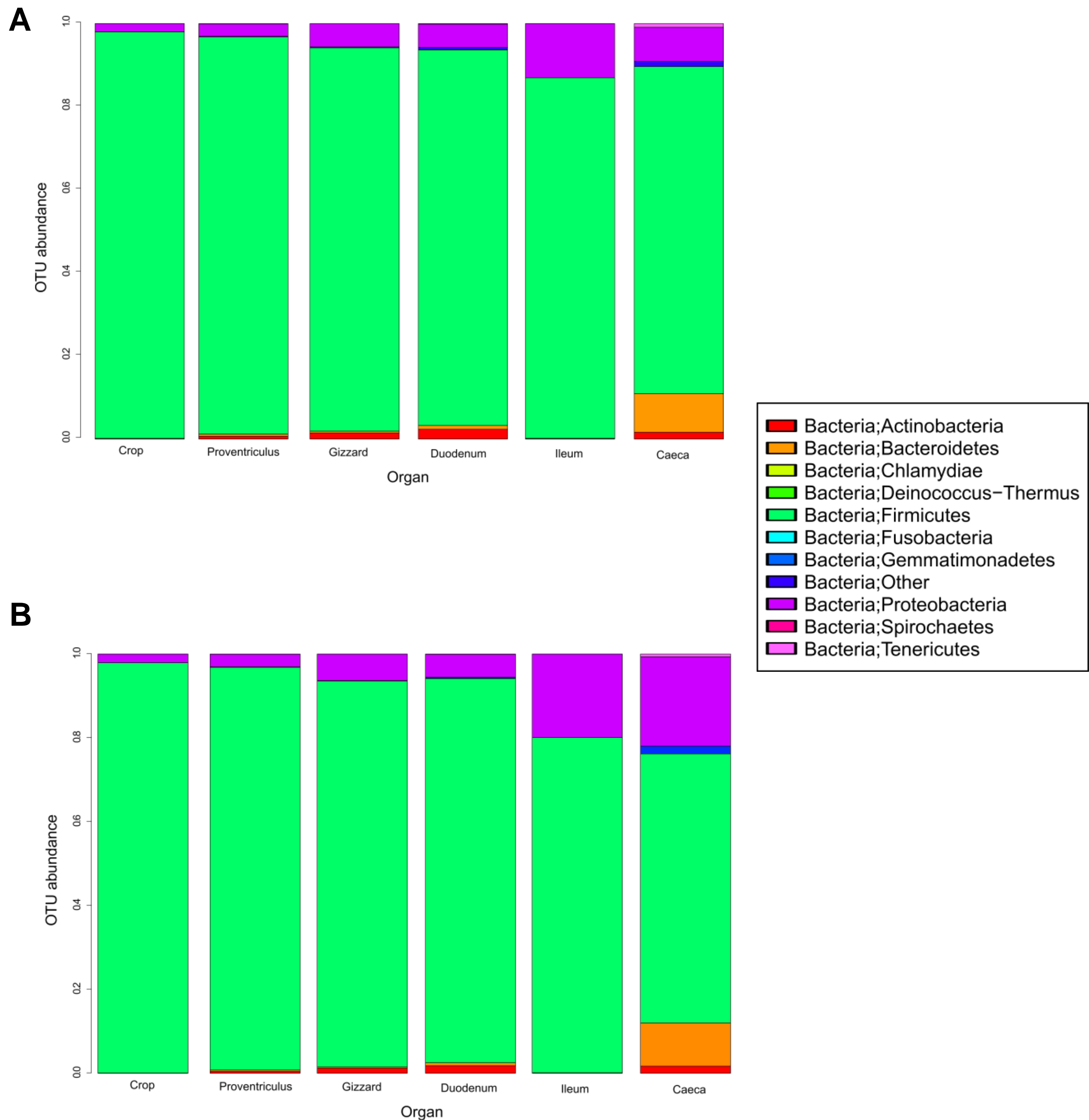


Figure 4.2 – **a)** Taxonomic composition of control-diet organs at phylum level before 16S rRNA gene copy number normalisation.

b) Taxonomic composition of control-diet organs at phylum level after 16S rRNA gene copy number normalisation.

Organ	Number of samples	Total reads	Lowest number of reads within organ	Number of OTUs	Number of core OTUs	Number of core OTUs between diets
Crop	3	200,738	35,774	443	101	36
Crop	3	214,998	47,593	323	44	
Proventriculus	3	126,159	33,655	551	134	73
Proventriculus	3	152,817	35,160	577	112	
Gizzard	3	238,950	62,161	692	255	93
Gizzard	3	250,410	36,476	632	112	
Duodenum	3	380,608	94,151	597	245	99
Duodenum	3	212,794	37,844	359	105	
Ileum	3	234,772	36,711	425	136	60
Ileum	3	127,198	31,478	282	74	
Caecum	3	132,252	40,598	403	238	207
Caecum	3	231,691	42,920	460	289	

Table 4.1 – Summary of control (red) and phytase (blue) diet organs showing number, total reads, lowest sample count per organ, number of OTUs and number of core OTUs identified from V4-V6 16S rRNA gene-fragment sequencing.

4.3.2 Crop

200,378 reads were obtained from three control-diet samples. These were assigned to 443 OTUs, which belonged to eight bacterial phyla and one unassigned bacterial phylum. The majority of reads were assigned to *Lactobacillus* (83.1%) and Bacilli (14%). 101 OTUs were present in all crop control-diet samples. Of the most abundant OTUs, four were assigned to *Lactobacillus* (OTU 1, OTU 0, OTU 595 and OTU 820) with one OTU unassigned past Bacilli (OTU 579). OTU 1 was the most abundant and contributed ~56% of total reads (Table 4.2a).

214,998 reads were obtained from three crop phytase-diet samples. These were assigned to 323 OTUs, which belonged to six bacterial phyla and one unassigned bacterial phylum. *Lactobacillus* formed the most abundant genus with 96.7% of total reads. The next most abundant named genus was *Escherichia/Shigella* with 0.1% of total reads. 44 OTUs were present in all crop phytase-diet samples. Four of the five most abundant OTUs were assigned to *Lactobacillus*. OTU 0 was the most abundant (~83%) and was assigned to a *Lactobacillus*. The second most abundant OTU (OTU 1) was responsible for 9.9% of reads and the remaining OTUs represented much lower percentages (Table 4.2a).

Alpha rarefaction of the crop samples from the two diets was used at a depth of 35,000 reads (Table 4.2b). Figure 4.3a showed the number of observed species begins to level off at 35,000 reads and therefore the majority of OTUs had been discovered in both diets. The Simpson's diversity metric indicated that on average

the microbiota associated with the control-diet (0.56±0.12) was more diverse than the phytase-diet (0.30±0.25) (Figure 4.3b).

There was no significant difference in diversity of the microbiota between the control and phytase-diets in the crop ($p > 0.05$). Furthermore, no significantly abundant OTUs between the diets in crop samples were identified.

Jackknife beta diversity showed the samples did not cluster by diet (Figure 4.3c). Control 1 crop and control 2 crop formed a branch with high bootstrap values (75%-100%); however, these were the only samples from the same diet that clustered together.

A

OTU ID	Taxonomy	% of total OTU reads in control-diet	% of total OTU reads in phytase-diet
0	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	23	83
1	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	56	9.9
579	Bacteria; Firmicutes; Bacilli	14	2.3
595	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	2.8	2.2
820	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	1.6	1.6

B

Diet	Seqs/Sample	Chao1 Ave.	Chao1 Err.	Observed species Ave.	Observed species Err.	Shannon Ave.	Shannon Err.	Simpson Ave.	Simpson Err
Control	35,000	161.63	20.46	102.70	18.75	1.72	0.35	0.56	0.12
Phytase	35,000	107.34	40.88	76.40	28.96	0.97	0.61	0.30	0.25

Table 4.2 – **a)** Top five abundant OTUs after V4-V6 16S rRNA gene-fragment sequencing of crop control-diet samples.

b) Alpha rarefaction results of the crop from control and phytase-diet V4-V6 16S rRNA gene-fragment sequencing.

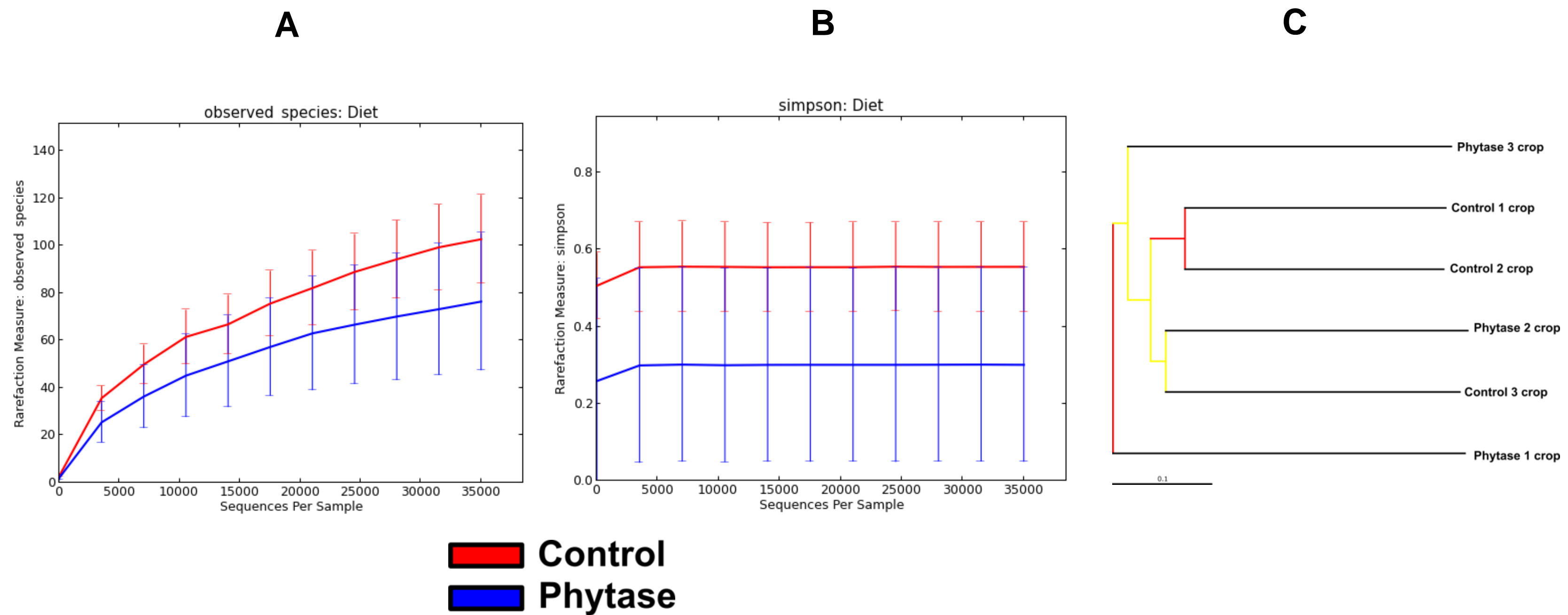


Figure 4.3 – a) Number of observed species against the number of sequences in both diets in the crop from V4-V6 16S rRNA gene-fragment sequencing.

b) Simpson diversity index against number of sequences in both diets in the crop from V4-V6 16S rRNA gene-fragment sequencing.

c) Jackknife beta diversity bootstrap tree of crop samples.

Red=75-100%, yellow=50-75%, green=25-50% and blue =< 25%

*error bars are S.E.M

4.3.3 Proventriculus

126,159 reads were obtained from three proventriculus control-diet samples. These were assigned to 551 OTUs, which belonged to seven named bacterial phyla and one unassigned phylum. *Lactobacillus* was again the most abundant genus with 84.2%; the next highest named genera were *Escherichia/Shigella* (0.8%) and *Alistipes* (0.4%). 134 OTUs were present in all proventriculus control-diet samples. A single *Lactobacillus* OTU (OTU 0) was responsible for ~56% of total reads with the second most abundant OTU (OTU 1) forming ~28%. Two Enterobacteriaceae (OTU 2 and OTU 61) were also in the top five most abundant OTUs although they contributed significantly less to the total number of reads with both <1% (Table 4.3a).

152,817 reads were obtained from three proventriculus phytase-diet samples. These were assigned to 577 OTUs, which belonged to eight bacterial phyla and one unassigned bacterial phylum. There were only two named genera with >1% total abundance; *Lactobacillus* (79%) and *Pseudomonas* (1.5%). 112 OTUs were present in all proventriculus phytase-diet samples. The two most abundant OTUs (OTU 0 and OTU 1) belonged to the *Lactobacillus* genus and accounted for 78% of total reads between them. A *Pseudomonas* (OTU 8) and Enterobacteriaceae (OTU 61) were equally abundant at 1.5% total reads (Table 4.3a).

Alpha rarefaction was completed at a depth of 34,000 reads (Table 4.3b). Figure 4.4a showed the number of observed species begun to level off at 34,000 reads and therefore the majority of OTUs had been discovered in both diets. The

Simpson's diversity index showed the microbiota associated with the phytase-diet had more diversity on average (Figure 4.4b). Both of the figures showed large error bars, indicating high variance in the samples within their respective diets.

There were no significantly abundant OTUs or difference in diversity between the diets within the proventriculus ($p > 0.05$).

Jackknife beta diversity showed there was no distinct clustering of samples by diet, with control 1 and control 3 the only samples from the same diet to cluster together (Figure 4.4c). There was high bootstrap support for each placement within the tree (75-100%).

A

OTU ID	Taxonomy	% of total OTU reads in control-diet	% of total OTU reads in phytase-diet
0	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	56	50
1	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	28	28
2	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia/Shigella	0.83	N/A
8	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	N/A	1.5
61	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	0.49	1.5
579	Bacteria; Firmicutes; Bacilli	5.6	5.0

B

Diet	Seqs/Sample	Chao1 Ave.	Chao1 Err.	Observed species Ave.	Observed species Err.	Shannon Ave.	Shannon Err.	Simpson Ave.	Simpson Err
Control	34,000	263.25	100.10	208.87	110.55	1.98	1.39	0.44	0.27
Phytase	34,000	299.11	105.08	246.17	113.62	2.38	1.54	0.50	0.33

Table 4.3 – **a)** Top five abundant OTUs from V4-V6 16S rRNA gene-fragment sequencing of the proventriculus control and phytase-diet samples.

b) Alpha rarefaction results of the proventriculus from control and phytase-diet V4-V6 16S rRNA gene-fragment sequencing.

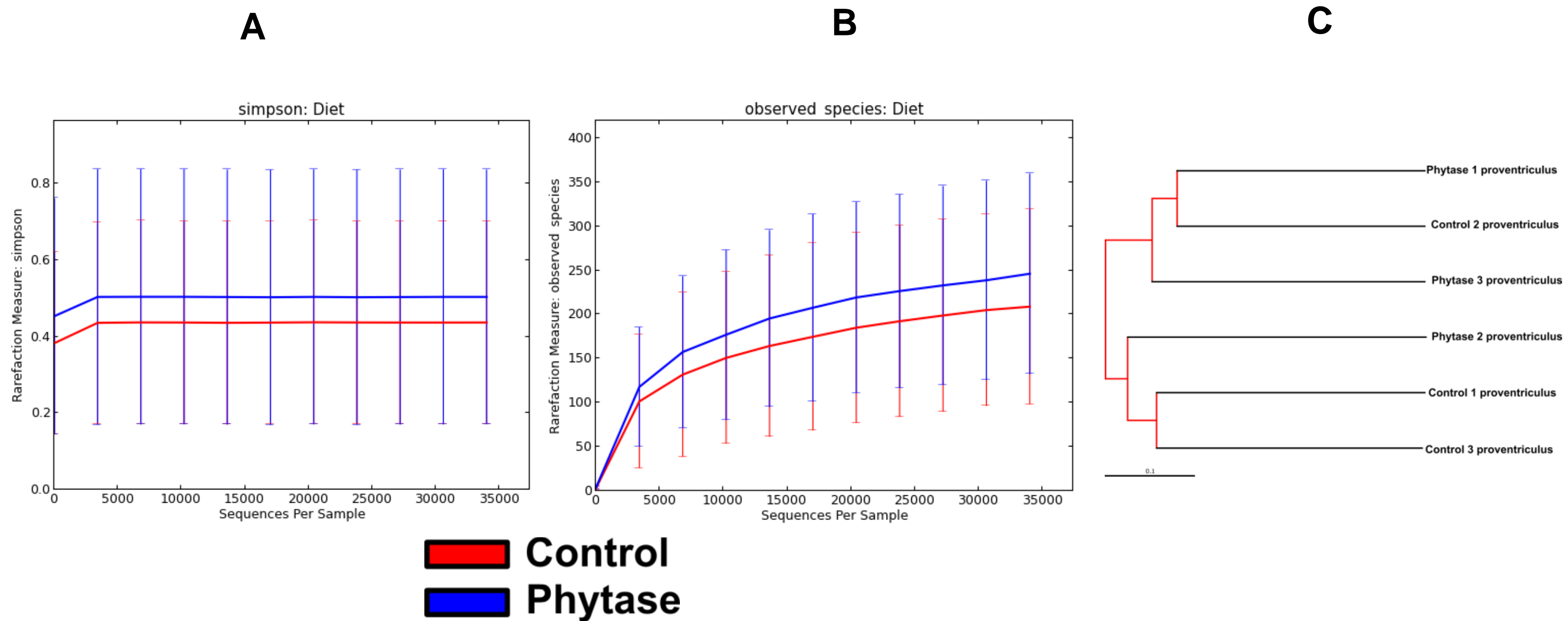


Figure 4.4 – a) Number of observed species against the number of sequences in both diets in the proventriculus from V4-V6 16S rRNA gene-fragment sequencing.

b) Simpson diversity index against number of sequences in both diets in the proventriculus from V4-V6 16S rRNA gene-fragment sequencing.

c) Jackknife beta diversity bootstrap tree of proventriculus samples.

Red=75-100%, yellow=50-75%, green=25-50% and blue =< 25%

*error bars are S.E.M

4.3.4 Gizzard

238,950 reads were obtained from three gizzard control-diet samples. These were assigned to 692 OTUs, which belonged to nine named bacterial phyla and one unassigned phylum. Similar to the crop and proventriculus, *Lactobacillus* (76.7%) and *Escherichia/Shigella* (1.7%) were the most abundant genera. Other abundant OTUs were unable to be assigned to a genus. 255 OTUs were present in all gizzard control-diet samples. Two of the most abundant OTUs (OTU 0 and OTU 1) were *Lactobacillus* with >70% of total OTU reads combined. The other most abundant OTUs belonged to *Bacilli* (OTU 579), *Clostridiales* (OTU 33) and *Escherichia/Shigella* (OTU 2) (Table 4.4a).

250,410 reads were obtained from three gizzard phytase-diet samples. The reads were assigned to 632 OTUs, which belonged to eight named bacterial phyla and one unassigned phylum. There were only two named genera in the gizzard >1% of total reads, *Lactobacillus* (91.9%) and *Pseudomonas* (1.4%). 112 OTUs were present in all gizzard phytase-diet samples. The most abundant OTUs were dominated by *Lactobacillus*, of which the top two (OTU 0 and OTU 1) accounted for 89.5% of total reads. The next three OTUs shared a similar level of abundance from 1.1-1.5% of total OTU reads (Table 4.4a).

Alpha rarefaction of gizzard samples was completed at a depth of 40,000 reads (Table 4.5b). Figure 4.5a showed the number of observed species levelled off at 40,000 reads and therefore the majority of OTUs had been discovered in both diets. The control samples contained a higher number of observed species than

the phytase samples on average. The Simpson's diversity index showed the microbiota associated with the control-diet had a higher level of diversity than the phytase-diet (Figure 4.5b).

There were no significantly abundant OTUs or significant difference in diversity of the microbiota between the diets within the gizzard. Jackknife beta diversity analysis showed the samples did not cluster by diet (Figure 4.5c).

A

OTU ID	Taxonomy	% of total OTU reads in control-diet	% of total OTU reads in phytase-diet
0	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	42	82
1	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	31	7.2
2	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia/Shigella	1.7	N/A
8	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	N/A	1.4
33	Bacteria; Firmicutes; Clostridia; Clostridiales	2.1	N/A
579	Bacteria; Firmicutes; Bacilli	6.3	1.5
595	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	N/A	1.1

B

Diet	Seqs/Sample	Chao1 Ave.	Chao1 Err.	Observed species Ave.	Observed species Err.	Shannon Ave.	Shannon Err.	Simpson Ave.	Simpson Err
Control	40,000	383.30	64.96	317.60	55.28	2.75	0.56	0.62	0.08
Phytase	40,000	281.21	177.76	213.30	159.90	1.67	1.41	0.37	0.31

Table 4.4 – **a)** Top five abundant OTUs from V4-V6 16S rRNA gene-fragment sequencing of gizzard control and phytase-diet samples.

b) Alpha rarefaction results of the gizzard from control and phytase-diet V4-V6 16S rRNA gene-fragment sequencing.

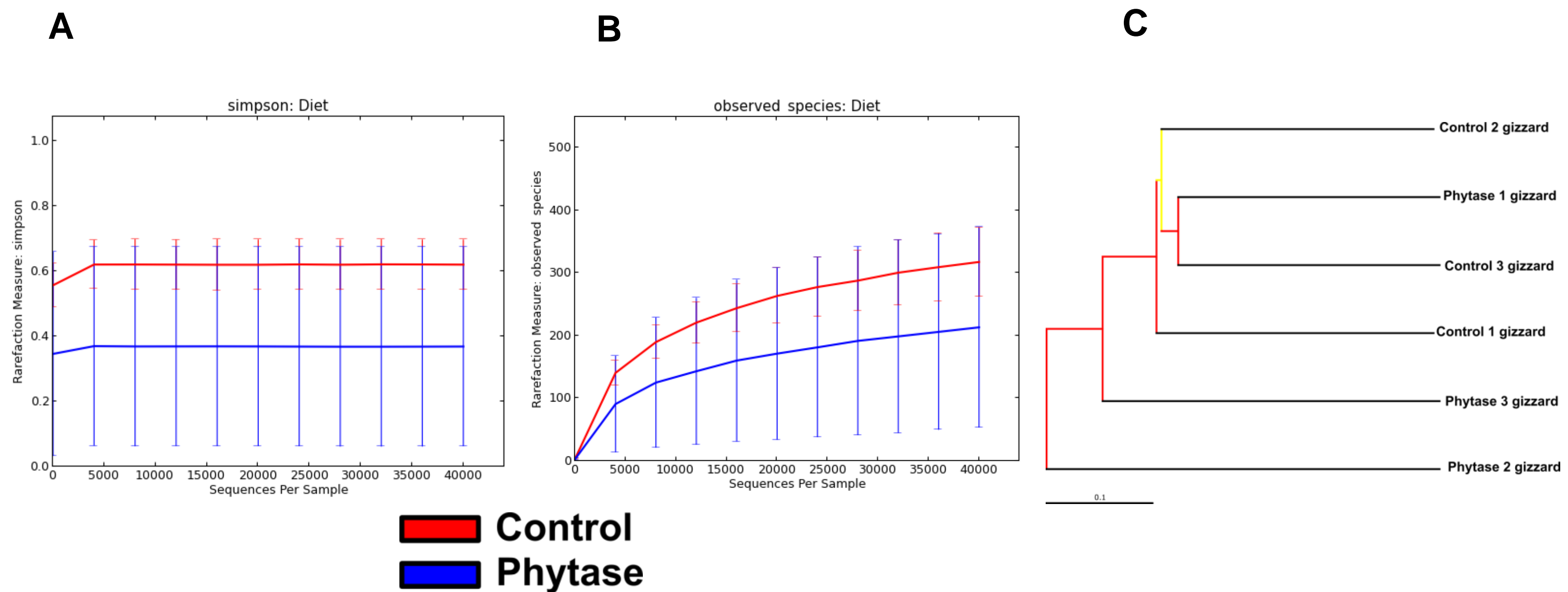


Figure 4.5 – a) Number of observed species against the number of sequences in both diets in the gizzard from V4-V6 16S rRNA gene-fragment sequencing.

b) Simpson diversity index against number of sequences in both diets in the gizzard from V4-V6 16S rRNA gene-fragment sequencing.

c) Jackknife beta diversity bootstrap tree of gizzard samples.

Red=75-100%, yellow=50-75%, green=25-50%, and blue =< 25%

*error bars are S.E.M

4.3.5 Duodenum

380,608 reads were obtained from three duodenum control-diet samples. These were represented by 597 OTUs, which belonged to eight named bacterial phyla and one unassigned phylum. The majority of reads were assigned to Firmicutes (90.4%) with Proteobacteria (5.6%) and Actinobacteria (2.4%) the only other phyla to account for >1% of total reads. Again, the most abundant genus was *Lactobacillus,* which was responsible for all of the Bacilli reads (55.8%). The only assigned genus in Lachnospiraceae was *Dorea* (1.6%) with all other reads unable to be assigned. *Herbaspirillum* (2.8%), *Bifidobacterium* (1.6%) and *Escherichia/Shigella* (1%) were the other genera responsible for ≥1% of total reads. 245 OTUs were present in all duodenum control-diet samples. The first and second most abundant OTUs (OTU 0 and OTU 1) in the duodenum were assigned to *Lactobacilli,* which were responsible for 30% and 24% of total reads respectively. A Clostridiales (OTU 3) and Bacilli (OTU 579) OTU were the next most abundant, with a *Herbaspirillum* OTU (OTU 7) being the fifth most abundant (Table 4.5a).

212,794 reads were obtained from three duodenum phytase-diet samples. These were assigned to 359 OTUs, which belonged to seven named bacterial phyla and one unassigned phylum. Firmicutes (99.6%) was the only phylum that had reads >1%. The only assigned genus with >1% of reads was *Lactobacillus* (97.9%). The next most abundant named genera were *Alistipes, Escherichia/Shigella* and *Dorea,* which were all present at 0.1%. 105 OTUs were present in all duodenum samples; the majority of these were Clostridia (n=59). The most abundant OTU (OTU 0) was

responsible for ~95% of all reads within the duodenum. The third, fourth and fifth most abundant OTUs (OTU 820, OTU 579 and OTU 595) contributed <1% to the total reads (Table 4.5a).

Alpha rarefaction of the duodenum samples was completed at a depth of 37,000 (Table 4.5b). Figure 4.6a showed the number of observed species levelled off at 37,000 and therefore the majority of OTUs had been discovered in both diets. The control-diet contained more observed species than the phytase-diet; the difference was also evident when observing the Simpson's diversity index, with the plot indicating the microbiota associated with the control-diet was more diverse than the phytase-diet (Figure 4.6b).

Interestingly, there was one significantly abundant OTU (OTU 470, assigned to Polyangiaceae) in the microbiota associated with the control-diet. There was no significant difference in diversity of the microbiota between the control and phytase-diets with a p-value of >0.05.

Jackknife beta diversity showed that two of the phytase samples (phytase 2 duodenum and phytase 3 duodenum) clustered with each other with high bootstrap support in contrast to the other phytase organ samples. Control 1 duodenum and control 2 duodenum clustered with 50-75% bootstrap values and were more similar to control 3 duodenum and phytase 1 duodenum in diversity than the other samples (Figure 4.6c).

A

OTU ID	Taxonomy	% of total OTU reads in control-diet	% of total OTU reads in phytase-diet
0	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	30	95
1	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	24	2.2
3	Bacteria; Firmicutes; Clostridia; Clostridiales	5.8	N/A
7	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Herbaspirillum	2.8	N/A
579	Bacteria; Firmicutes; Bacilli	4.4	0.42
595	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	N/A	0.32
820	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	N/A	0.53

B

Diet	Seqs/Sample	Chao1 Ave.	Chao1 Err.	Observed species Ave.	Observed species Err.	Shannon Ave.	Shannon Err.	Simpson Ave.	Simpson Err
Control	37,000	342.14	22.27	299.87	16.10	3.53	1.14	0.70	0.15
Phytase	37,000	156.48	82.22	116.17	77.82	0.62	0.55	0.14	0.14

Table 4.5 – **a)** Top five abundant OTUs from V4-V6 16S rRNA gene-fragment sequencing of duodenum control and phytase-diet samples.

b) Alpha rarefaction results of the duodenum from control and phytase-diet V4-V6 16S rRNA gene-fragment sequencing.

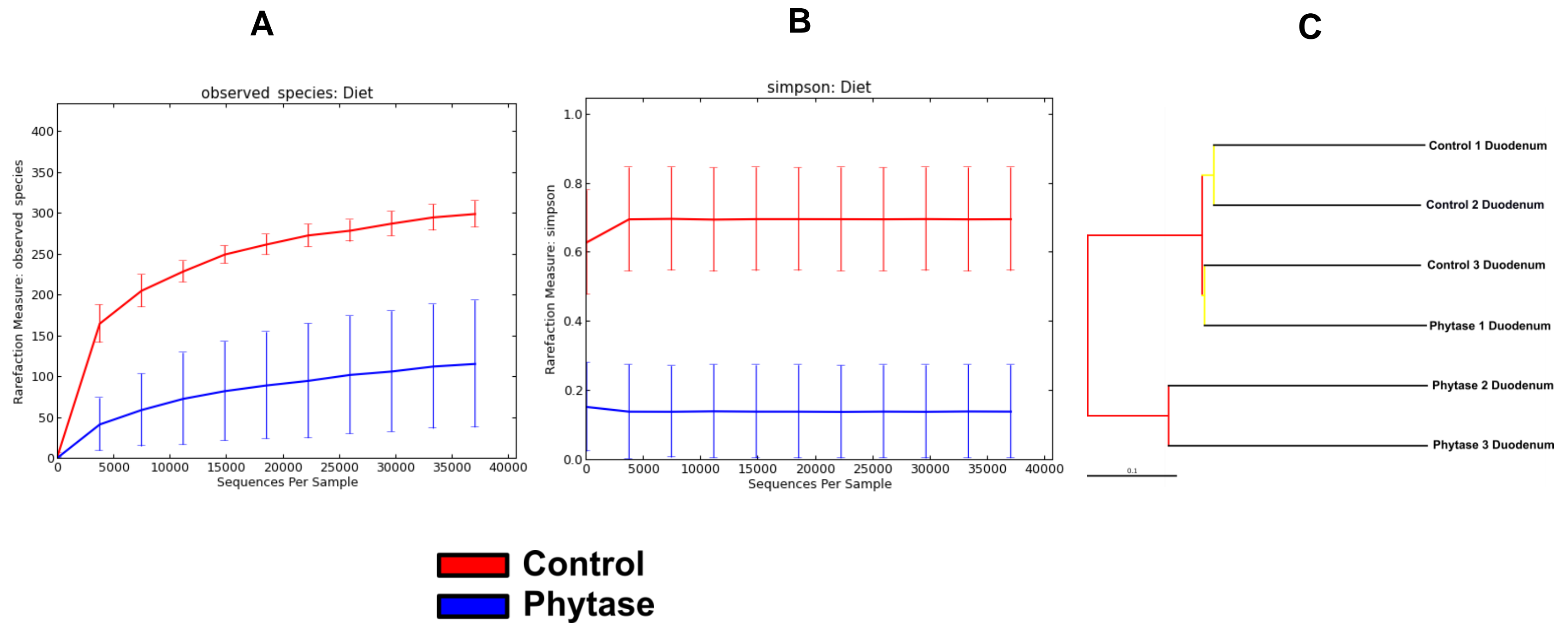


Figure 4.6 – a) Number of observed species against the number of sequences in both diets in the duodenum from V4-V6 16S rRNA gene-fragment sequencing.

b) Simpson diversity index against number of sequences in both diets in the duodenum from V4-V6 16S rRNA gene-fragment sequencing.

c) Jackknife beta diversity bootstrap tree of duodenum samples.

Red=75-100%, yellow=50-75%, green=25-50% and blue =< 25%

*error bars are S.E.M

4.3.6 Ileum

234,772 reads were obtained from three ileum control-diet samples. These were assigned to 425 OTUs, which belonged to eight bacterial phyla and one unassigned phylum. A large proportion of the total reads (57.1%) could not be assigned below Clostridiales. At genus level, *Lactobacillus* was the most abundant with 26.3%; *Escherichia/Shigella* accounted for 10.1% with 2.7% of Enterobacteriaceae reads unable to be assigned below family level. 136 OTUs were present in all ileum control-diet samples; the majority of these were Clostridiales and specifically Ruminococcaceae. The most abundant OTU belonged to the Clostridiales (OTU 3); accounting for 53% of total reads. There were two *Lactobacillus* OTUs (OTU 0 and OTU 1), *Escherichia/Shigella* (OTU 2) and another Clostridiales (OTU 883) in the top five most abundant OTUs (Table 4.6a).

127,198 reads were obtained from three ileum phytase-diet samples. These were assigned to 282 OTUs, which belonged to six named bacterial phyla and one unassigned phylum. A large proportion of Clostridiales reads (13.7%) could not be assigned a family; with Ruminococcaceae (0.2%) and Lachnospiraceae (0.1%) the only families with ≥0.1% of total reads. There were only two assigned genera that accounted for >0.1% of reads; *Lactobacillus* (72.8%) and *Escherichia/Shigella* (7%). 74 OTUs were present in all ileum phytase-diet samples. The most abundant OTU (OTU 0) was a *Lactobacillus,* which formed 55.8% of total reads within the ileum. The second and third most abundant OTUs (OTU 0 and OTU 3) were similar

in total reads and belonged to *Lactobacillus* (12. 9%) and Clostridiales (12.3%) (Table 4.6a).

Alpha rarefaction was completed on the ileum samples at a depth of 31,000 reads (Table 4.6b). This showed the control-diet samples contained more observed species than the phytase-diet samples; this was illustrated in Figure 4.7a. However, the Simpson's diversity index showed the microbiota associated with both diets had a similar level of diversity (Figure 4.7b).

There were no significantly abundant OTUs or any difference in diversity in the microbiota between diets within ileum samples. The jackknife beta diversity tree had two phytase samples (phytase 1 ileum and phytase 2 ileum) clustered together with high bootstrap support values. Control 1 ileum and control 3 ileum also clustered together with a bootstrap support value of 50-75% (Figure 4.7c).

A

OTU ID	Taxonomy	% of total OTU reads in control-diet	% of total OTU reads in phytase-diet
0	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	17	55.8
1	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	8.3	12.9
2	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia/Shigella	10	7.0
3	Bacteria; Firmicutes; Clostridia; Clostridiales	53	12.3
579	Bacteria; Firmicutes; Bacilli	N/A	2.9
883	Bacteria; Firmicutes; Clostridia; Clostridiales	3.5	N/A

B

Diet	Seqs/Sample	Chao1 Ave.	Chao1 Err.	Observed species Ave.	Observed species Err.	Shannon Ave.	Shannon Err.	Simpson Ave.	Simpson Err
Control	31,000	190.51	31.96	127.27	18.96	2.03	0.21	0.61	0.08
Phytase	31,000	113.69	36.04	82.30	26.82	1.90	0.45	0.57	0.16

Table 4.6 – **a)** Top five abundant OTUs from V4-V6 16S rRNA gene-fragment sequencing of the ileum control and phytase-diet samples.

b) Alpha rarefaction results of the ileum from control and phytase-diet after V4-V6 16S rRNA gene-fragment sequencing.

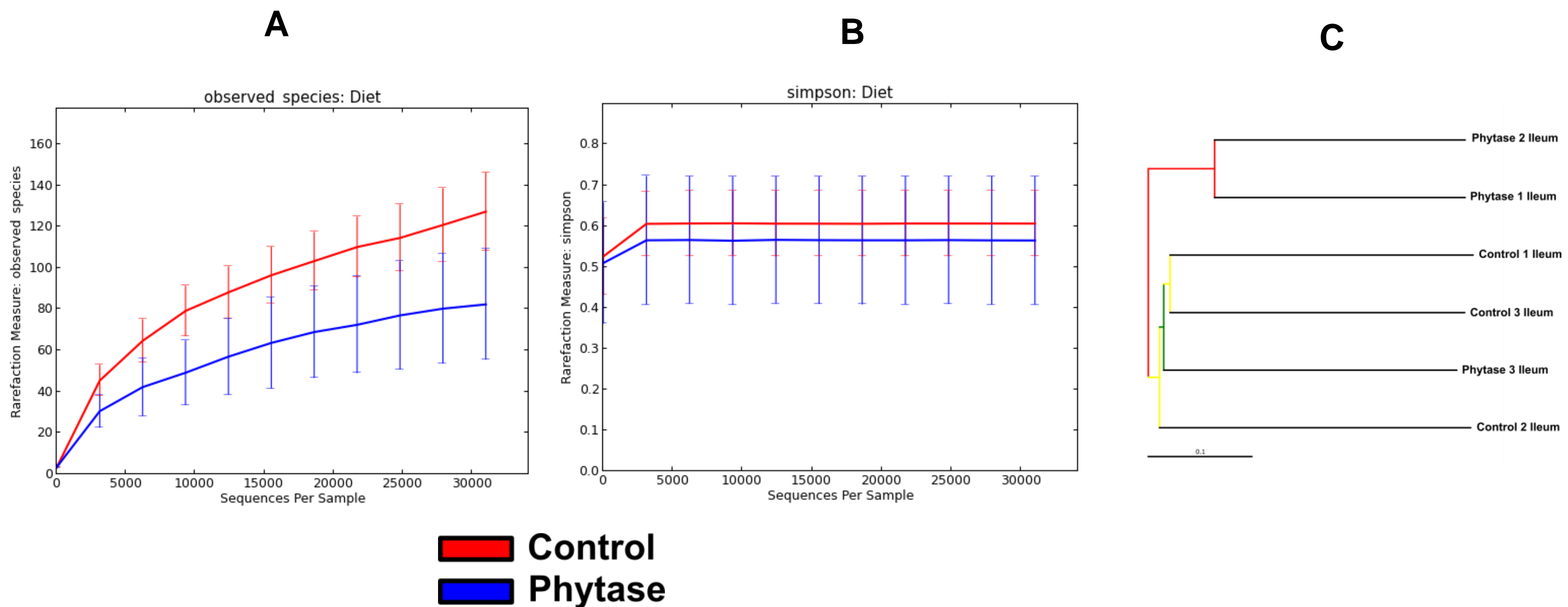


Figure 4.7 – a) Number of observed species against the number of sequences in both diets in the ileum from V4-V6 16S rRNA gene-fragment sequencing.

b) Simpson diversity index against number of sequences in both diets in the ileum from V4-V6 16S rRNA gene-fragment sequencing.

c) Jackknife beta diversity bootstrap tree of ileum samples.

Red=75-100%, yellow=50-75%, green=25-50%, and blue =< 25%

*error bars are S.E.M

4.3.7 Caecum

132,252 reads were obtained from three caecum control-diet samples. These were assigned to 403 OTUs, which belonged to five bacterial phyla and one unassigned phylum. The majority of OTU reads were assigned to Firmicutes (78.8%), followed by Bacteroidetes (9.3%) and Proteobacteria (8.2%). Actinobacteria accounted for 1.6%; Tenericutes 0.9% and 1.3% of reads could not be assigned a phylum. Firmicutes consisted of 73.9% Clostridia with 1.1% assigned to Bacilli (Figure 4.8a).

Clostridia reads were mainly assigned to Clostridiales; however 1.3% could not be assigned beyond class level. Bacilli reads were due to Lactobacillales (1%) and Bacteroidia reads were only assigned to Bacteroidales (9.3%). The next most abundant orders were Enterobacteriales (8.2%) and Bifidobacteriales (1.5%). Anaeroplasmatales were the sole constituent of the Mollicutes (0.9%).

Clostridiales contained three main families, Lachnospiraceae (28.5%), Ruminococcaceae accounted for 17.4% and 6.9% were Incertae Sedis XIV (Clostridiales family XIV of uncertain placement). Rikenellaceae was the most abundant family outside the Clostridiales order with 9.3% of total reads, followed by Enterobacteriaceae (8.2%), Bifidobacteriaceae (1.5%), Lactobacillaceae (1%) and Aneroplasmataceae (0.9%). The Lachnospiraceae family primarily comprised of unassigned reads (22.7%) and the *Dorea* genus (5.8%). *Alistipes* formed the most abundant named genus with 9.3% of reads followed by *Escherichia/Shigella* (8%),

Blautia (6.9%), *Oscillibacte*r (3.8), *Bifidobacterium* (1.5%) and *Anaeroplasma* (0.9%).

238 OTUs were present in all caecum control-diet samples. Of these core OTUs, 180 were assigned to Clostridiales of which 67 were Ruminococcaceae and 33 Lachnospiraceae. The most abundant OTU was an *Alistipes* with 9.3% of total reads (OTU 5). There were seven Clostridiales OTUs in the top ten most abundant OTUs, of which four were assigned to Lachnospiraceae (OTU 22, OTU 19, OTU 13 and OTU 54) (Table 4.7a).

231,691 reads were obtained from three caecum phytase-diet samples. These were assigned to 460 OTUs, which belonged to five named bacterial phyla and one unassigned phylum. The most abundant phylum was Firmicutes (83.6%), followed by Bacteroidetes (3.1%), Proteobacteria (1.5%), Tenericutes (1.4%) and Actinobacteria (1.1%). The unassigned bacterial phylum was responsible for 2.2% of caecum reads. At class level, Clostridia had the highest number of reads (76.7%) with Bacilli (2.5%) and an unassigned class forming the rest of Firmicutes reads (Figure 4.8b).

Clostridia consisted of Clostridiales (75%) and 1.7% of unassigned reads. Lactobacillales (2.2%) was the main constituent of Bacilli reads, with Bacillales (0.2%) and an unassigned order (0.1%) forming the remaining total reads. Bacteroidales (3.1%) and Anaeroplasmatales (1.4%) were the only orders within Bacteroidia and Mollicutes respectively with Enterobacteriales responsible for 1.5% of total reads.

Clostridiales reads were formed from three main families and a smaller one; Lachnospiraceae (20.1%), Ruminococcaceae (17.7%), unassigned families (35.9%) and Incertae Sedis XIV (Clostridiales family XIV of uncertain placement, 1.2%). The next most abundant families were Rikenellaceae (3.1%), Lactobacillaceae (2.1%), Enterobacteriaceae (1.5%), Anaeroplasmataceae (1.4%), Coriobacteriaceae (0.6%) and Bifidobacteriaceae (0.5%). At genus level, the most abundant named genera were *Dorea* (6.4%), *Alistipes* (3.1%), *Lactobacillus* (2.1%), *Anaeroplasma* (1.4%), *Blautia* (1.2%), *Oscillibacter* (1.1%), *Escherichia/Shigella* (1.1%) and *Bifidobacterium* (0.5%).

289 OTUs were present in all caecum phytase-diet samples, the majority of these were Firmicutes with 74 Ruminococcaceae and 41 Lachnospiraceae assigned OTUs. Of the top ten most abundant OTUs, seven were assigned to Clostridiales, with one OTU (OTU 17) unable to be assigned to a phylum (Table 4.7a).

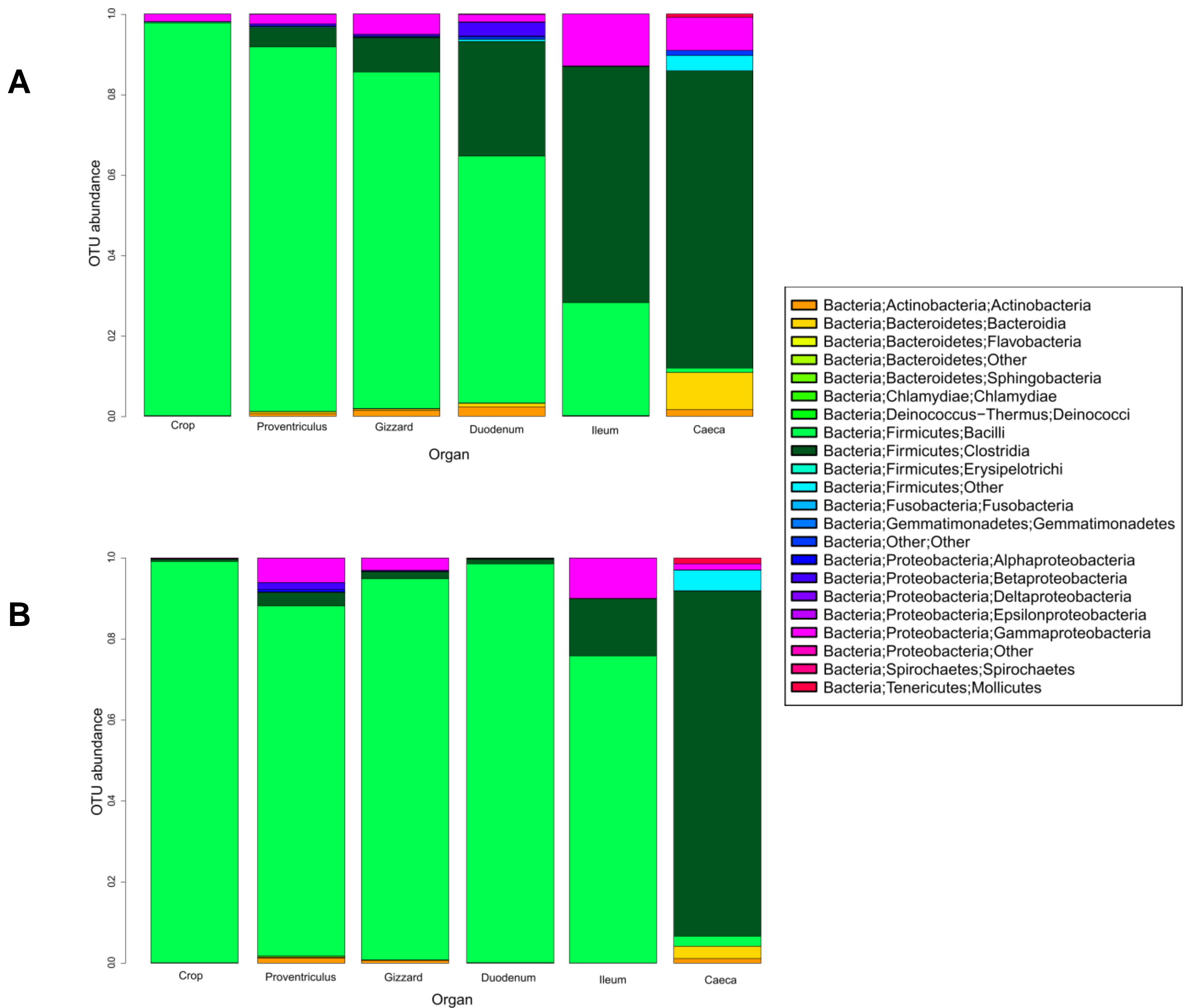


Figure 4.8 – **a)** Summary of taxonomic composition at class level for control-diet organs after V4-V6 16S rRNA gene-fragment sequencing.

b) Summary of taxonomic composition at class level of phytase-diet organs after V4-V6 16S rRNA gene-fragment sequencing.

A

OTU ID	Taxonomy	% of total OTU reads in control-diet	% of total OTU reads in phytase-diet
2	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia/Shigella	8.0	N/A
5	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; Alistipes	9.3	3.2
6	Bacteria; Firmicutes	N/A	3.4
9	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium	1.5	N/A
10	Bacteria; Firmicutes; Clostridia; Clostridiales	N/A	10
11	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	N/A	2.2
13	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	4.3	3.7
16	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillibacter	3.6	N/A
17	Bacteria	N/A	2.2
19	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea	5.3	6.5
22	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	6.2	N/A
28	Bacteria; Firmicutes; Clostridia; Clostridiales; Incertae Sedis XIV; Blautia	6.7	N/A
30	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	N/A	3.7
33	Bacteria; Firmicutes; Clostridia; Clostridiales	1.4	N/A

OTU ID	Taxonomy	% of total OTU reads in control-diet	% of total OTU reads in phytase-diet
54	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	4.1	N/A
139	Bacteria; Firmicutes; Clostridia; Clostridiales	N/A	2.7
908	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	N/A	7.1

B

Diet	Seqs/Sample	Chao1 Ave.	Chao1 Err.	Observed species Ave.	Observed species Err.	Shannon Ave.	Shannon Err.	Simpson Ave.	Simpson Err
Control	40,000	277.32	8.51	258.03	6.63	5.59	0.19	0.95	0.01
Phytase	40,000	333.88	14.42	299.57	10.59	5.81	0.21	0.96	0.01

Table 4.7 – **a)** Top ten abundant OTUs from V4-V6 16S rRNA gene-fragment sequencing of caecum control and phytase-diet samples.

b) Alpha rarefaction results of the caecum from control and phytase-diet V4-V6 16S rRNA gene-fragment sequencing.

Alpha rarefaction was completed at a depth of 40,000 (Table 4.7b). This showed that on average the phytase-diet contained more observed species (Figure 4.9a), had a higher richness and higher Shannon and Simpson's diversity index values (Figure 4.9b). The caecal samples from both diets had the highest Simpson's diversity index values in their respective guts.

There were no significantly abundant OTUs observed in the microbiota associated from either diet, however there was a significant difference in diversity of the microbiota with a bonferroni corrected p-value of 0.0004.

Jackknife beta diversity showed that the samples clustered by diet with high bootstrap support values (Figure 4.9c). Within the diets, phytase 2 caecum and phytase 3 caecum shared a closer diversity than phytase 1 caecum and control 2 caecum and control 3 caecum were closer than control 1 caecum.

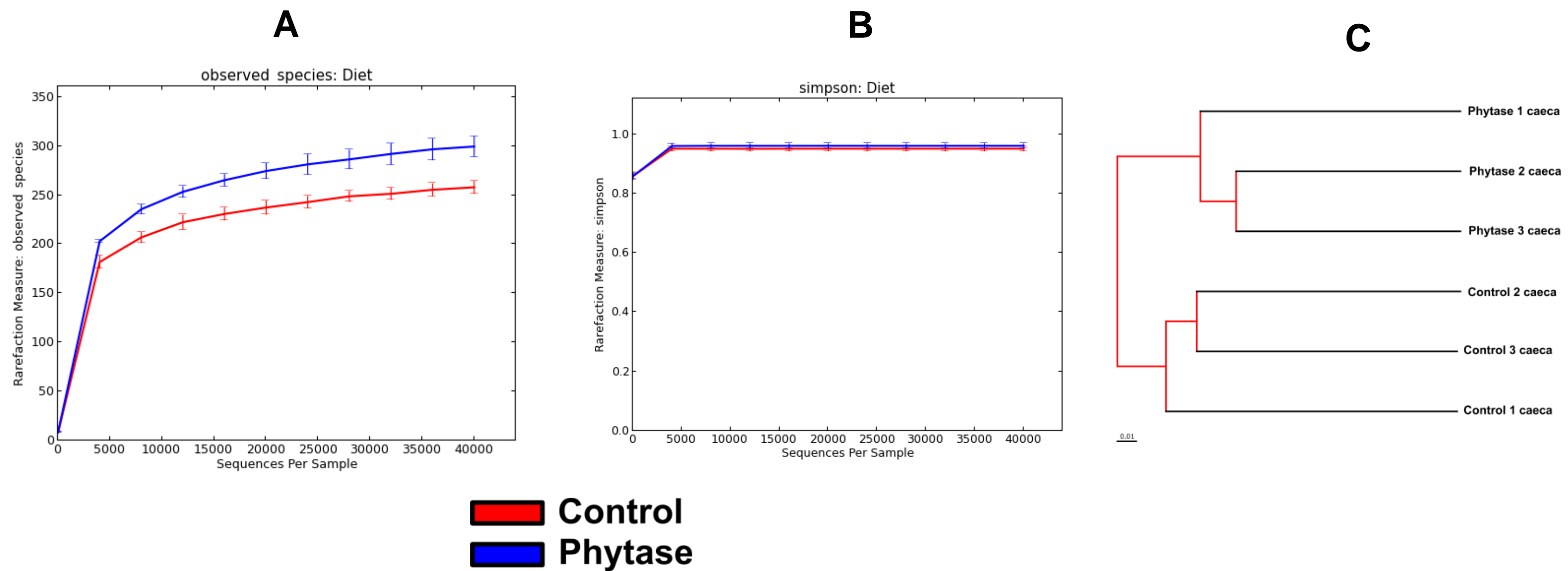


Figure 4.9 – a) Number of observed species against the number of sequences in both diets in the caecum after V4-V6 16S rRNA gene-fragment sequencing.

b) Simpson diversity index against number of sequences in both diets in the caecum after V4-V6 16S rRNA gene-fragment sequencing.

c) Jackknife beta diversity bootstrap tree of caecum samples.

Red=75-100%, yellow=50-75%, green=25-50%, and blue =< 25%

*error bars are S.E.M

4.3.8 Alpha rarefaction and beta diversity of the control-diet gut

Alpha rarefaction of the control-diet gut was completed to a depth of 32,000 reads (Table 4.8). The gizzard had the highest average number of observed species (297±53) followed by the duodenum (291±16) (Figure 4.10a). The caecum had the least amount of variance in the number of observed species (252±5). The Chao1 richness index indicated the gizzard was the richest organ with a value of 364±61.

The Simpson's diversity plot showed the caecum (red) was the most diverse organ in the control-diet gut with a value of 0.95±0.01 (Figure 4.10b). This was mirrored by the Shannon index, which gave the caecum samples a value of 5.58±0.2; the next most diverse organ was the duodenum (3.46±1.2).

Statistical analyses showed there were no significant OTUs or significance in diversity between crop and proventriculus, proventriculus and gizzard or gizzard and duodenum ($p > 0.05$). However, OTU 97 (Clostridiales) was determined to be significant between the crop and gizzard with a p-value of 0.034 and there was also a significant difference in diversity with a p-value of 0.00054.

Between the duodenum and ileum no significant OTUs were identified, however there was a significant difference in diversity with a p-value of 0.000031. There was a significant difference in diversity between the ileum and the caecum with a bonferroni p-value of 0.011 however there were no significantly abundant OTUs. Furthermore, there were no significant differences between any of the chickens within each organ.

Jackknifed beta diversity was completed on the control-diet gut samples at 32,000 reads (Figure 4.11). This showed the grouping of the caecum samples together with a bootstrap value of 75-100% however there was limited other grouping of samples originating from the same organ or the same chicken.

The control-diet organs were compared to a depth of 127,000 reads (Figure 4.11). The tree indicated strong bootstrap support for organ placement with all of the branches except from the caecum coloured red (75-100% support).

Organ	Chao1 Ave.	Chao1 Err.	Observed species Ave.	Observed species Err.	Shannon Ave.	Shannon Err.	Simpson Ave.	Simpson Err.
Crop	160.29	25.98	97.87	18.75	1.68	0.38	0.55	0.12
Proventriculus	261.03	96.48	204.73	108.24	1.89	1.37	0.42	0.26
Gizzard	364.01	61.40	297.47	53.07	2.64	0.48	0.60	0.06
Small Intestine First	334.03	26.51	291.67	15.96	3.46	1.18	0.68	0.16
Small Intestine Last	181.32	30.47	126.03	18.50	2.02	0.21	0.61	0.08
Caecum	273.70	6.41	252.03	5.77	5.58	0.19	0.95	0.01

Table 4.8 – Alpha rarefaction statistics of control-diet organs at a depth of 32,000 reads.

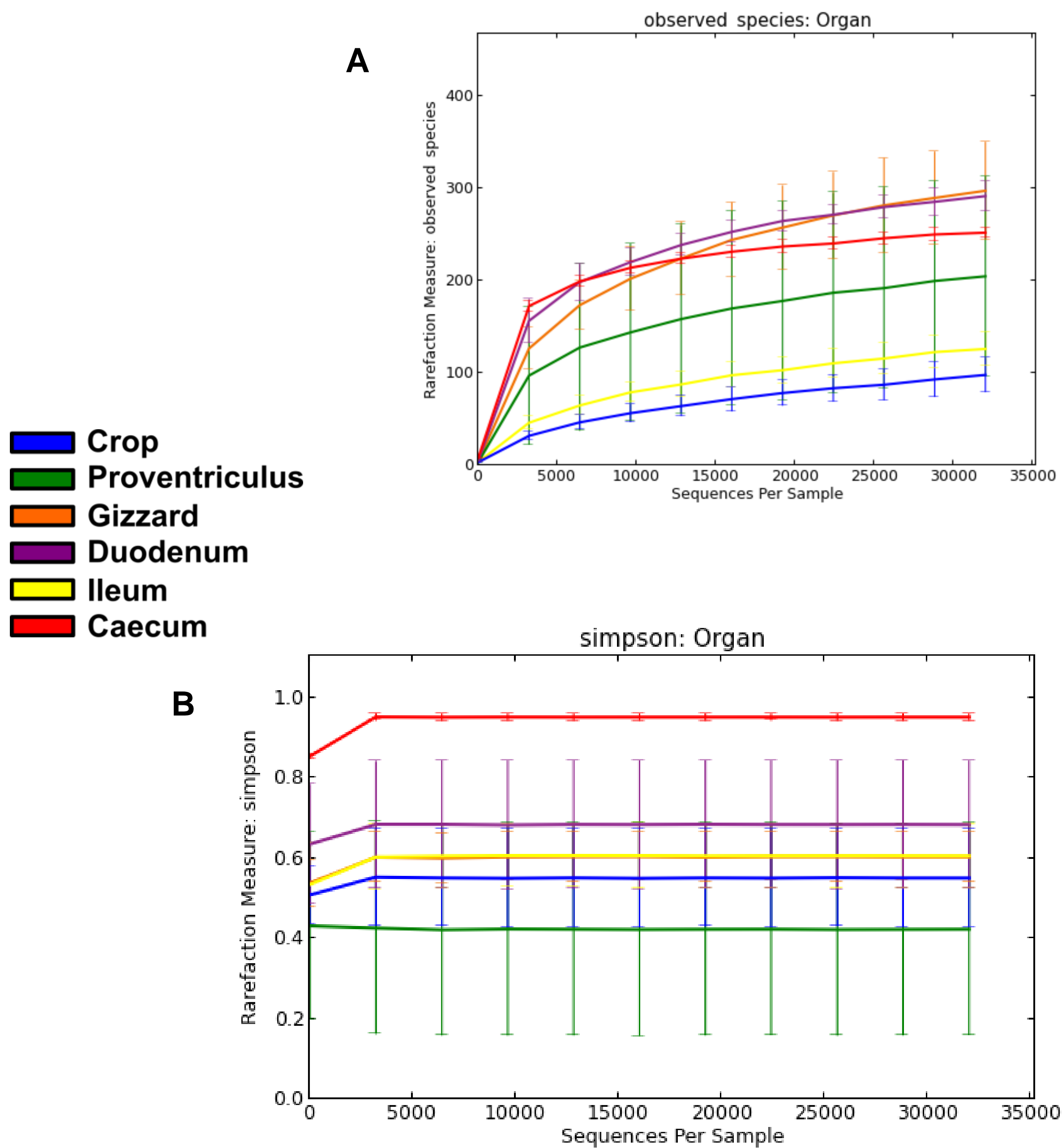


Figure 4.10 – a) Number of observed species against the number of sequences from control-diet organs from V4-V6 16S rRNA gene-fragment sequencing.

b) Simpson diversity index against number of sequences in the control-diet organs from V4-V6 16S rRNA gene-fragment sequencing.

*error bars are S.E.M

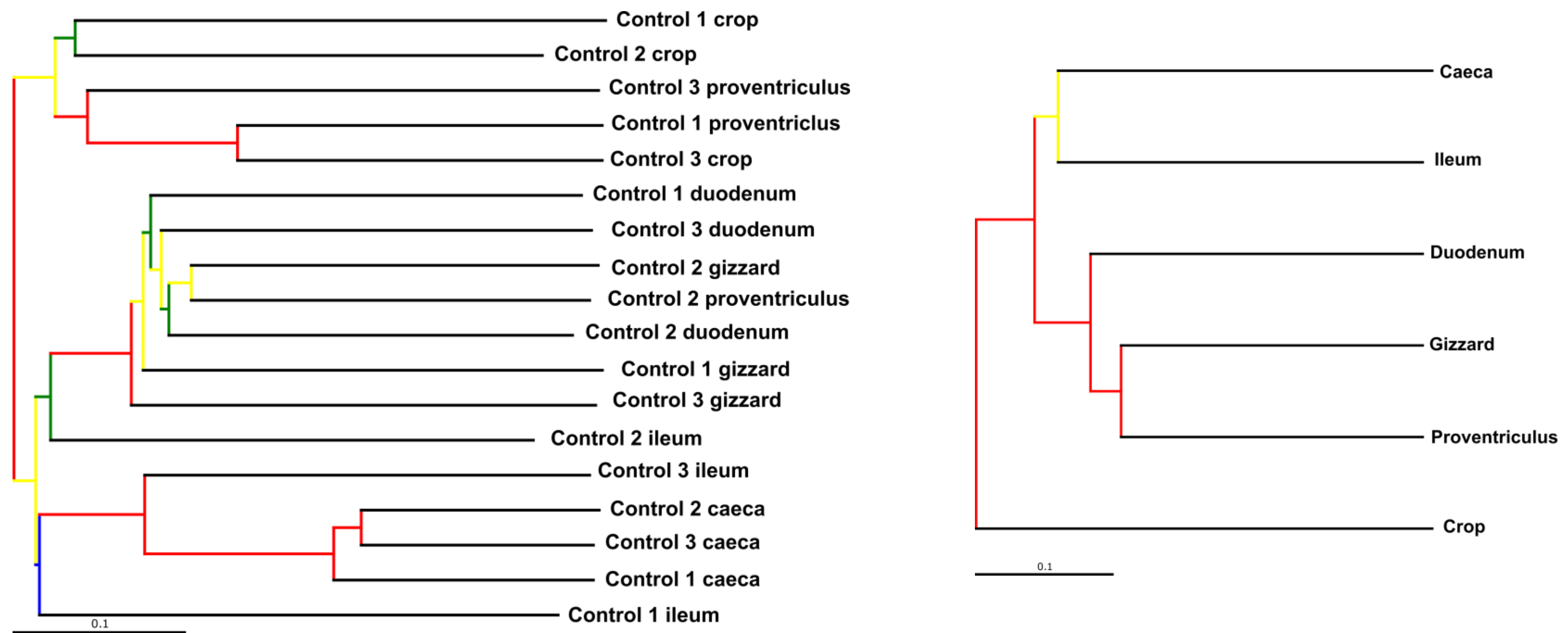


Figure 4.11 – Jackknife beta diversity bootstrapped tree of control-diet samples from V4-V6 16S rRNA gene-fragment sequencing.

Red=75-100%, yellow=50-75%, green=25-50%, and blue =< 25%

4.3.9 Alpha rarefaction and beta diversity of the phytase-diet gut

Alpha rarefaction of the control-diet gut was completed to a depth of 31,000 reads (Table 4.9). Figure 4.12a showed that the number of observed species had levelled off at 31,000 reads and therefore extra sampling would probably not result in the observation of further species. The gizzard had the greatest error value of 197±155 followed by the proventriculus 241±113. The lowest error value was assigned the caecum with 291±10, indicating the lowest amount of variance in the number of species between samples.

The Chao1 richness index indicated the caecum was the richest organ with a value of 322±15. The Simpson's diversity plot showed the caecum (red) was the most diverse organ in the phytase-diet gut with a value of 0.96±0.01 (Figure 4.12b). The Shannon index also showed the caecum was the most diverse with a value of 5.81±0.22, which was more than double the next nearest organ (proventriculus 2.2±1.22).

There were no significant OTUs or difference in diversity between crop and proventriculus, proventriculus and gizzard, crop and gizzard, gizzard and duodenum or duodenum and ileum. However, there was a significant difference between ileum and caecum with a bonferroni corrected p-value of 0.00068. Furthermore, there were no significant differences between any of the chickens within each organ.

Jackknife beta diversity was completed on the phytase-diet gut samples at 31,000 reads (Figure 4.13). There was high bootstrap support (75-100%) for the placement of the majority of the samples within the tree. The tree showed the grouping of the caecum samples together with a bootstrap value of 75-100% however, there was limited other grouping of samples originating from the same organ or the same chicken which mirrors what was observed in the control-diet tree in Section 4.3.8.

The phytase-diet organs were compared to a depth of 127,000 reads and the tree indicated high bootstrap support for every organ placement (Figure 4.13). The tree showed that the crop is more closely related to the duodenum and ileum than the proventriculus and gizzard.

4.3.10 Phylogenetic analysis of abundant OTUs from both diets

ARB was used to analyse the phylogeny of the V4-V6 16S rRNA gene-fragment OTUs. Many trees failed to provide more taxonomic resolution for OTUs that were assigned previously with the RDP classifier. In total, nine of the 28 most abundant OTUs formed trees with confidence levels ≥50% (Table 4.10).

Organ	Chao1 Ave.	Chao1 Err.	Observed species Ave.	Observed species Err.	Shannon Ave.	Shannon Err.	Simpson Ave.	Simpson Err
Crop	103.45	41.91	70.33	27.46	0.97	0.61	0.30	0.25
Proventriculus	289.08	109.52	241.60	113.80	2.2	1.41	0.47	0.31
Gizzard	262.74	172.15	197.40	155.33	1.58	1.30	0.35	0.26
Small Intestine First	151.94	78.66	108.33	72.45	0.62	0.55	0.14	0.14
Small Intestine Last	118.28	35.59	82.77	26.87	1.91	0.45	0.57	0.16
Caecum	322.49	14.56	291.97	10.27	5.81	0.22	0.96	0.01

Table 4.9 – Alpha rarefaction statistics of phytase-diet organs at a depth of 31,000 reads.

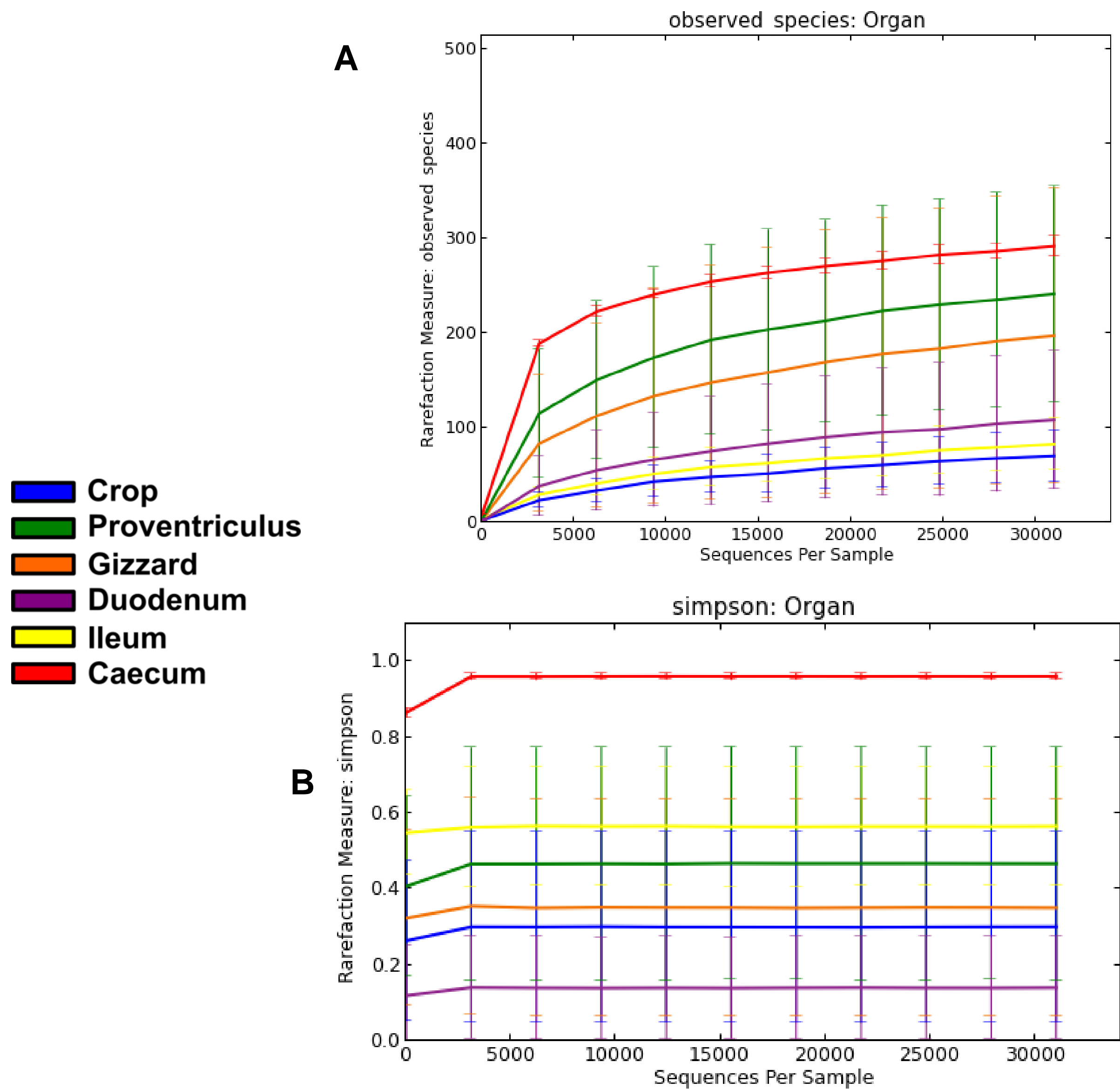


Figure 4.12 – a) Number of observed species against the number of sequences from phytase-diet organs from V4-V616S rRNA gene-fragment sequencing.

b) Simpson diversity index against number of sequences in the phytase-diet organs from V4-V616S rRNA gene-fragment sequencing.

*error bars are S.E.M

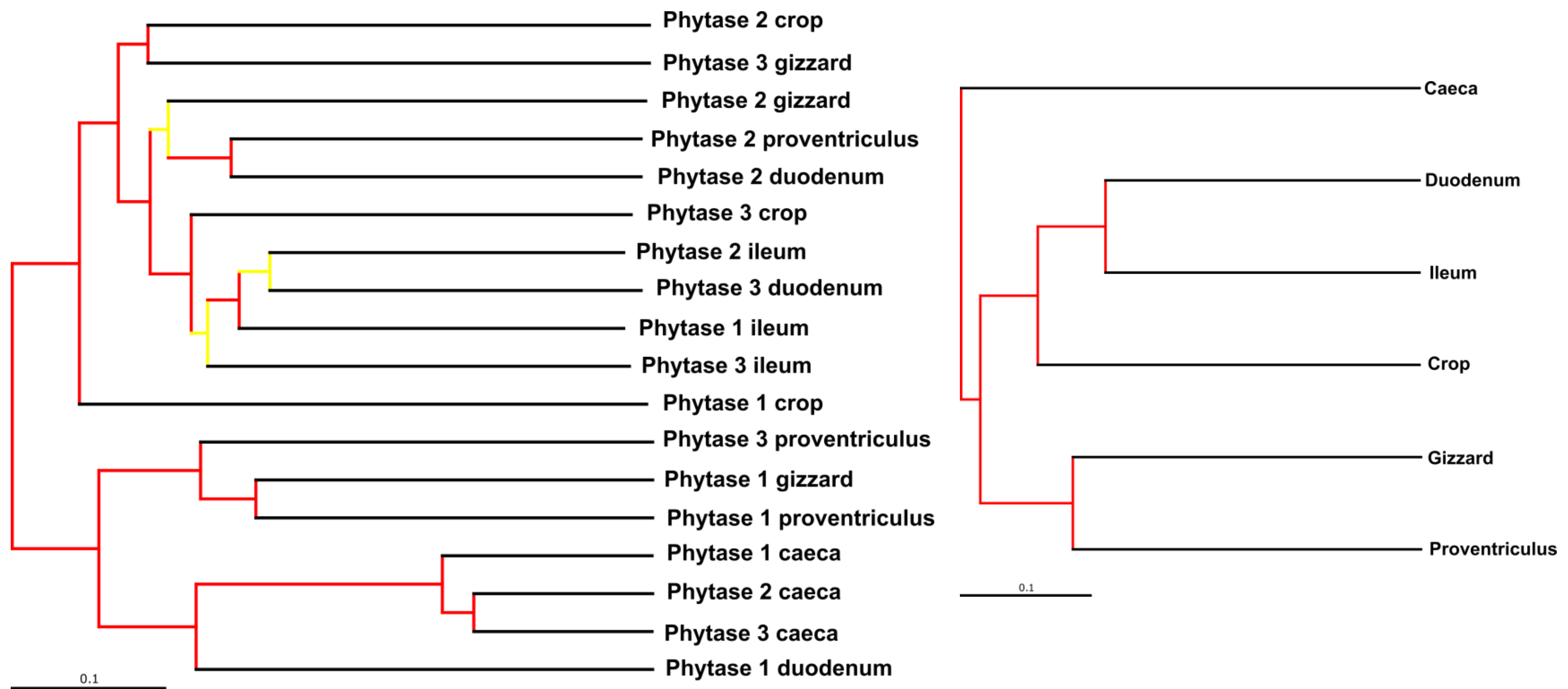


Figure 4.13 – Jackknife beta diversity bootstrapped tree of phytase-diet samples after V4-V6 16S rRNA gene-fragment sequencing.

Red=75-100%, yellow=50-75%, green=25-50%, and blue =< 25%

OTU	Nearest named bacterial species
3	*Clostridium bartlettii*
16	*Oscillibacter valericigenes*
17	*Elusimicrobium minutum*
19	*Ruminococcus lactaris*
22	*Clostridium glycyrrhizinilyticum*
28	*Blautia glucerasea*
33	*Marvinbryantia formatexigens*
139	*Blautia hansenii*
908	*Faecalibacterium prausnitzii*

Table 4.10 - The nine most abundant OTUs from V4-V6 16S rRNA gene-fragment sequencing which were placed in an ARB tree with ≥50% confidence levels and their nearest species.

4.4 Discussion

To the authors' knowledge this was the first study of the microbiota of the crop, proventriculus, gizzard, duodenum, ileum and caecum from a single chicken using V4-V6 16S rRNA gene-fragment sequencing. This is not the first use of the V4-V6 region to study the microbiota of the chicken; it was used by Zhao *et al.* 2013 to study faecal samples from 15 chickens however the taxonomic data was not published [206].

Although a published study used Roche 454 FLX Titanium pyrosequencing of the 16S rRNA gene V1-V3 hypervariable region to study the crop, gizzard, duodenum, jejunum, ileum, caecum and large intestine of three chickens, they generated only 111,970 sequences. The authors' study generated over 900 times more sequences than the published study [119]. Furthermore, their sequences were assigned to 2,803 OTUs within 1,500 genera; this was three times more OTUs than were reported by Wei *et al.* (2013) when they collected all sequences of chicken origin from GenBank, Silva database and RDP and over ten times more genera [24]. The vastly inflated taxonomic diversity renders the results from that previous study implausible.

The increased depth of sequencing resulted in the discovery of 307 genera and 1,153 OTUs which is almost double the species and genera found by Apajalahti *et al*. (2004) and more than identified by Wei *et al.* (2013) [24, 38]. Wei *et al.* (2013) used rarefaction to estimate the number of OTUs in the chicken gut and this is closer to the number observed by the authors' study [24]. However, both are significantly lower than the species and genera found by Choi *et al.* 2014 [119].

Firmicutes sequences dominated in both diets however the abundance was almost 20% higher than found in previous studies [24, 115, 119]. Furthermore, Proteobacteria and Bacteroides were the next most abundant phyla in both diets, which is the opposite of what was found in a study by Wei *et al.* (2013) [24]. An explanation for these differences in abundance could be that the majority of publically available sequences were from studies of the small intestine and caecum; which coincides with the lack of published sequencing studies on the foregut. Hence, when just the caecal samples from both diets are summarised the abundances are closer to those found by other studies.

The domination of lactobacilli in the crop, gizzard and small intestine found in this study complements previous studies of these organs by both culture-dependent [12, 106, 109-111, 120] and culture-independent techniques [115, 116, 119]. The identification of these lactobacilli to species level was not possible through 16S rRNA gene-fragment sequencing of the V4-V6 region. However, culturing efforts in Chapter Five led to the isolation and identification of some of these species.

The proventriculus was dominated by *Lactobacillus*, which supports the findings from the only previous study of this organ [106]. Both diets had a similar abundance of the same *Lactobacillus* OTUs and no significant difference was identified, indicating the addition of phytase does not affect the microbial population in this organ. Furthermore, the microbiota associated with the proventriculus was not significantly different from the crop or gizzard microbiota in either diet, agreeing with the hypothesis of Oakley *et al.* (2014) that the proventriculus was similar to the gizzard [44].

It has been demonstrated that the addition of phytase to the chicken diet causes the proliferation of lactobacilli in the small intestine [112]. This hypothesis was supported in this study by the dominance of *Lactobacillus* species in the phytase-diet ileum samples. In contrast, within the control-diet ileum samples, Clostridiales dominated, becoming the first class to displace Lactobacillaceae as the most abundant. High abundance of *Clostridium* has been found in the ileum in previous studies [94, 118]. The abundance of *Lactobacillus* species, caused by phytase, could be why there is no significant difference in the microbiota associated with organs of the phytase-diet between the duodenum and ileum, however there is a significant difference in the control-diet.

The abundance of *Herbaspirillum* in the duodenum control samples raises an interesting question of whether this is a true or spurious result. *Herbaspirillum*, among other genera, have been identified as a contaminant in DNA extraction kits and laboratory reagents [207]. *Herbaspirillum* has not been documented in the chicken gut previously; it is a soil-dwelling, plant-associated genus and thus could have been associated with the feed given to the chickens and was probably transient, rather than an established species in the gut [208]. Although it was identified in all organs it was not identified in every sample and was most abundant in the proventriculus and duodenum samples. This could be due to the influx of acid, bile acids and enzymes reducing the bacterial load in these sections of the gut, resulting in OTUs from environmental sources (such as extraction kits) becoming more abundant than OTUs originating from the chicken gut [100].

In the microbiota associated with the phytase-diet, the duodenum had more observed species than the ileum but had lower diversity as shown by the Shannon and Simpson values. The more observed species matched the results of Gong *et al.* (2007) however, they did not employ diversity statistics to their data therefore a true comparison cannot be made [115].

In the control-diet, the duodenum had more observed species and was more diverse than the ileum. This result is similar to Choi *et al.* (2014) where they found the duodenum was much more diverse than the ileum, however, there are large variations in the number of reads used to calculate the values reported in Choi *et al.* (2014), therefore these results could be unreliable.

The caecum was the most diverse organ in both diets with no OTU dominating and high Simpson diversity index values which agrees with previous studies of the chicken caecum and the results detailed in Chapter Three [68, 91-93, 115, 116, 119, 120]. The number of observed species in each diet was similar to that found by Sergeant *et al.* (2014) although lower than that found by Danzeisen *et al.* (2011) [2, 68]. Clostridiales was the most abundant order, with Lachnospiraceae and Ruminococcaceae the most abundant families; this mirrors the findings of Chapter Three and published data [29, 118-120, 209]. The abundance of Bacteroides in the control-diet was similar to that in culture-dependent and culture-independent studies [24, 91-93, 119], however, the abundance in the phytase-diet was closer to that found by Zhu *et al.* (2002) [24, 67]. The abundance of lactobacilli in the caecal samples from both diets was considerably lower than some previous studies [5, 90, 94, 115, 118, 125, 210] although it was closer to that of others [24, 67, 93, 119]. The varying counts of

lactobacilli in the studies occur in both culture-dependent and culture-independent studies; therefore it is unlikely to be a methodology limitation and is variability between birds.

Comparison of the results in Chapter Three and Chapter Four was not always possible for all organs due to the depth of coverage gained in the V3-V4 16S rRNA gene-fragment sequencing. Furthermore, because the different regions of the 16S rRNA gene can result in different levels of taxonomic refinement (due to reference sequence quality or the number of variable parts in a region) this led to another problem of direct comparison. Within the caecum, OTUs that were identified in both Chapters Three and Four often had different relative abundances. An example of this is an OTU that represented *Escherichia/Shigella* that represented 4.8% of total reads in Chapter Three but 8% in Chapter Four. In addition, the OTU with the highest abundance in the Chapter Three was not replicated in Chapter Four, with the most abundant OTU from the same family having almost three times less relative abundance. The differences were not isolated to just the most abundant organisms; although the observed species graphs from both chapters are similar, the jackknife beta diversity tree places different samples together in each chapter. This difference between the same samples but using different 16S rRNA regions poses a question about the reliability of comparing results when different methods and regions of the 16S rRNA gene are used.

If the identified OTUs are genotypically and phenotypically similar to their identified closest bacterial relatives, then it could be assumed they would have similar traits to those discussed.

Clostridium bartlettii was one of the most abundant organisms in the control-diet duodenum and ileum samples and in the phytase-diet ileum samples. It has been found in the ileum previously, however was not found in the duodenum and the abundance was not reported [211]. The abundance of this organism in the small intestine could be explained by its high resistance to bile, therefore it is able to proliferate where other organisms may not. It produces large quantities of acetate, valerate and butyrate, which are beneficial for chicken health [18, 183, 212]. High abundance of this organism has been associated with higher FCR in turkeys [213]. Furthermore, *C. bartlettii* has been shown to grow better at pH 6.7 [214] which could explain the higher abundance in the control-diet as a previous study on the effects of phytase in the ileum showed a significant increase in pH (from pH 6.7 to pH 7.2) with the addition of phytase [112].

Oscillibacter valericigenes was abundant in the control-diet caecal samples. It is a strict anaerobe that is able to ferment a range of sugars into the volatile fatty acid valerate and has been found in high abundance in the chicken caecum in a previous study [209, 215, 216]. However, previous studies on this organism have found the optimum temperature of growth for isolated strains to be 30 ^{o}C with no growth observed over 35 ^{o}C, which might indicate this strain is different from those previously isolated as the internal temperature of the chicken is ~42 ^{o}C [215, 216].

Faecalibacterium prausnitzii was in the most abundant OTUs in the caecum of both diets. It has been identified from the caecum previously [5, 120]. The majority of strains are unable to ferment common plant polysaccharides [201].

In humans and murine models, it has been shown that *F. prausnitzii* is an anti-inflammatory commensal and it produces volatile fatty acids such as propionate and butyrate. However, its role in chickens is currently unknown [209, 217, 218].

Elusimicrobium minutum was in the most abundant organisms in the phytase caecal samples and although the species has not been identified in the chicken before the phylum has been identified in the caecum previously. Elusimicrobia formed ~0.1% of total V3-V4 16S rRNA gene-fragment sequences during a study of the caecum of egg laying hens over their life [219]. Originally isolated and whole genome sequenced from a beetle larva gut, it is a strict anaerobe that produces moderate amounts of acetate, ethanol and hydrogen through fermentation [220, 221].

Ruminococcus lactaris was identified as one of the most abundant in both diets in the caecal samples and has not been reported in chickens before, however it is common in the human gut [222-224]. The ability of *R. lactaris* to colonise mucin is unknown, however species in the same cluster possess this trait [225]. It proliferates in the presence of fermentable carbohydrates and produces acetate and formate, but not butyrate [224]. The production of these volatile fatty acids could have positive effects on the chicken health [18, 183].

Clostridium glycyrrhizinilyticum was identified in this study and was most abundant in the control-diet caecal samples. Although it has not been reported as being identified from previous chicken gut studies, an isolate with 96.3% similarity was found in broilers that were fed a diet with no added vitamins [226]. In the only study of the bacterium, it was found to produce volatile-fatty acids from a range of carbohydrates, however the specific acids produced were not

reported [227]. The bacterium is capable of hydrolysing glycyrrhizin (a glycoside of glycyrrhetic acid) which is not common in the chicken diet therefore, the benefits of this organism are unknown [227].

Blautia glucerasea was identified as one of the most abundant organisms in the control caecal samples. This species has not been reported in chickens previously, however the *Blautia* genus is commonly found in caecal samples [24, 68]. *B. glucerasea* is an obligate anaerobe that can ferment a range of polysaccharides, including xylan, in addition to producing beneficial volatile fatty acids [228]. These traits could be why it is so abundant in the caecum and not found in other parts of the gut.

Marvinbryantia formatexigens is able to ferment cellulose and CMC and was most abundant in the control-diet gizzard and caecal samples [229]. It has been identified in the chicken caecal microbiota through 16S rRNA gene sequencing previously [206]. It requires formate to grow, therefore could be part of a co-culture in the chicken gut [229]. It is a strict anaerobe that produces the volatile-fatty acids: acetate, succinate and lactate through fermentation, grows at pH 7 and has multiple glycoside hydrolases [229, 230]. As it is a strict anaerobe, it requires a neutral pH and the presence of formate (a product of fermentation), it could be suggested that it is transient in the gizzard and not residing there.

Blautia hansenii (formally known as *Streptococcus hansenii* and *Ruminococcus hansenii)* is an obligate anaerobe that was abundant in the phytase-diet caecal samples [231]. Through fermentation it mainly produces lactate with lower amounts of acetate and succinate; it is unable to ferment cellobiose [231]. It

was found in the caecal mucus layer of 17 out of 18 chickens in one of the first culture-independent studies of the caecal microbiota [67].

The jackknife beta diversity trees of the control-diet showed a clustering of caecal and ileum samples and weak clustering of crop and proventriculus samples, however the phytase-diet had little clustering of organs and chickens beyond the caecal samples. This spatial heterogeneity in the phytase-diet is further illustrated by the abundance of three OTUs in each of the organs up to the ileum however; there is less spatial heterogeneity in the control-diet. These findings are similar to those found by Choi e*t al.* (2014) where they noted that low numbers of shared OTUs can still form the majority of bacterial abundance between each organ [119].

There was a single significant OTU between the diets, found in the duodenum. However, this was in such a low abundance that any affect this would have is negligible. Compared to the V3-V4 16S rRNA gene-fragment sequencing results from Chapter Three, deeper sequencing of the chicken gut uncovered a significant difference in microbiota between the caecal samples. As there has only been one study that looked at the change in microbiota through the addition of phytase and this focussed on the ileum, there are no direct results to compare those found in this study to [112]. This study was also the first to apply copy-number correction to the generated sequences, although this could inflate the abundance of OTUs that could not be assigned to a phylum because all other OTU abundance will be reduced. This has provided a more accurate and deeper analysis of the crop, proventriculus, gizzard, duodenum, ileum and caecum of the chicken than has previously been achieved.

CHAPTER FIVE

5. Isolation of bacteria from the chicken gut

5.1 Introduction

The first study of the chicken gut microbiota was published in 1897 by Heinrich Kern [36]. It was determined by Kern (through microscopic preparations of the stomach and intestinal contents) that a large number of bacterial species could not be isolated using standard cultivation techniques. Kern cultured and identified 21 bacterial species from the avian intestine [36, 40]. However, early studies of the microbiota were restricted by the use of highly selective media and aerobic storage of samples prior to culturing [232]. Therefore, the assumption that the intestinal conditions of a healthy chicken were not conducive for growth of obligate anaerobes by Gage in 1911 was probably due to the lack of non-selective media and anaerobic technology.

It was proposed by King in 1905 that the mucosa of the caecum had a higher microbial concentration than other parts of the gut [233]. This was confirmed by Barnes in 1972 as approximately 10^{11}/g and it has been documented that a large proportion of these microbes have yet to be cultured in the laboratory [5, 90]. The shift towards culture-independent methods to study the gut microbiota resulted in the discovery of more complex and diverse communities than previously imagined [24, 74]. However, there is a limit to how useful omics-based techniques can be when trying to establish physiology and metabolism of particular species from a complex community, leading to a renewed interest in culturing [25, 69, 234].

The importance of culturing bacteria is paramount; natural products and derivatives from bacteria are thought to form 50% of commercially available pharmaceuticals [41]. Furthermore, culturing allows for the study of

pathogenicity, physiology and genetics in addition to the discovery of industrially relevant enzymes [234].

A study into the human microbiome using 212 different culture conditions and metagenomics on the same samples identified only 51 overlapping species in the data [25]. This indicates sequencing and culturing together identifies more organisms within the microbiota and therefore would provide a more accurate bacterial census.

The aim of this research was to isolate bacterial species from the chicken gut to further knowledge, and form a census of bacterial species that reside in this habitat and to complement sequencing efforts of the same samples. The majority of culturing efforts focussed on the caeca due to microbial density with particular interest in novel bacterial species that have not been cultured before.

5.2 Methods

To isolate bacteria an anaerobic and microaerophilic chamber, a range of media and antibiotics were used (Table 2.2). The use of FAA with 5% horse blood as the base medium for the cultivation of facultative and obligate anaerobes was influenced by Heginbothom *et al.* (1990), where they deemed it superior to other anaerobic media [235]. Heating and ethanol use on the digesta prior to serial dilution selected for spore forming bacteria. OSA selected for acid tolerant bacteria such as *Lactobacillus* and culturing aerobically selected for facultative anaerobes and aerobes (full details of bacterial culturing is provided in Chapter Two).

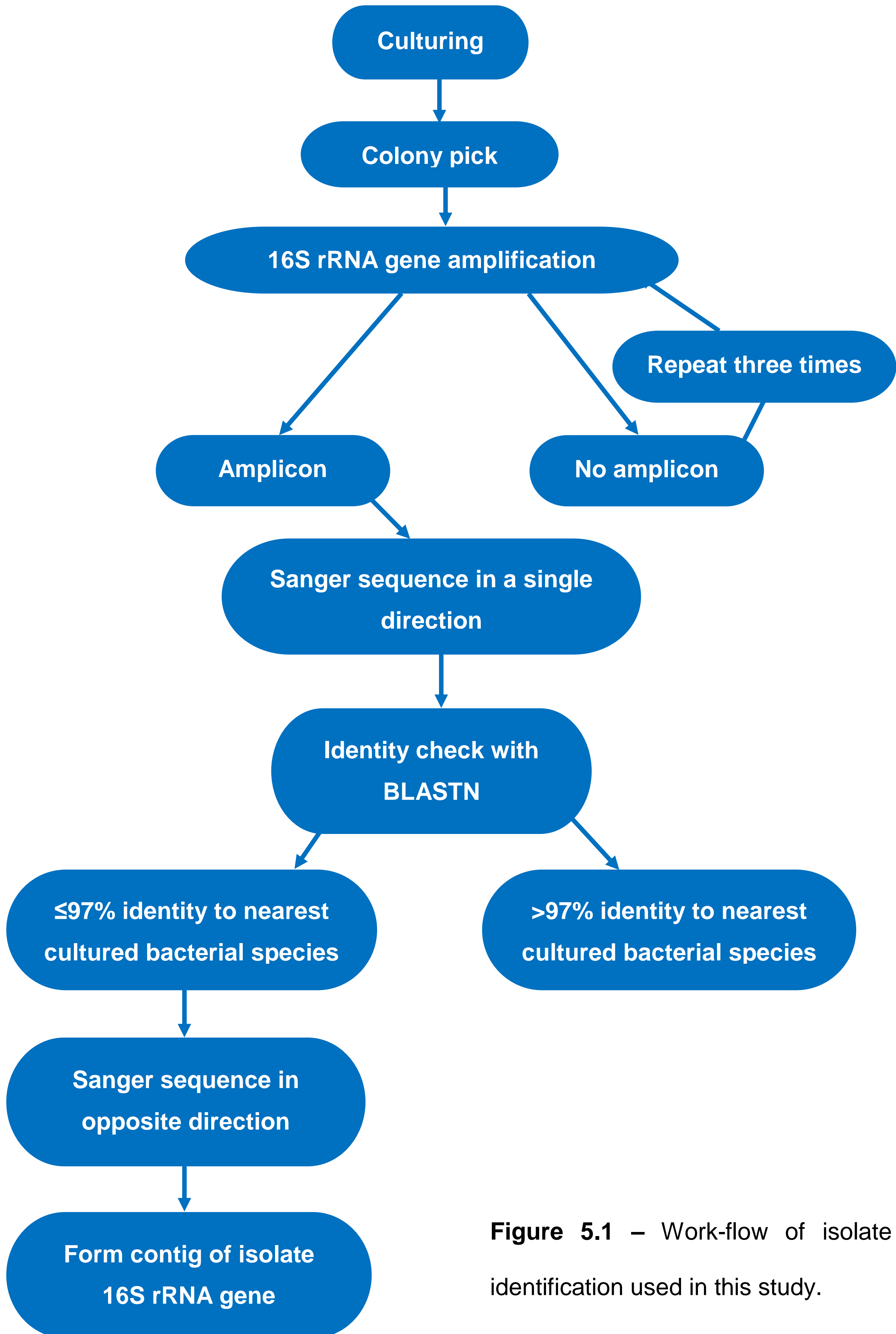


Figure 5.1 – Work-flow of isolate identification used in this study.

5.3 Results

35 isolates were cultured on FAA plates with 5% horse blood under anaerobic conditions at 37 °C. The majority of these isolates (17/35) were grown after serial dilution (to yield single colonies) without the addition of antibiotics or other supplements (Table 5.1).

A further seven isolates were cultured after pre-treating caecal samples with 80% ethanol for 30 minutes prior to serial dilution; four on Mueller-Hinton agar (MHA) with sodium thioglycolate and three on FAA plates with 5% horse blood supplemented with sodium thioglycolate under anaerobic conditions at 37 °C. A single isolate was cultured using the same method but with the addition of vancomycin at 30 µg/ml to the agar (Table 5.1).

Heating caecal samples to 65 °C for 15 minutes prior to serial dilution resulted in four isolates being cultured on FAA plates with 5% horse blood supplemented with sodium thioglycolate under anaerobic conditions at 37 °C. A further isolate was cultured after heating the sample to 80 °C and placed into a static blood culture medium for three days prior to serial dilution on FAA plates with 5% horse blood supplemented with sodium thioglycolate under anaerobic conditions at 37 °C (Table 5.1).

FAA plates supplemented with antibiotics enabled five different bacterial isolates to be cultured. A single isolate was cultured on an FAA plate supplemented with colistin sulphate and two isolates each were cultured on FAA plates supplemented with gentamicin and rifampicin (Table 5.1).

Under aerobic conditions at 37 °C, four isolates were cultured on LBA plates and two on OSA. BHI was used under anaerobic conditions and 37 °C and two isolates were cultured (Table 5.1).

5.3.1 Bacterial identification of cultured isolates

Isolates from the gut were identified using Sanger sequencing of the 16S rRNA gene and placement into ARB. In total, 43 bacterial isolates were cultured from the gut. For sixteen, the 16S rRNA gene was sequenced in both directions, providing a single contig for almost the entire gene, while 27 had the 16S rRNA gene sequenced just in a single direction.

The majority of bacterial species isolated were Gram-positive organisms with five isolates assigned to Gram-negative organisms (*E. coli,* two *Alistipes, Bacteroides fragilis* and *B. intestinalis*).

Caecal samples yielded the majority of isolates with ≤97% identity to the nearest named bacterial species with 36 isolates cultured from a single sample (phytase 2 caecum). *Bifidobacterium pullorum* was the only bacterial species cultured from another caecal sample (control 1 caecum).

Staphylococcus warneri, a *Bifidobacterium* and a *Bacillus species* were identified from isolates of proventriculus samples. *E. coli* was isolated from all organ samples. *Lactobacillus crispatus* and *Lactobacillus salivarius* were identified from cultured isolates from crop samples.

Isolate ID	Medium	Condition	Isolation source	Taxonomic assignment
Isolate 1[2]	BHI	Serial dilution	Caecum	*Bifidobacterium pullorum*
Isolate 2[2†]	BHI	Serial dilution	Caecum	*Clostridium spiroforme*
Isolate 3[1†]	FAA	Serial dilution	Caecum	*Enterococcus faecalis*
Isolate 4[1†]	FAA	Serial dilution	Caecum	*Clostridium*
Isolate 5[1†]	FAA	Serial dilution	Caecum	Ruminococcaceae
Isolate 6[2†]	FAA	Serial dilution	Caecum	*Bacteroides fragilis*
Isolate 7[2†]	FAA	Serial dilution	Caecum	*Bacillus subtilis*
Isolate 8[2†]	FAA	Serial dilution	Caecum	*Enterococcus*
Isolate 9[2†]	FAA	Serial dilution	Caecum	Coriobacteriaceae
Isolate 10[2†]	FAA	Serial dilution	Caecum	*Corynebacterium stationis*
Isolate 11[2†]	FAA	Serial dilution	Caecum	*Clostridium*
Isolate 12[2†]	FAA	Serial dilution	Caecum	*Alistipes*
Isolate 13[2†]	FAA	Serial dilution	Caecum	*Bacteroides intestinalis*
Isolate 14[2†]	FAA	Serial dilution	Caecum	*Lactobacillus*
Isolate 15[2†]	FAA	Serial dilution	Caecum	*Propionibacterium granulosum*
Isolate 16[2†]	FAA	Serial dilution	Caecum	*Clostridium*
Isolate 17[2†]	FAA	Serial dilution	Caecum	*Clostridium lactatifermentans*
Isolate 18[2†]	FAA	Serial dilution	Caecum	*Staphylococcus*
Isolate 19[2†]	FAA	Serial dilution	Caecum	*Propionibacterium acnes*
Isolate 20[2]	LBA	Serial dilution	Proventriculus	*Staphylococcus warneri*
Isolate 21[2]	LBA	Serial dilution	Proventriculus	*Bacillus*
Isolate 22[2]	LBA	Serial dilution	Proventriculus	*Bifidobacterium*
Isolate 23[2]	LBA	Serial dilution	Small intestine first	*Escherichia coli*
Isolate 24[2]	OSA	Serial dilution	Crop	*Lactobacillus salivarius*

Isolate ID	Medium	Condition	Isolation source	Taxonomic assignment
Isolate 25[2]	OSA	Serial dilution	Crop	*Lactobacillus crispatus*
Isolate 26[1†]	FAA	Heat	Caecum	*Bacillus*
Isolate 27[1†]	FAA	Heat	Caecum	Lachnospiraceae
Isolate 28[1†]	FAA	Heat	Caecum	*Clostridium*
Isolate 29[1†]	FAA	Heat	Caecum	*Gordonibacter pamelaeae*
Isolate 30[1†]	MHA	Heat	Caecum	Clostridiales
Isolate 31[1†]	FAA	Ethanol	Caecum	*Eubacterium hallii*
Isolate 32[1†]	FAA	Ethanol	Caecum	*Faecalibacterium*
Isolate 33[2†]	FAA	Ethanol	Caecum	*Flavonifractor plautii*
Isolate 34[2†]	FAA	Ethanol	Caecum	*Ruminococcus*
Isolate 35[2†]	MHA	Ethanol	Caecum	*Clostridium*
Isolate 36[1†]	MHA	Ethanol	Caecum	*Clostridium*
Isolate 37[1†]	MHA	Ethanol	Caecum	*Eubacterium*
Isolate 38[2†]	MHA	Ethanol + vancomycin	Caecum	*Clostridium*
Isolate 39[1†]	FAA	Colistin sulphate	Caecum	*Alistipes*
Isolate 40[1†]	FAA	Rifampicin	Caecum	*Coprobacillus*
Isolate 41[1†]	FAA	Rifampicin	Caecum	*Clostridium*
Isolate 42[2†]	FAA	Gentamicin	Caecum	*Clostridium*
Isolate 43[2†]	FAA	Gentamicin	Caecum	*Clostridium innocuum*

Table 5.1 – Identification of bacterial species after culturing (medium, condition and isolation source detailed).

[1] Indicates isolate was sequenced in both directions [2] Indicates isolate was sequenced in a single direction

[†] Indicates the isolate was cultured from sample phytase 2 caecum

5.3.2 Isolates from the Clostridiales

From the Clostridiales, isolate 5 formed a sister group of *Gemmiger formicilis* and *Subdoligranulum variabile*. Isolate 32 was placed with *Faecalibacterium prausnitzii*, however the branch length indicated this was a different species, and isolate 38 was placed as an outgroup of two *Clostridium* species (Figure 5.2a). Isolate 37 grouped with *Eubacterium coprostanoligenes;* however, the branch lengths indicated it is not the same species. Isolate 33 clustered with *Flavonifractor plautii* and isolate 30 formed an outgroup of these species. Isolate 36 formed a sister group of multiple *Clostridium* species (Figure 5.2a).

Isolate 42 formed a sister group of *Clostridium* species, of which isolate 2 was within and placed next to *C. spiroforme*. Isolate 40 was placed with *Coprobacillus cateniformis,* however the branch length indicates this is a different species. Isolates 28 and 41 were placed together as an outgroup of Clostridiaceae, Erysipelotichaceae and Lactobacillaceae species. Through BLASTN it was determined isolates 28 and 41 were different species. Isolate 43 was placed with *C. innocuum* (Figure 5.2b).

Isolate 31 grouped with *Eubacterium hallii*, isolate 34 was placed outside a clade of three *Ruminococcus* species and isolate 17 was placed with *Clostridium lactatifermentans* (Figure 5.3). In the same tree, isolate 27 formed an outgroup to a clade containing two *Anaerostipes* species and a *Eubacterium*. Isolate 35 was placed as an outgroup to *Clostridium schirmacherense* and *Clostridium argentinense*. Isolate 16 formed a sister group of *Clostridium botulinum*, *Eubacterium comesii* and *Clostridium sporogenes*. Isolate 4 was placed within a group of six *Clostridium* species and isolate 11 formed an

outgroup to *Clostridium xylanolyticum* and *Clostridium aerotolerans* (Figure 5.3).

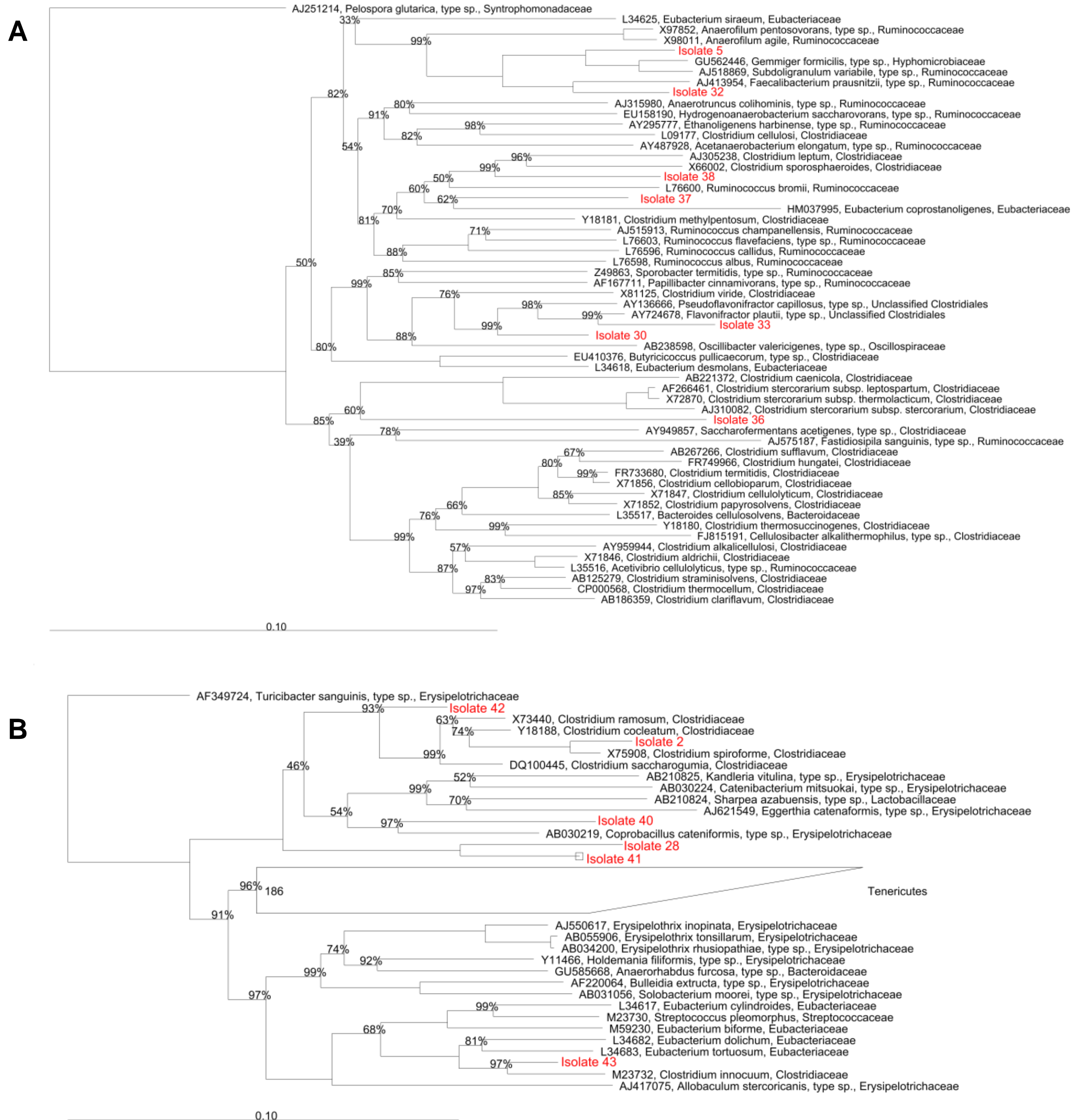


Figure 5.2 - A consensus of 1,000 bootstrap trees created using the ARB neighbour joining distance matrix showing the placement of

a) Seven isolates within the Ruminococcaceae

b) Six isolates within the Clostridiaceae and Erysipelotrichaceae (the 186 Tenericutes sequences have been condensed into a single branch)

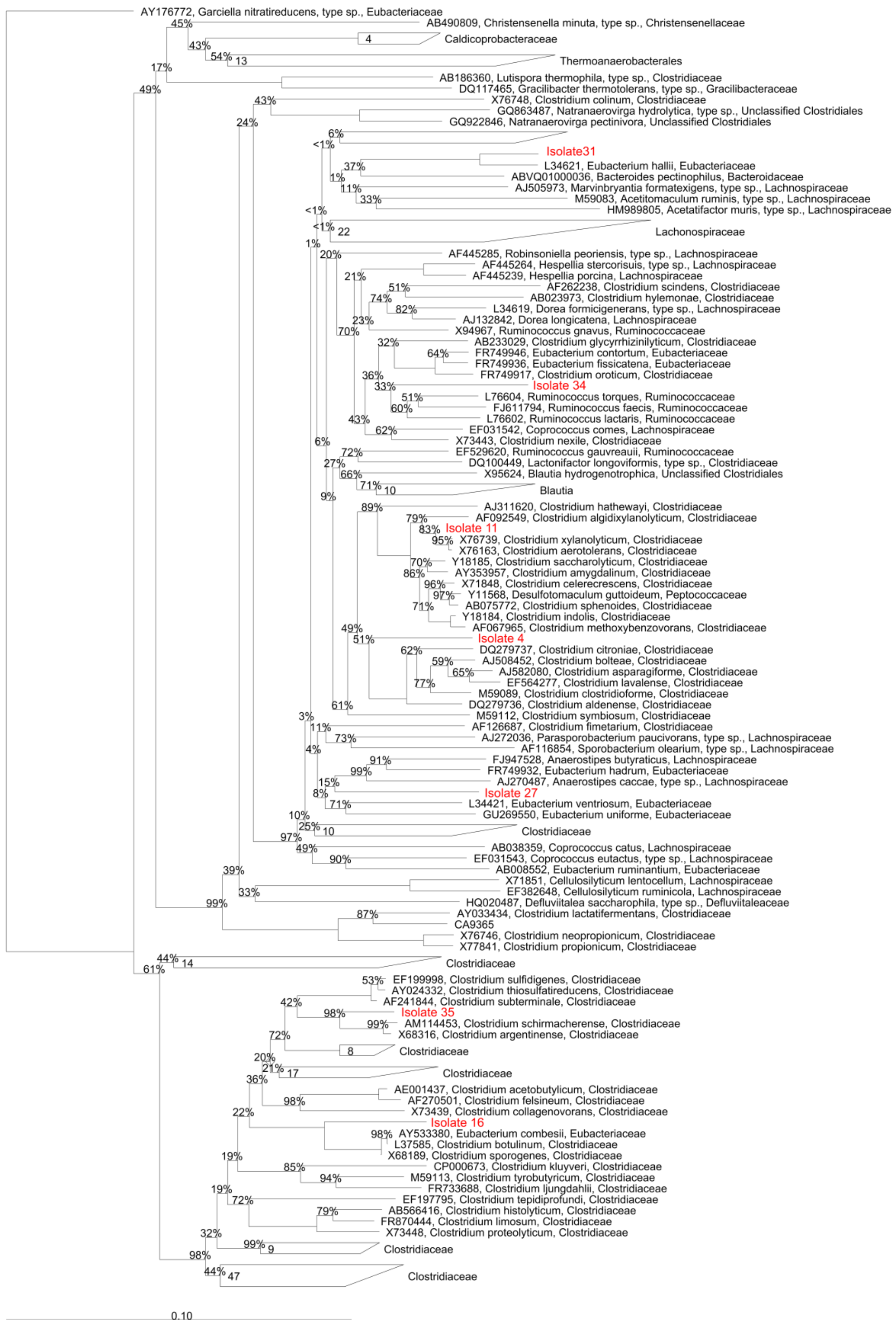


Figure 5.3 - A consensus of 1,000 bootstrap trees created using the ARB neighbour joining distance matrix showing the placement of eight isolates in Clostridiaceae, Lachnospiraceae and Ruminococcaceae

5.3.3 Isolates from the Bacilli

Within the isolates that were Bacilli, isolate 21 was placed as an outgroup of three *Bacillus* species, isolate 7 was placed with *B. subtilis* and isolate 26 formed a sister group of 18 *Bacillus* species (Figure 5.4). Isolate 20 placed with *Staphylococcus warneri* and although isolate 18 grouped with *S. gallinarum*, the branch lengths indicate they might not be the same species (Figure 5.5a).

Isolate 25 nested in a clade with *L. crispatus*, isolate 24 placed with *L. salivarius* and isolate 14 placed as an outgroup of seven *Lactobacillus* species (Figure 5.5b). There were two isolates that placed within Enterococcaceae; isolate 8, formed a sister group of multiple *Enterococcus* species and isolate 3, placed with *E. faecalis* (Figure 5.6).

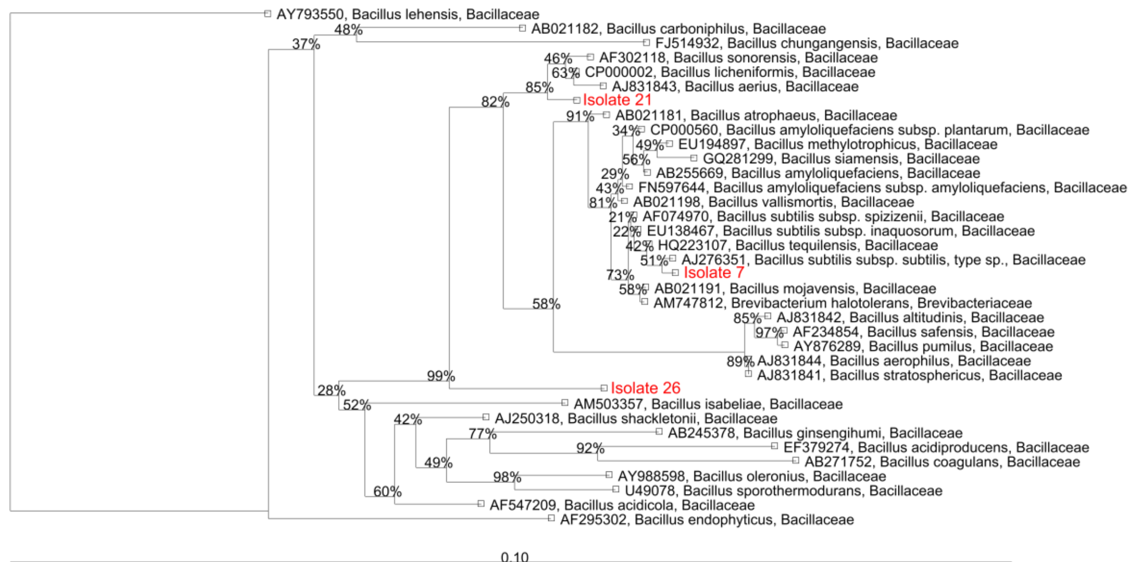


Figure 5.4 - A consensus of 1,000 bootstrap trees created using the ARB neighbour joining distance matrix showing three isolates within the Bacillaceae

Figure 5.5 - A consensus of 1,000 bootstrap trees created using the ARB neighbour joining distance matrix

a) Two isolates within the Staphylococcaceae

b) Three isolates within the Lactobacillaceae

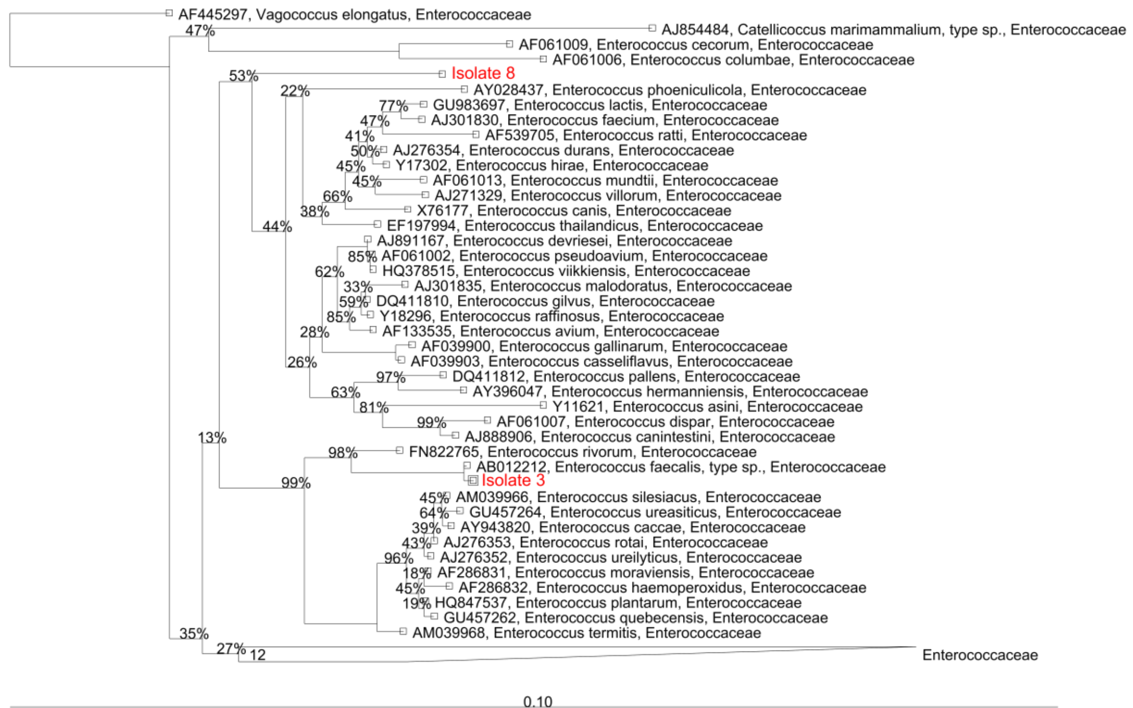


Figure 5.6 - A consensus of 1,000 bootstrap trees created using the ARB neighbour joining distance matrix showing two isolates with *Enterococcus* species

5.3.4 Isolates from the Actinobacteria

Isolate 15 was placed with *Propionibacterium granulosum* and Isolate 19 was placed with *P. acnes*, although the branch lengths indicate this could be a different species (Figure 5.7a). Isolate 10 was placed with *Corynebacterium stationis* and was the only isolated Corynebacteriaceae (Figure 5.7b). Isolate 29 placed with *Gordonibacter pamelaeae* and Isolate 9 formed an outgroup of four species (Figure 5.7c). Isolate 1 placed with *Bifidobacterium pullorum* and Isolate 22 placed with *B. bohemicum*, although had a long-branch length suggesting it was a new species (Figure 5.7d).

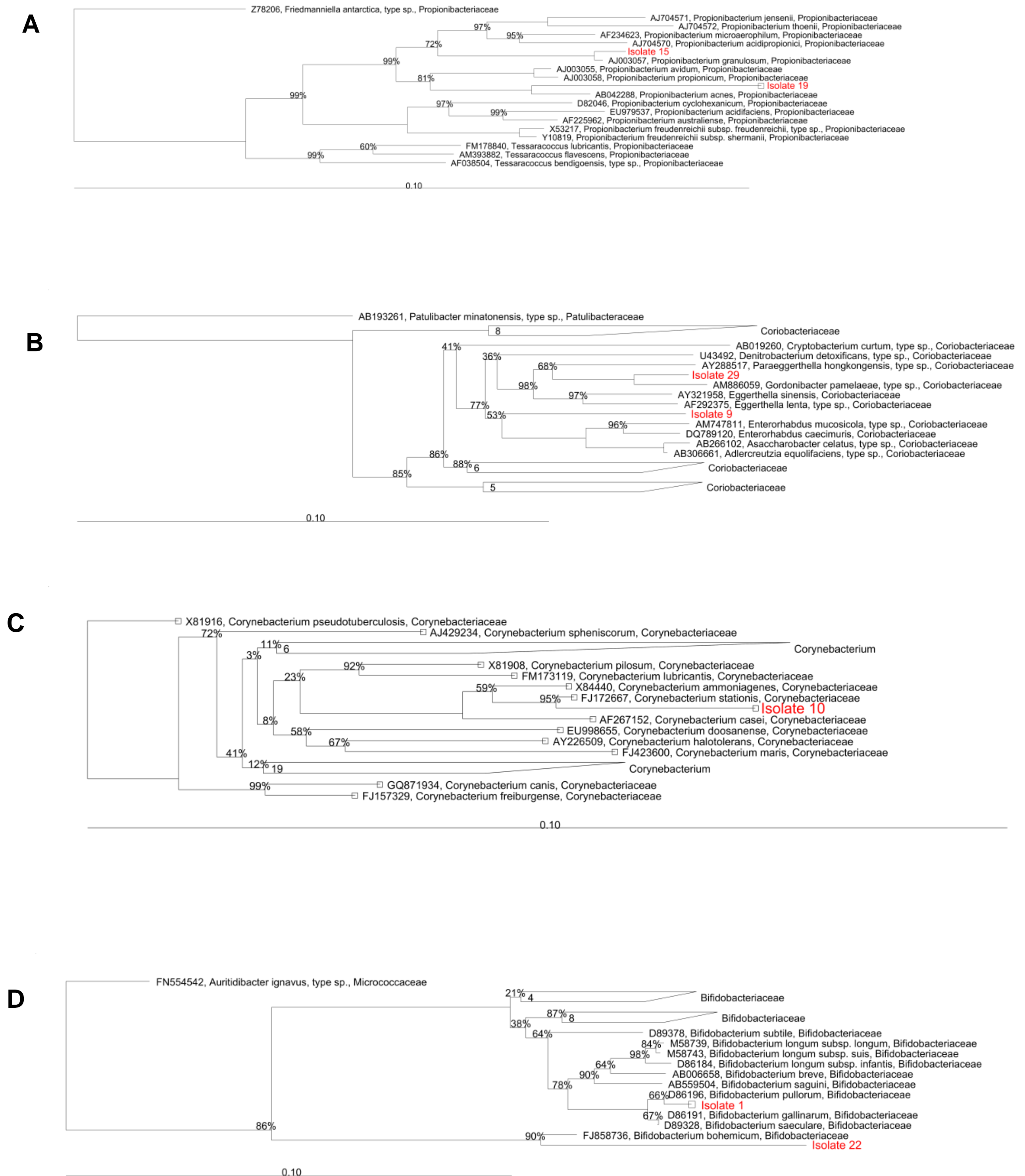


Figure 5.7 - A consensus of 1,000 bootstrap trees created using the ARB neighbour joining distance matrix **a)** Two isolates within the Propionibacteriaceae **b)** Two isolates within the Coriobacteriaceae **c)** A single *Corynebacterium* isolate **d)** Two isolates within the *Bifidobacterium*

5.3.5 Gram-negative isolates

Within the *Bacteroides*, Isolate 6 placed with *B. fragilis* and Isolate 13 placed with *B. intestinalis* (Figure 5.8b). Isolate 39 placed with *Alistipes putredinis* but the branch length indicated this is a new species and Isolate 12 placed with two *Alistipes* species; *A. finegoldii* and *A. shahii* (Figure 5.8c). Isolate 23 placed with *E. coli* (Figure 5.8c).

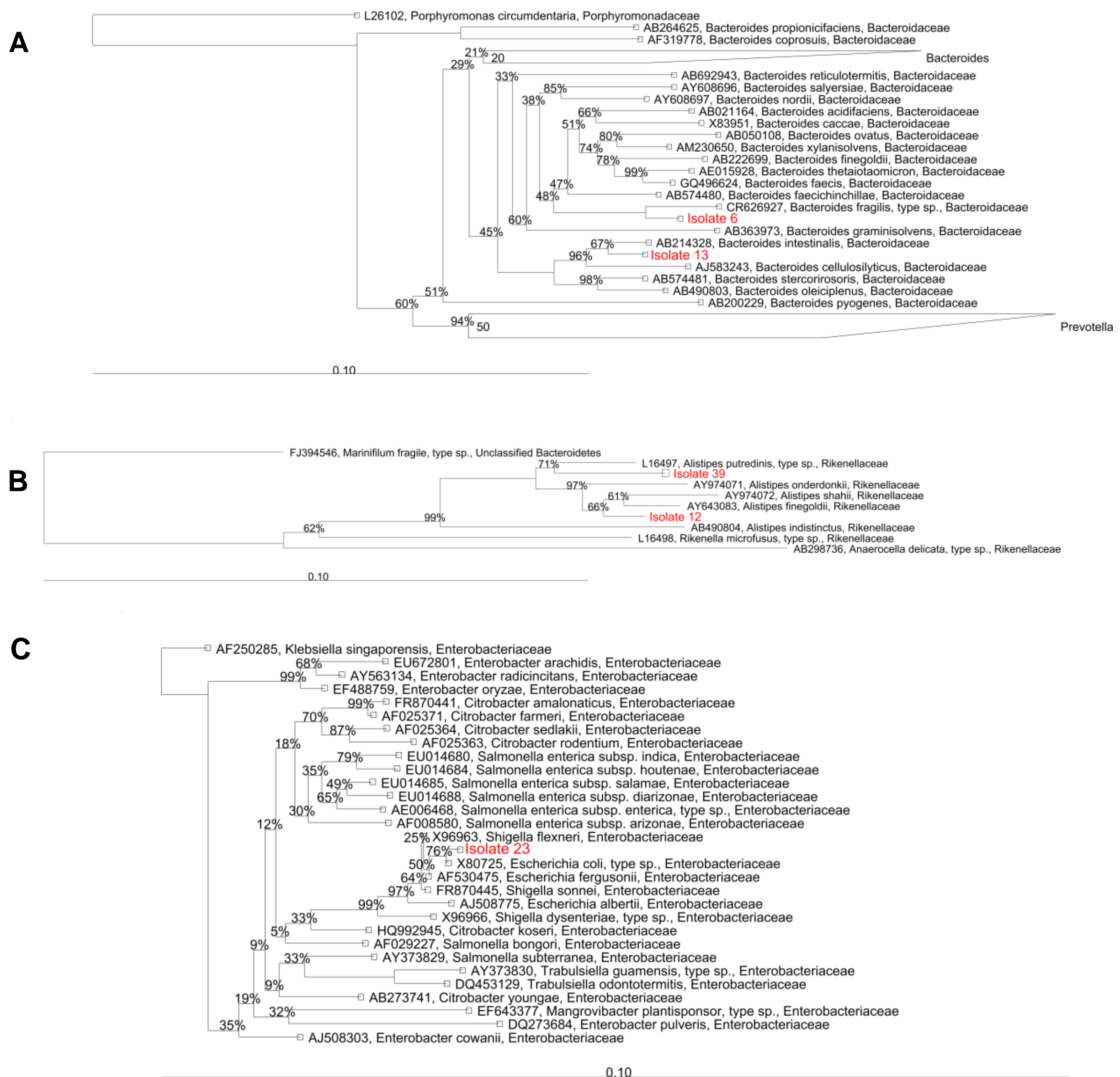


Figure 5.8 - A consensus of 1,000 bootstrap trees created using the ARB neighbour joining distance matrix **a)** Two isolates within the *Bacteroides* **b)** Two isolates with *Alistipes* **c)** A single *Escherichia* isolate

The reference sequences of the species the isolates were closest to in the phylogenetic trees were downloaded and aligned on BLASTN to confirm the taxonomy. An example of an alignment is shown below.

Isolate 7 – *Bacillus subtilis*

Score	Identities	Gaps
1478 bits (800)	806/811 (99%)	0/811 (0%)

```
Query  64   CTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGA  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGA  60

Query  124  CTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCA  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCA  120

Query  184  AACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTG  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  AACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTG  180

Query  244  GTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC  303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC  240

Query  304  ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA  363
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA  300

Query  364  TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGC  423
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGC  360

Query  424  TCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAAC  483
            |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TCTGTTGTYAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAAC  420

Query  484  CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG  543
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG  480

Query  544  TCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC  603
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC  540

Query  604  CCGGCTCA  611
            ||||||||
Sbjct  541  CCGGCTCA  548
```

5.3.6 Comparison against the V4-V6 16S rRNA gene-fragment sequenced dataset

To identify the relative abundance of cultured isolates in the sequencing dataset, they were compared against V4-V6 16S rRNA gene-fragment sequences from culture-independent methods (previously described in Chapter Four) using BLASTN. This allowed cross-referencing of which assigned OTU number corresponded to the isolated bacterial species. Any isolates that shared ≤97% identity with the 16S rRNA gene-fragment sequences were considered not to have been identified in the culture-independent experiments.

In the comparison of full-length 16S rRNA gene sequences, Isolate 32 (*Faecalibacterium*) was assigned to OTU 908 and had 3.2% relative abundance. Isolate 38 (*Clostridium*, OTU 29) and isolate 39 (*Alistipes*, OTU 5) were the only other isolates to have >1% of total reads with 1.5% and 1.3% respectively. Isolate 3 (*Enterococcus faecalis*) was assigned to OTU 68 and had the least relative abundance (0.00018%). Isolates 27, 28, 31, 36 and 37 shared ≤97% identity to the V4-V6 16S rRNA gene-fragment sequences and therefore were not represented in the amplicon library. Isolate 41 (Clostridium, OTU 1104) and Isolate 40 (*Coprobacillus*, OTU 706) were assigned no reads in the sample they were cultured from (Table 5.2).

From cultured isolates with the 16S rRNA gene sequenced in a single direction, seven (Isolates 6, 16, 17, 20, 35, 42 and 43) shared ≤97% identity to V4-V6 16S rRNA gene-fragment sequences and therefore suggested they were not present in the amplicon library. Furthermore, there were six isolates (11, 12,13,14,15 and 19) that were assigned no reads within the sample.

Isolate ID	V4-V6 rep OTU	Identity (%)	Relative abundance (%)
Isolate 3	68	99	0.00018
Isolate 4	75	100	0.51
Isolate 5	212	100	0.19
Isolate 26	23	100	0.00044
Isolate 27	862	97	N/A
Isolate 28	1104	94	N/A
Isolate 29	213	99	0.02
Isolate 30	32	100	0.18
Isolate 31	117	97	N/A
Isolate 32	908	98	3.2
Isolate 36	1072	97	N/A
Isolate 37	127	97	N/A
Isolate 38	29	99	1.5
Isolate 39	5	99	1.3
Isolate 40	706	100	0
Isolate 41	1104	99	0

Table 5.2 – Comparison of full-length 16S rRNA gene sequenced isolates with representative OTUs from V4-V6 16S rRNA gene-fragment sequencing

The isolate with the highest abundance in the V4-V6 16S rRNA gene-fragment sequencing was Isolate 25 (*L. crispatus*), which was assigned to OTU 0 and the source of ~96% of total reads within the phytase 2 crop sample. There were three isolates that were assigned >1% relative abundance: Isolate 33 (*Flavonifractor plautii*, 10%), Isolate 24 (*L. salivarius*, 1.5%) and Isolate 1 (*Bifidobacterium pullorum*, 1.2%). The isolate of lowest abundance present in the sequencing dataset was Isolate 2 (*Clostridium spiroforme*), which was assigned to OTU 500 and contributed 0.0004% relative abundance within the sample. Isolates, 7, 10 and 18 could not be assigned a closest OTU as the quality of the isolate Sanger sequence deteriorated at the end of the 16S rRNA gene and failed to remain after quality trimming (Table 5.3).

The cultured isolates that had ≤97% identity to the representative OTU sequences were also checked against the original sequences (prior to singleton removal) to see if they were present. Isolates 27, 36 and 37 were the only species that had ≥97% similarity to a V4-V6 16S rRNA gene-fragment sequence that were removed because they were singletons.

The overall relative abundance of the isolated species in each organ and in total of each diet was also calculated (Table 5.4). This was achieved by adding the abundance of each cultured isolate from the 16S rRNA gene fragment sequencing data and comparing to the total number of reads. This showed that despite the majority of species having been isolated from the caecum, the relative abundance of these organisms was still low. In comparison, the foregut relative abundances were much higher with 97% of the abundance in the duodenum from the phytase diet recovered by culturing.

Isolate ID	V4-V6 rep OTU	Identity (%)	Relative abundance (%)
Isolate 1	9	99	1.2
Isolate 2	500	99	0.0004
Isolate 6	636	95	N/A
Isolate 7	N/A	N/A	N/A
Isolate 8	68	100	0.0018
Isolate 9	115	99	0.22
Isolate 10	N/A	N/A	N/A
Isolate 11	475	100	0
Isolate 12	218	99	0
Isolate 13	169	100	0
Isolate 14	79	99	0
Isolate 15	342	100	0
Isolate 16	186	93	N/A
Isolate 17	47	96	N/A
Isolate 18	N/A	N/A	N/A
Isolate 19	82	99	0
Isolate 20	15	97	N/A
Isolate 21	23	99	0.012
Isolate 22	112	100	0.007
Isolate 23	2	100	0.04
Isolate 24	1	99	1.7
Isolate 25	0	100	96
Isolate 33	10	99	10
Isolate 34	130	99	0.32
Isolate 35	392	94	N/A
Isolate 42	370	95	N/A
Isolate 43	943	94	N/A

Table 5.3 – Comparison of single-sequenced only 16S rRNA gene sequenced isolates with representative OTUs from V4-V6 16S rRNA gene-fragment sequencing

	Crop (%)	Proventriculus (%)	Gizzard (%)	Duodenum (%)	Ileum (%)	Caecum (%)	Total (%)
Control-diet	79	85	76	60	36	23	60
Phytase-diet	93	80	90	97	76	26	77

Table 5.4 – Relative abundance of isolated species from V4-V6 16S rRNA gene-fragment sequencing in each organ and in total from each diet

5.4 Discussion

Culture-dependent approaches to the microbiota can lead to the acquisition of rare or previously uncultured organisms that could go undetected by molecular methods [234]. The study of sequencing data and subsequent hypotheses often relies on previously isolated organisms for interpretation and therefore is integral to the investigation of microbial diversity [234, 236]. This chapter focussed on the culturing of bacterial isolates from the chicken gut.

A large-scale culture study of the human microbiota utilised 212 different culture conditions to isolate 32,500 colonies and compared this to 16S rRNA gene-fragment sequencing [25]. This led to the discovery of 340 species, 31 of which were novel and only 51 of the isolates were found to also be in the DNA sequencing results. However, they did not publish the relative abundance of the species that overlapped [25]. This renewed approach to culturing shows there is potential to isolate previously uncultured species if enough effort is applied.

There has been a single study that used culture-dependent and culture-independent techniques to study the ileum and caecum of conventional and organic raised broilers [5] However, to the authors' knowledge this is the first study to directly compare isolates with those found with high-throughput 16S rRNA gene-fragment sequencing.

The culturing of 43 different bacterial species from the chicken gut led to the identification of many species that could not be identified from high-throughput 16S rRNA gene-fragment sequencing alone. One of these, *L. crispatus*, was one of the most dominant species throughout the gut and has been identified in the chicken previously [101, 117, 118]. The ability of *L. crispatus* strains to adhere to the stratified squamous epithelium helps it to proliferate in the crop and has been shown to competitively exclude *Salmonella enterica* serovar Enteritidis, when present with *Clostridium lactatifermentans* (which was also isolated in the authors' study) [237, 238].

Of the other *Lactobacillus* species isolated; *L. salivarius* has been identified in chickens previously and was also found to be one of the most abundant species in the crop [239]. Some strains of *L. salivarius* can produce a bacteriocin with an inhibitory effect on *C. jejuni* and other strains can out-compete *E.coli* and *Salmonella*, similarly to *L. crispatus*. [240]. The other *Lactobacillus* isolate could not be identified beyond genus-level. The cultured isolate was found in V4-V6 16S rRNA gene-fragment sequencing and the inability to determine the species could have been because the isolate did not have the entire 16S rRNA gene sequenced, therefore there was not enough sequence to differentiate it from other *Lactobacillus* species.

The majority of caecal isolates were Firmicutes and were within the Ruminococcaceae and Lachnospiraceae families, corroborating the sequencing results and the findings of previous culturing studies [90-93]. Facultative anaerobes such as *E. coli* and lactobacilli were commonly isolated in this study despite their low relative abundance in the sample and this could be why levels of these groups were over-estimated in early culturing attempts [90-93].

To the authors' knowledge this is the first time *Corynebacterium stationis* has been identified in the chicken gut. However, the genus has been found before both within the gut and on the surface of chicken meat during processing [241, 242]. Furthermore, it was not detected in the 16S rRNA gene sequencing analysis in Chapter Three and could not be compared to the deeper V4-V6 (detailed in Chapter Four) data due to the low quality of Sanger sequence in the V4-V6 16S rRNA region.

Of the cultured *Bacteroides* species, *B. fragilis* has been identified from previous studies using both culture-dependent and culture-independent methods on the chicken gut. However, the effect on the chicken is unknown [91, 118]. *B. fragilis* has previously been identified as the most virulent *Bacteroides* species with fimbriae and agglutinins acting as adhesins. The polysaccharide capsule and enzymes suite protects it from host immune response and mediate tissue destruction [243]. It is the most commonly isolated *Bacteroides* species despite its low relative abundance in the host, this is in part due to the ability to tolerate and use oxygen [243]. *B. intestinalis* was originally isolated and identified from human faeces but has not been reported in chickens before [244]. It can ferment a range of polysaccharides to produce acids, but the

identity of these acids and the amounts are not described [244]. It was found in very low abundance in the caecal sample it was isolated from, therefore any effect this species would have on the chicken could be reduced.

Clostridium spiroforme was originally isolated from a chicken in 1979 and has been implicated in the cause of the enteritis-complex in rabbits [245, 246]. Similarly to *C. perfringens*, it contains an iota-toxin however, this is on the chromosome of *C. spiroforme* rather than a plasmid like *C. perfringens* [247]. The effect of *C. spiroforme* on chickens has not been fully investigated although it has been isolated from healthy chickens and those with coccidiosis (the same as C*. perfringens*) [248]. In the authors' study, this species was isolated from the heaviest chicken; therefore any negative effects imposed on the bird must have been limited because it did not influence weight gain.

Flavonifractor plautii (formerly *Clostridium orbiscindens*/*Eubacterium plautii*) has been found in multiple previous studies of the chicken gut [115, 118, 137, 210]. A reason behind the discovery of this species in many other studies could be due to the anaerobic degradation of flavonoids [249]. Flavonoids are widely distributed secondary metabolites in most plant seeds and grains and therefore ingested naturally [249]. As an obligate anaerobe, it is unable to ferment the majority of common saccharides. However, in a broth produces large amounts of butyric and acetic acid, with a smaller amount of propionic acid [250]. This suggests this species could be very beneficial to the host; the inability to ferment saccharides means it is not competing for nutrients whilst still producing beneficial volatile fatty acids.

E. coli has been identified in chickens since the first study in 1896 (known as *Bacillus coli*) [36]. It has been found in multiple culture-dependent and culture-independent studies of the chicken gut since then and is the main source of Proteobacteria assigned reads [5, 24, 90-93, 109-111]. Avian pathogenic *Escherichia coli* (APEC) causes a range of extra-intestinal diseases in chickens including colibacillosis, which can result in significant economic losses to the poultry industry [251, 252]. However, with sequencing of the 16S rRNA gene it is difficult to provide identification below species level, therefore the strains of *E. coli* that were isolated still need to be determined [253].

A number of bacterial species were isolated from the proventriculus that have not been observed before. *E. coli* was commonly isolated from this organ, in addition to *L. crispatus*, *L. salivarius* and a *Bacillus* species. This is the first time an organism has been specifically identified, with the only published study observing lactobacilli however failed to identify a species [106].

From the 43 isolates, 25 could not be assigned to a bacterial species. This could have been due to the lack of full-length 16S rRNA gene sequences for some of the isolates, resulting in the lack of differentiation. However, 12 of the isolates that had a full-length sequence failed to cluster with a known bacterial species, indicating they could be new species. The isolates sequenced from a single direction should have the full length of the 16S rRNA gene sequenced to allow for better assignment.

Unlike the culturomics study of human faeces, this was not a comprehensive study of the chicken gut using culturing. The authors' study relied upon phenotypic traits for differentiation and therefore many different isolates could

have been missed. Furthermore, the selection of spores by using heat and ethanol could result in the germination and subsequent identification of species that were in a vegetative state in the gut, hence reducing the probability of being lysed during the isolation of DNA for sequencing and thus not detected. From the limited culturing applied to the chicken gut, a number of potential novel species were isolated; therefore there is scope for a more comprehensive study to identify multitudes of novel species. Six of the isolates were chosen for whole-genome sequence analysis and this is presented in Chapter Six.

CHAPTER SIX

6. Genome sequence analysis of novel bacterial species isolated from the chicken gut

6.1 Introduction

Large polymers from NSPs often become entangled and increase the viscosity of the intestine [172]. This causes a slow-down in digesta transit time, leading to bacterial overgrowth. Furthermore, this viscosity prevents enzymes from breaking down the polymer efficiently [99, 180, 184, 195]. The addition of NSP-degrading enzymes reduces large polymers before they can become entangled and thus increases the digestibility of the feed which has been shown to increase the FCR [174, 191]. Exogenous enzymes such as xylanase and cellulase have also shown to reduce the abundance of pathogens such as *C. perfringens* and *Campylobacter* species in the caeca of chicks, this is attributed to oligosaccharides that are released after NSP degradation by the enzymes being fermented by these species [174, 254].

The treatment of bacterial infections is becoming increasingly more difficult with the emergence of multi-antibiotic resistant organisms [163, 255, 256]. From whole genome sequencing of some organisms (such as MRSA) it was found that the majority of the resistance genes were acquired through horizontal gene transfer as opposed to intra-genomic evolution of resistance [132, 163]. Antibiotic growth promoters were routinely fed to chickens before they were banned in 2005 and therapeutic antibiotics are still given in large quantities promoting the emergence of resistant zoonotic pathogens [132, 160, 161].

In metagenomic studies, antibiotic resistance genes are regularly identified and multi-drug resistant bacteria have been isolated from the chicken on multiple occasions [2, 29, 68, 167, 168]. This indicates that microbial communities (such as the chicken-gut microbiome) are potential reservoirs for antibiotic resistance,

which could be particularly important if human pathogens were exposed to these communities. Therefore the identification of antibiotic resistance in novel species found in chickens could have potential importance to human health.

The aim of this chapter was to perform whole genome sequence analysis on six isolates; the objectives were to determine if they were novel through comparison of closest bacterial species, identify antibiotic resistance genes and potential industrially relevant enzymes.

6.2 Methods

From the cultured isolates discussed in chapter five, six were whole genome sequenced using an Illumina MiSeq 2x250 bp run. To check the quality of the sequences, they were initially loaded into fastqc before they were trimmed using SICKLE. *De novo* assembly was performed using SPAdes3.1. The contigs file output from SPAdes3.1 was used to map the reads against using BWA mem. Sequences were then converted with samtools and loaded into qualimap to check the quality of the assembly and the coverage of each contig. Contigs with <5x coverage were removed from the contig files and the process was repeated from the BWA step. To annotate the contig sequences, PROKKA1.9 was utilised.

Genome sequences were uploaded to SPECI which compares the genome sequences against 40 clusters of orthologous groups and found the nearest bacterial species cluster [257] and the 16S rRNA genes were inserted into ARB to determine taxonomy. An *in silico* DNA-DNA hybridization (DDH) analysis of

the genomes was completed using the Genome-to-Genome Distance Calculator (GGDC 2.0) [258] and average nucleotide identity (ANI) was performed with JSpecies [259] on the bacterial species that were closest to the isolate after ARB taxonomy placing. A threshold value of 70% (DDH) and 95% (ANI) were set for a genome belonging to the same species as per convention [258, 259]. Genomes were checked for antibiotic resistance genes using ResFinder2.1 [260]. As previously described, polysaccharide degrading enzymes are industrially relevant, therefore to identify any within these novel isolates BLASTKOALA [261] was used and PROKKA annotations further searched in Artemis. A custom python script was used to determine the number of sporulation genes present using the genes referred to in Abecasis *et al.* [108].

6.3 Results

6.3.1 Clostridiales isolate one

Clostridiales isolate one was cultured from a chicken caecum by heating the digesta to 65 ^{o}C for 15 minutes prior to serial dilution on FAA supplemented with sodium thioglycolate for three-five days in an anaerobic chamber at 37 ^{o}C. A total of 498,389 reads were produced, which resulted in 31x mean genome coverage. *De novo* assembly resulted in 48 contigs (max length, 346,860 bp) which totalled 3,426,140 bp with a G+C content of 36.8% (Table 6.1). Within the genome there were 52 tRNAs, five rRNA operons, 80 ncRNA operons, 3081 CDS and five CRISPR structures.

Isolate ID	Number of reads	Coverage (x)	Number of contigs	Max contig length (bp)	Genome size (bp)	G+C content (%)	Number of CDS	Number of tRNAs	Number of rRNA operons	Number of ncRNAs	Number of CRISPRs
Clostridiales isolate one	498,389	31	48	346,860	3,426,140	36.8	3,081	52	5	80	5
Coriobacteriaceae isolate	374,771	24	264	97,786	3,482,123	62.9	2,936	47	3	11	2
Alistipes isolate	348,213	26	105	265,881	2,870,012	55.8	2,506	42	3	10	0
Eubacteriaceae isolate one	476,955	29	87	423,250	2,870,012	45.5	3,686	43	5	41	7
Eubacteriaceae isolate two	448,415	44	46	373,141	2,492,634	50.2	2,248	41	6	26	3
Clostridiales isolate two	348,213	26	165	241,690	3,341,113	43.1	3,295	54	10	46	1

Table 6.1 – Isolate statistics, including sequencing output and PROKKA results

ResFinder revealed no antibiotic resistance genes. There were 86% of genes required for sporulation. BLASTKOALA annotated 47.8% of the CDS and indicated the isolate had a Sec-secretion pathway (Figure 6.1). The closest bacterial species cluster assigned by SPECI was Lachnospiraceae with a value of 74% which was below the species cut off. Using ARB, the 16S rRNA gene of the isolate was placed as an outgroup of three *Eubacterium* species, two *Anaerostipes* species and an *Acetivibrio* (Figure 6.2a). As there were no publicly available genome sequences of *Acetivibrio ethanolgignens*, *Anaerostipes butyraticus* and *Eubacterium uniforme*, DDH and ANI was unable to be completed on these. However, none of the remaining nearest bacterial species had a DDH value of >70%. *Anaerostipes caccae* had the highest DDH value (27.4% ± 2.5); the highest ANI value was 67.28% (against *E. ventriosum*), indicating this was a novel species (Table 6.2). Clostridiales isolate one was predicted to contain three copies of beta-glucosidase genes, which are involved in the breakdown of cellobiose and 1,4-β-D-glucan into β-D-glucose. Furthermore, the isolate was predicted to contain a pectinase gene which can break down pectin, a component of plant cell walls, a bi-functional xylanase/deacetylase and acetylxylan esterase (Figure 6.3).

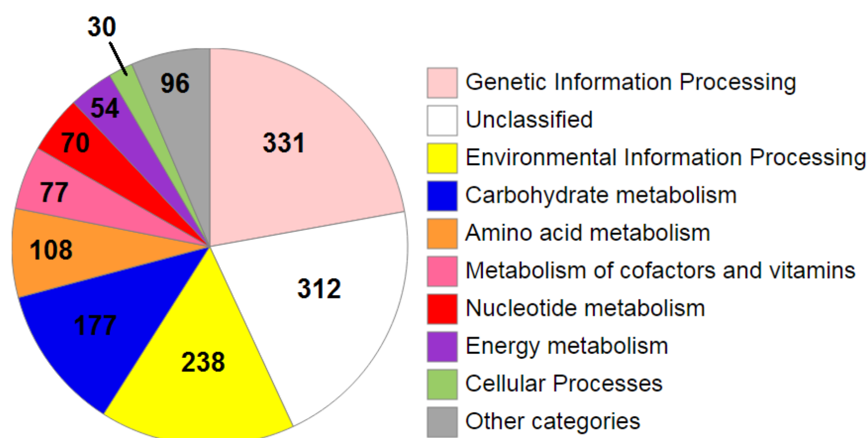


Figure 6.1 – Functional categories of CDS from Clostridiales isolate one after BLASTKOALA annotation

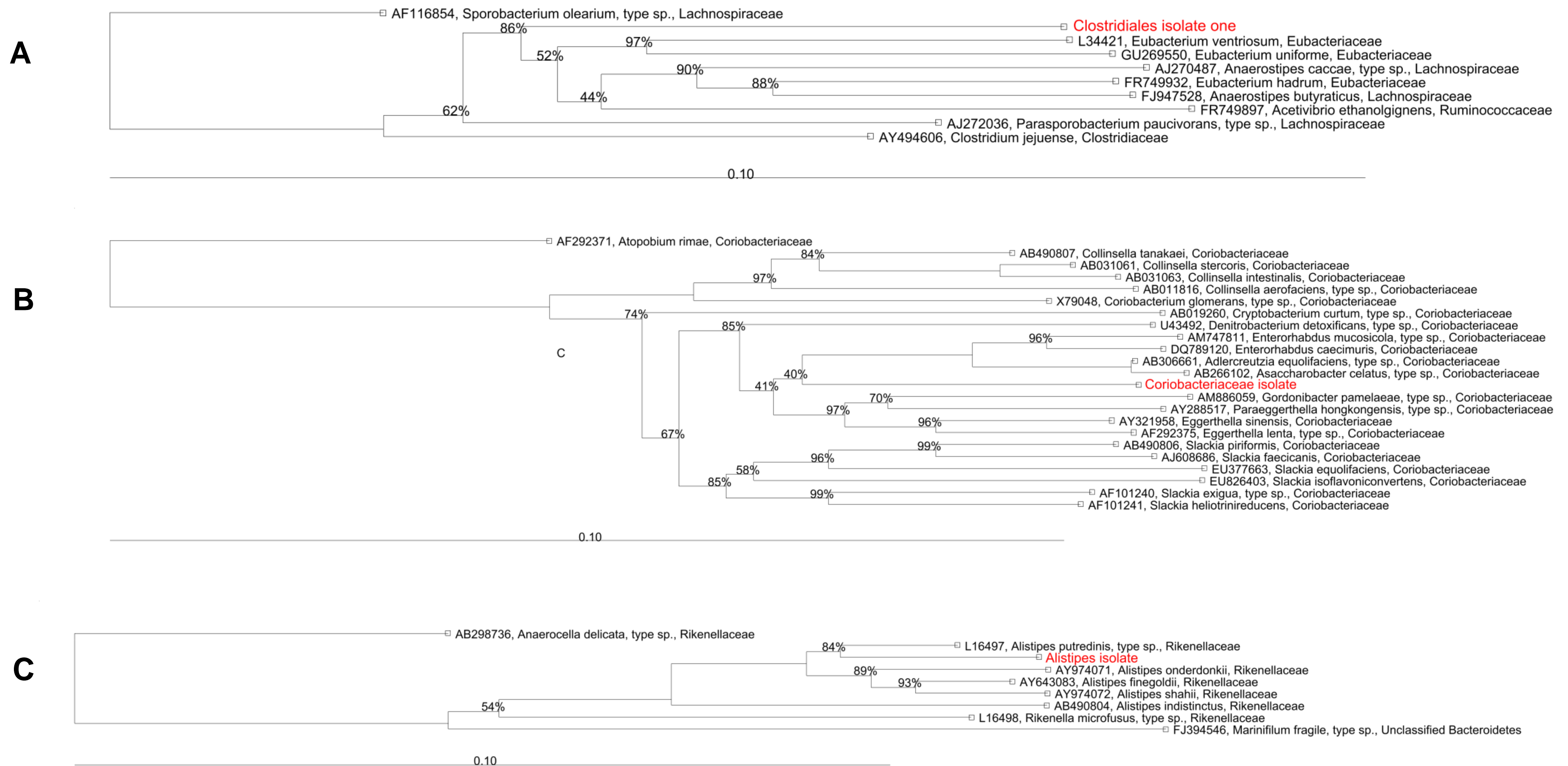


Figure 6.2 - A consensus of 1,000 bootstrap trees created using the ARB neighbour joining distance matrix **a)** Clostridiales isolate one **b)** Coriobacteriaceae isolate **c)** *Alistipes* isolate

Isolate ID	Species tested against	DDH (%)	ANI (%)
Clostridiales isolate one	*Anaerostipes hadrus*	20.7 ± 2.5	66.8
	Anaerostipes caccae	27.4 ± 2.5	66.6
	Eubacterium ventriosum	24.6 ± 2.5	67.3
Coriobacteriaceae isolate	*Adlercreutzia equolifaciens*	21.2 ± 2.7	73.8
	Enterorhabdus caecimuris	21.6 ± 2.7	74.2
	Enterorhabdus mucosicola	21.2 ± 2.7	74.5
	Gordonibacter pamelaeae	27.0 ± 2.4	80.7
	Eggerthella lenta	25.5 ± 2.4	80.0
Alistipes isolate	*Alistipes putredinis*	21.8 ± 2.7	74.6
Eubacteriaceae isolate one	*Eubacterium hallii*	22.3 ± 2.6	70.4
Eubacteriaceae isolate two	*Ruminococcus bromii*	34.5 ± 2.5	63.9
	Clostridium sporosphaeroides	18.4 ± 2.3	65.7
	Clostridium leptum	22.7 ± 2.5	67.4
Clostridiales isolate two	*Clostridium saccharogumia*	19.5 ± 2.5	66.5
	Clostridium ramosum	20.7 ± 2.5	66.1
	Clostridium spiroforme	22.2 ± 2.5	66.4

Table 6.2 – Results of DDH and ANI analysis of isolates tested against closest bacterial relatives determined by ARB

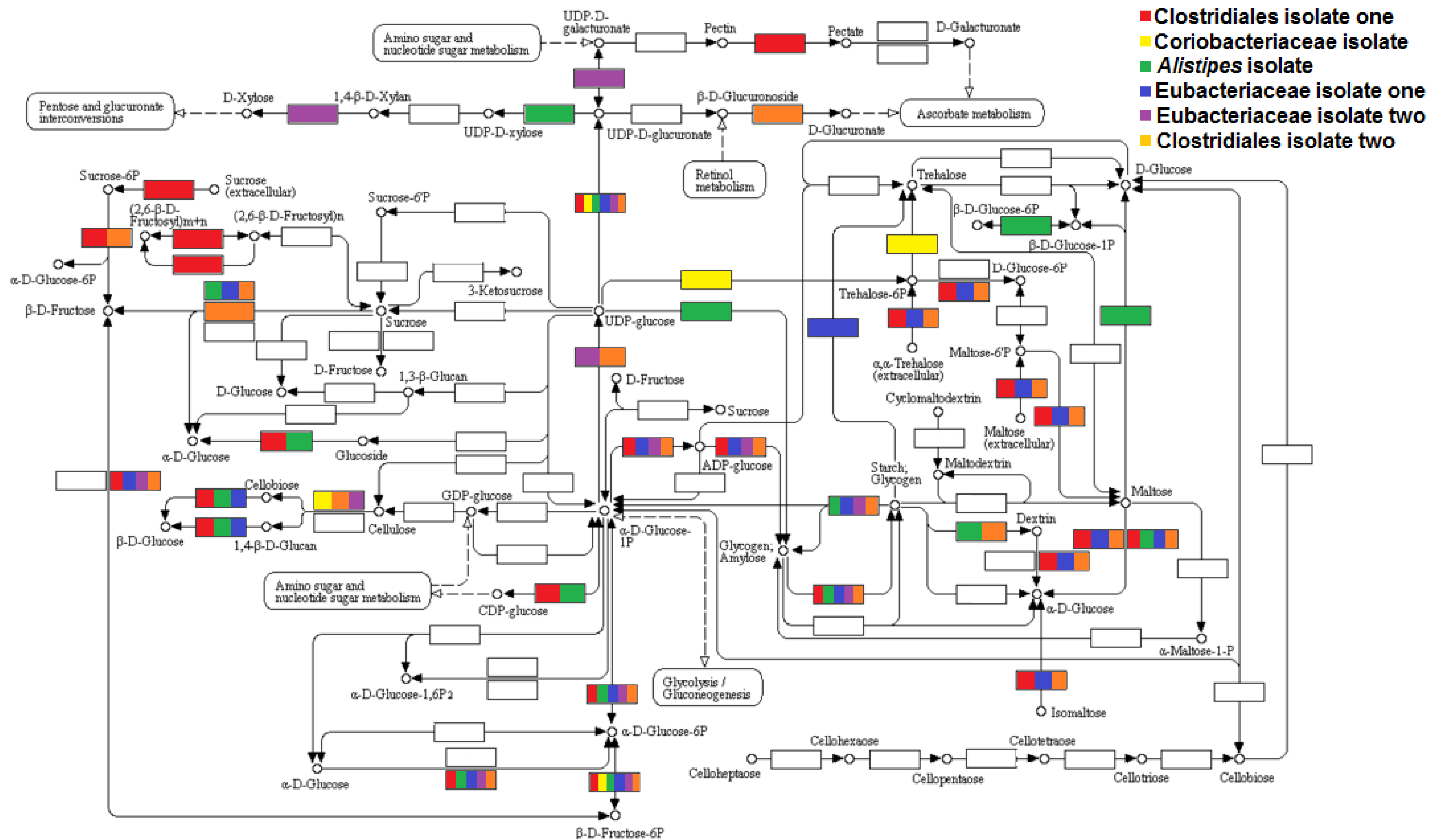


Figure 6.3 – BLASTKOALA output overlap of each isolate showing the starch and sugar metabolism pathways. Only the genes annotated by the program are shown here, all genes found in Artemis are not shown

6.3.2 Coriobacteriaceae isolate

The Coriobacteriaceae isolate was cultured from a chicken caecum using serial dilution and plated on FAA supplemented with 5% defibrinated horse blood in an anaerobic chamber at 37 ^{o}C for three-five days. A total of 374,771 reads were produced, which resulted in 24x mean genome coverage. *De novo* assembly resulted in 264 contigs (max length, 97,786 bp) which totalled 3,482,123 bp with a G+C content of 62.9% (Table 6.1). Within the genome there were 47 tRNAs, three rRNA operons, 11 ncRNA operons, 2,936 CDS and two CRISPR structures.

Antibiotic resistant genes were present against tetracycline (*tetW*), macrolides (*ermB*) and aminoglycosides (*aph(3')III*). BLASTKOALA annotated 42.4% of the CDS, revealing a Sec-dependant pathway and a partial twin-arginine pathway (Figure 6.4). SPECI output assigned *Eggerthella* as the closest bacterial species cluster with a value of 87%, which was below the species cut off. Using ARB, the Coriobacteriaceae isolate formed a sister group with four species (*Asaccharobacter celatus*, *Adlercreutzia equolifaciens*, *Enterorhabdus caecimuris* and *E. mucosicola*) with a bootstrap support of 40%, indicating weak placement (Figure 6.2b). Due to the weak support and the SPECI output, other bacterial species that had genome sequences and were in the same cluster as the Coriobacteriaceae isolate were also chosen for DDH and ANI analysis (*Gordonibacter pamelaeae* and *Eggerthella lenta*). DDH analysis revealed the highest value was 27% ± 2.4 to *G. pamelaeae*, which was also the closest after ANI comparison (Table 6.2). However, both of these values were below the threshold to be assigned to *G. pamelaeae* and therefore the Coriobacteriaceae

isolate was a novel species. The Coriobacteriaceae isolate was predicted to contain a single copy of an endoglucanase gene (K01104), which can degrade cellulose into cellobiose (Figure 6.3).

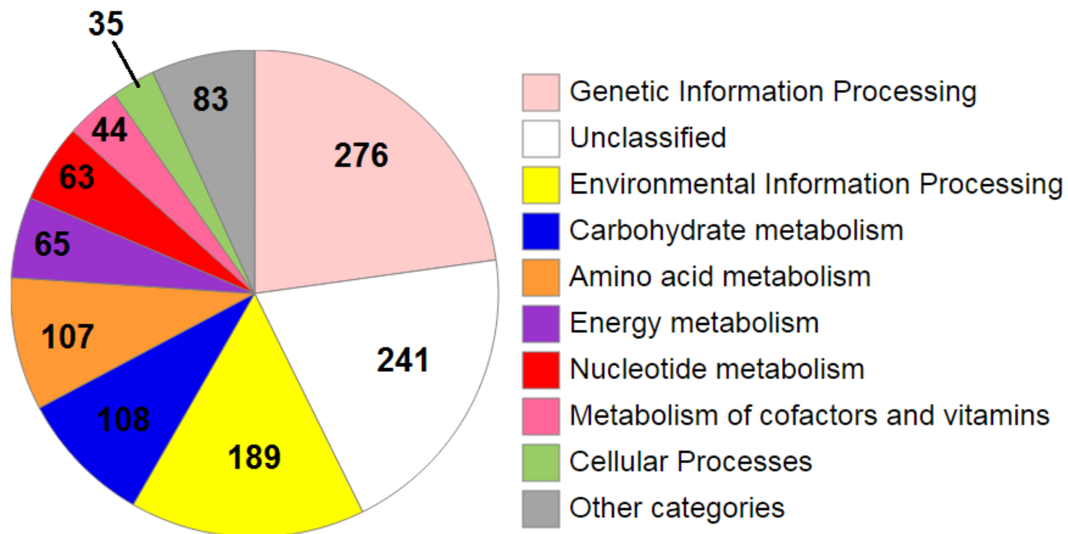


Figure 6.4 – Functional categories of CDS from Coriobacteriaceae isolate after BLASTKOALA annotation

6.3.3 *Alistipes* isolate

Alistipes isolate was cultured from a chicken caecum using serial dilution and culturing on FAA supplemented with 15 µg/ml of colistin sulphate in an anaerobic chamber for three-five days at 37 ^{o}C. A total of 348,213 reads were produced, which resulted in 26x mean genome coverage. *De novo* assembly resulted in 105 contigs (max length, 265,881 bp) which totalled 2,870,012 bp with a G+C content of 55.8% (Table 6.1). Within the genome there were 42

tRNAs, three rRNA operons, ten ncRNA operons, 2,506 CDS and no CRISPR structures.

The genome contained tetracycline resistance genes (*tetX, tetQ*) and a macrolide resistance gene (*ermF*). BLASTKOALA annotated 37.1% of the CDS, showing the isolate had a Sec-pathway (Figure 6.5). SPECI output found the closest bacterial species cluster was *Alistipes*, with an average identity of ~85%, which was below the species cut-off. This was confirmed by placement in ARB next to *A. putredinis* with a bootstrap support value of 84% (Figure 6.2c). DDH analysis found a similarity of 21.8% ± 2.7 and ANI analysis gave 74.6% to *A. putredinis* (Table 6.2). The *Alistipes* isolate was predicted to contain two copies of a beta-glucoside gene (Figure 6.3).

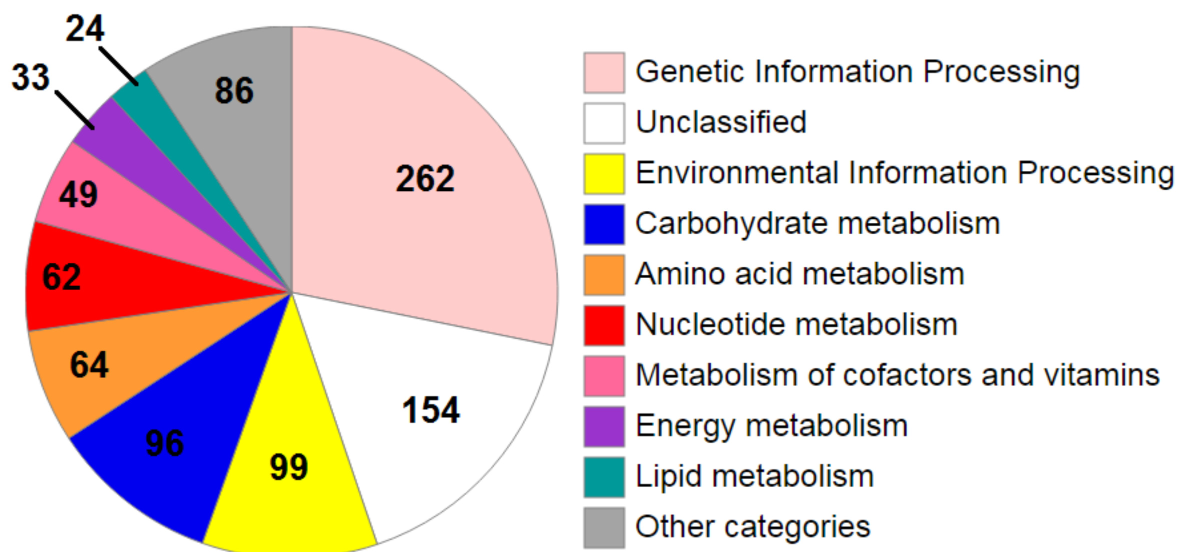


Figure 6.5 – Functional categories of CDS from the *Alistipes* isolate after BLASTKOALA annotation

6.3.4 Eubacteriaceae isolate one

Eubacteriaceae isolate one was cultured from a chicken caecum by mixing the digesta with 80% ethanol for 30 minutes prior to plating on FAA supplemented with sodium thioglycolate in an anaerobic chamber at 37 ^{o}C for three-five days. A total of 476,955 reads were produced, which resulted in 29x mean genome coverage. *De novo* assembly resulted in 87 contigs (max length, 423,250 bp) which totalled 2,870,012 bp with a G+C content of 45.5% (Table 6.1). Within the genome there were 43 tRNAs, five rRNA operons, 41 ncRNA operons, 3,686 CDS and seven CRISPR structures.

ResFinder revealed no antibiotic resistance genes. The ability to sporulate was identified due to the presence of 84% of the genes required for sporulation [108]. BLASTKOALA annotated 41.2% of CDS and a Sec-pathway was identified (Figure 6.6). SPECI found the nearest bacterial species cluster was *Eubacterium hallii*; however the average identity of 80.7% was below the species cut-off. This was the same result as ARB (Figure 6.7a). DDH and ANI analysis resulted in values of 22.3% ±2.6 and 70.4%; both lower than the threshold for designations as the same species as *E. hallii*, therefore isolated Eubacteriaceae isolate one was a new species (Table 6.2). Eubacteriaceae isolate one was predicted to have a beta-glucosidase gene and an endo 1, 4-beta xylanase Y gene (Figure 6.3).

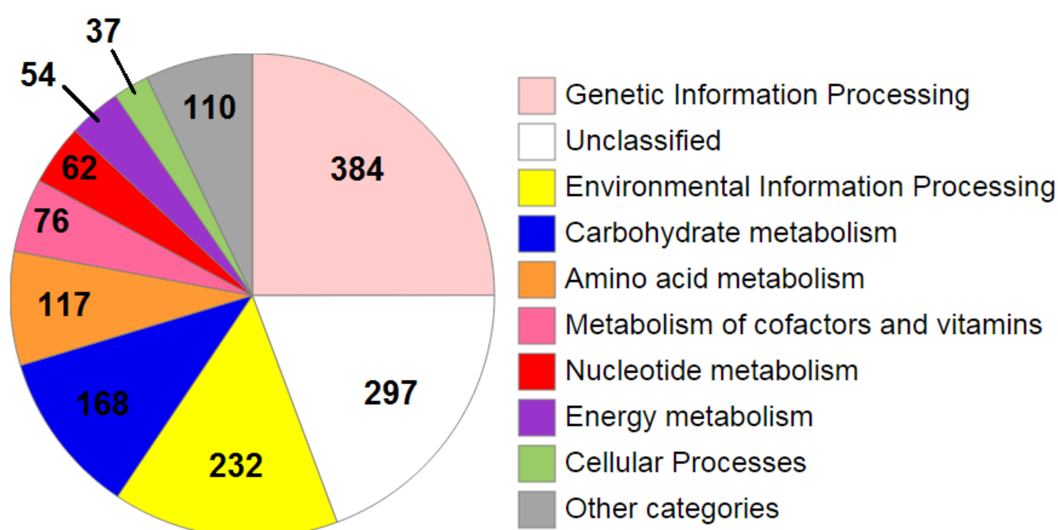


Figure 6.6 – Functional categories of CDS from Eubacteriaceae isolate one after BLASTKOALA annotation

6.3.5 Eubacteriaceae isolate two

Eubacteriaceae isolate two was cultured from a chicken caecum by mixing the digesta with 80% ethanol for 30 minutes prior to plating on Mueller-Hinton agar supplemented with sodium thioglycolate in an anaerobic chamber at 37 ^{o}C for three-five days. A total of 448,415 reads were produced, which resulted in 44x mean genome coverage. *De novo* assembly resulted in 46 contigs (max length 373,141 bp) which totalled 2,492,634 bp with a G+C content of 50.2% (Table 6.1). Within the genome there were 41 tRNAs, six rRNA operons, 26 ncRNA operons, 2,248 CDS and three CRISPR structures.

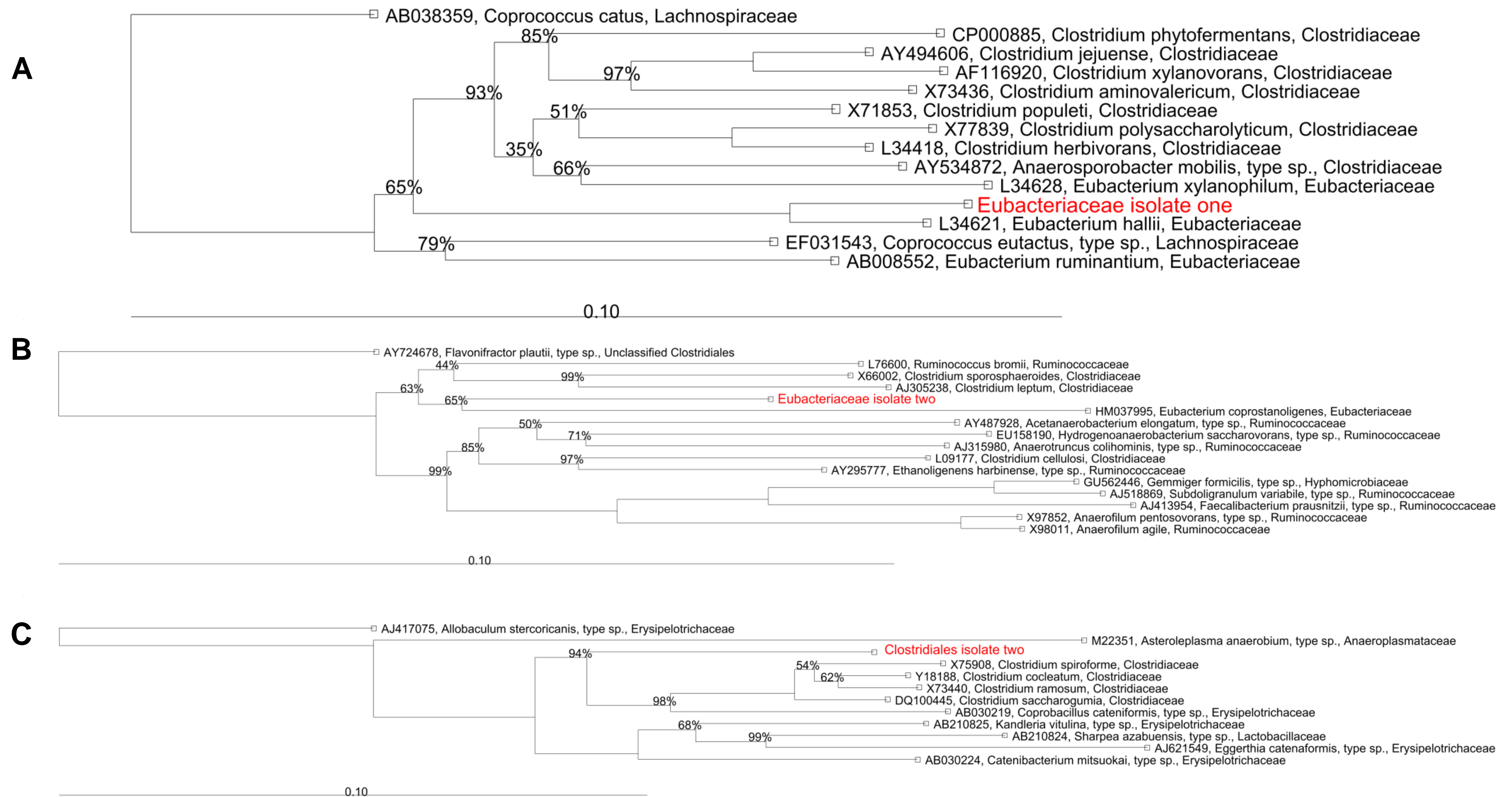


Figure 6.7 - A consensus of 1,000 bootstrap trees created using the ARB neighbour joining distance matrix **a)** Eubacteriaceae isolate one **b)** Eubacteriaceae isolate two **c)** Clostridiales isolate two

There was a tetracycline resistance gene (*tetW*) found after analysis. Furthermore, it was found the isolate contained 83% of the genes required for sporulation. BLASTKOALA annotated 47.1% of the CDS and a Sec-pathway was found (Figure 6.8). SPECI could not place the isolate with a species cluster however ARB placed the 16S rRNA gene of the isolate with *Eubacterium coprostanoligenes* with a bootstrap support value of 65% (Figure 6.7b). However, because there was no publicly available genome sequence for this organism, three other species that were in the same cluster were chosen for DDH and ANI analysis (*Ruminococcus bromii*, *Clostridium sporosphaeroides* and *C. leptum*). The closest bacterial species after DDH was *R. bromii* (34.5% ± 2.5) and the closest after ANI was *C. leptum* (67.4%) (Table 6.2). This indicated Eubacteriaceae isolate two was a new species. It was predicted to have a 1, 4-β-xylosidase gene, four endo-1, 4-β-xylanase A genes, an endo-1, 4-β-xylanase Z gene and two endo-glucanase genes (Figure 6.3).

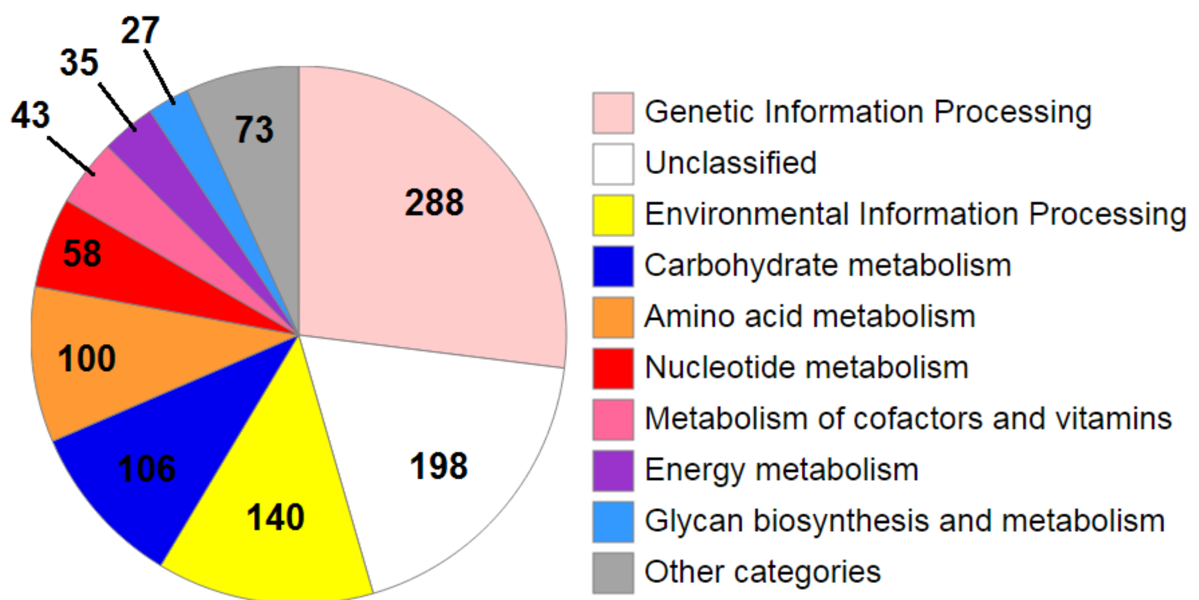


Figure 6.8 – Functional categories of CDS from Eubacteriaceae isolate two after BLASTKOALA annotation

6.3.6 Clostridiales isolate two

Clostridiales isolate two was cultured from a chicken caecum using serial dilution and FAA supplemented with 15 μg/ml rifampicin in an anaerobic chamber for three-five days at 37 ^{o}C. A total of 348,213 reads were produced, which resulted in 26x mean genome coverage. *De novo* assembly resulted in 165 contigs (max length, 241,690 bp) which totalled 3,341,113 bp with a G+C content of 43.1% (Table 6.1). Within the genome there were 54 tRNAs, ten rRNA operons, 46 ncRNA operons, 3,295 CDS and one CRISPR structure.

There was a lincosamide resistance gene (l*nuC*) and tetracycline resistance gene (*tetW*) present in the genome. Analysis of the sporulation genes identified 77% of the required genes which was below the threshold of 80%. BLASTKOALA annotated 39.9% of CDS and a Sec-pathway was identified (Figure 6.9). SPECI output found *Coprobacillus* was the nearest bacterial species cluster with an average identity of ~71% which was below the species cut-off. ARB placed the 16S rRNA gene as an outgroup of five species (*Clostridium spiroforme*, *C. cocleatum*, *C. ramosum*, *C. saccharogumia* and *Coprobacillus cateniformis*) with a bootstrap support value of 94% (Figure 6.7c). However, there are no published genome sequences of *C. cocleatum* or *C. cateniformis* so DDH and ANI could not be completed with them. Of the remaining species, *C. spiroforme* (22.2% ±2.5) was the closest after DDH analysis and *C. saccharogumia* (66.5%) was the closest after ANI analysis (Table 6.2). The genome of Clostridiales isolate two was predicted to contain four endo-1, 4-β-xylanase Z genes, an endo-1, 4-β-xylanase Y gene, an

endo-1, 4-β-xylanase/feruloyl esterase gene, a bi-functional xylanase/acetylase gene and an endoglucanase gene (Figure 6.3).

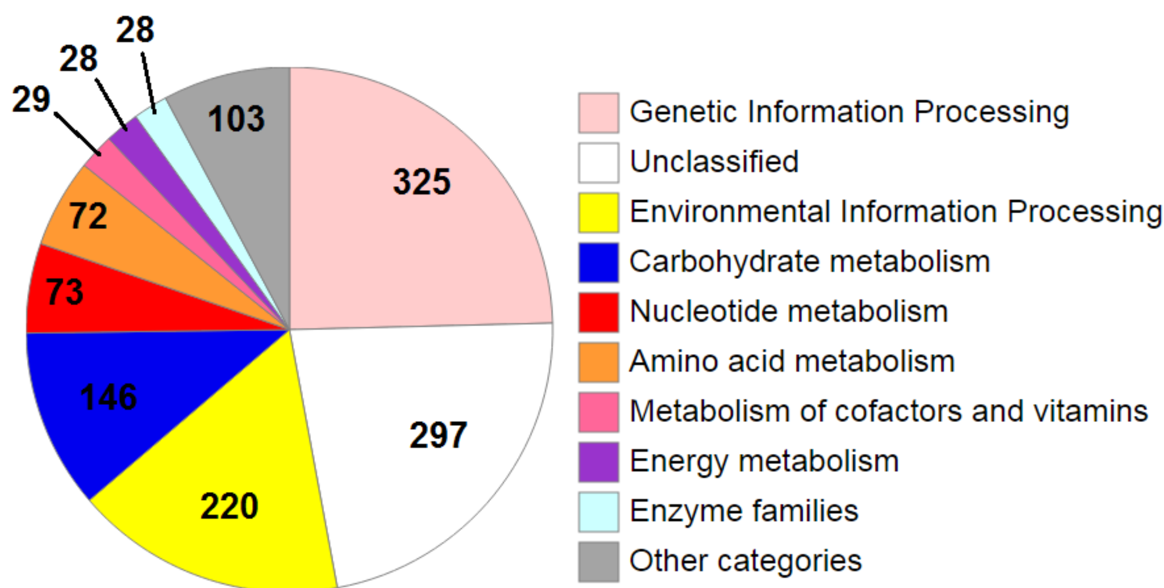


Figure 6.9 – Functional categories of CDS from Clostridiales isolate two after BLASTKOALA annotation

6.4 Discussion

The isolation, sequencing and analysis of six novel bacterial species revealed they had multiple antibiotic resistance genes and potential industrially relevant enzymes. However, there is a limitation to what can be observed or inferred through sequence analysis and full biochemical tests are required to determine volatile-acid production, fermentation profiles and to name the novel species. The presence of spore forming genes in Clostridiales isolate one and the Eubacterium isolates match the phenotypic properties of how these species

were isolated through selection and germination of spores. The isolation of novel species from the chicken gut can help future taxonomic assignments, not only because they can provide a reference, but also other species that are more taxonomically similar to them than currently available sequences.

Genes that confer tetracycline resistance were the most common antibiotic resistance from sequence analysis of the isolates which is the same result as in previous caecal microbiome studies [2, 29]. Qu *et al.* (2008) noted the abundance of horizontal gene transfer elements and virulence factors in the chicken caecum microbiome, therefore transfer events involving genes that confer resistance is likely [29]. However, the antibiotic resistance genes in some isolates were naturally occurring, for example resistance in the *Alistipes isolate* could be because it is closely related to the *Bacteroides* genus and the majority of species within the genus contain these resistance genes [262-264].

Eubacteriaceae isolate one could show potential as a probiotic to the chicken. It was found to be related to *E. hallii*, a lactate utilising and significant butyrate producing species [265]. Similarly *A. caccae* (related to Clostridiales isolate one) shared the same property [266]. Lactate is the major product of lactic acid bacteria such as lactobacilli, which are the most abundant organisms in the chicken gut [105, 266]. In humans, it has been shown that ulcerative colitis sufferers have increased concentrations of lactate [266]. *Lactobacillus* species have been studied as probiotics due to their ability to reduce pathogen load. However, the effect of excess lactate to chicken health has not been investigated [237, 238, 267-269]. It has been noted that increased lactate levels result in the stimulation of butyrate producing bacteria, therefore if isolates

Eubacteriaceae isolate one and Clostridiales isolate one were given as a probiotic at the same time as lactobacilli it could result in increased digestive health and weight gain [136, 270].

Industrially relevant enzymes such as xylanases were identified in the novel isolates. These are important to chicken gut health though reduced intestinal viscosity resulting in improved weight gain [99, 109, 174, 271]. Microbial xylanases also have potential for the biodegradation of biomass into fuels and the bleaching of pulp and paper [272]. Due to this multi-purpose potential, xylanases from microorganisms are often studied for activity and potential [272-276]. The discovery of two bi-functional xylanase could provide the greatest hope of a future feed additive. As the enzyme is bi-functional it does not require the presence of an additional enzyme to degrade xylan from grains [277]. These enzymes should be of particular interest and their activity should be tested in the future.

In addition to finding xylan-degrading enzymes, pectinase and cellulase genes were also discovered. The addition of cellulase to a broiler diet results in reduced feed consumption and an increase in FCR. It has also be found that calcium, phosphorus, iron, zinc and copper that were associated with cell walls are solubilised by cellulase [278]. Pectinase has also demonstrated a greater growth rate in chicks and egg production, both on its own and when mixed with cellulase and hemicellulose in a rye-based diet due to rye containing 8% pectin [279-281].

As the species in this study were isolated and sequenced, the enzymes identified can be observed in their natural host or primers can be designed to

amplify the genes into an expression host. If high enzyme activity was detected in the host, then other benefits the organism might confer to the host, such as the production of volatile-fatty acids or bacteriocins, could result in potential probiotic uses rather than cloning gene into other organisms for over-expression.

CHAPTER SEVEN

7. Discussion

This study used culture-dependent and culture-independent methods to establish the chicken gut microbiota. Chapter Three illustrates the problems of using the V3-V4 16S rRNA gene-fragment region to study the majority of organs in the chicken, due to chloroplast amplification. This issue has not been discussed in the literature before, despite previous studies using 16S rRNA gene sequencing to study organs other than the caecum [115, 119]. Chapter Four presents 16S rRNA gene-fragment data of the crop, proventriculus, gizzard, duodenum, ileum and caecum of six chickens fed different diets, something that has not been completed previously. This is the largest survey of the chicken gut using 16S rRNA gene sequencing to date.

The increase in the depth of sequencing in Chapter Four allowed for a more accurate bacterial census of the chicken gut. The minimum number of reads per sample was enough to saturate the number of OTUs present in each sample, something that was not achieved in Chapter Three. Furthermore, the increase in depth also revealed a significant difference in diversity in the caecum between the diets which was not found in the V3-V4 16S rRNA gene-fragment sequencing and might not have been found without the extra depth. This illustrates that significant differences could be missed in studies that do not sample to a great enough depth.

Chapter Five presents culturing data from the chicken gut and the discovery of multiple new species. This included the isolation of bacteria from the proventriculus, something that has not been previously published. The majority of novel species were isolated from the caecum, which matches the hypothesis-drawn from culture-dependent and culture-independent studies that the caecum

is populated with a multitude of uncultured species [38, 90-93, 120, 124]. Whilst the abundance of the isolated species in the chicken gut was high for the majority of organs, this was primarily due to the culturing of two *Lactobacillus* species. Even though 37 of the 43 species were isolated from the caecum, they conferred only a small proportion of the relative abundance in this organ. Many of the most abundant organisms found in Chapter Four were not isolated from the caecum, therefore the appropriate isolation techniques were not utilised, the respective colonies were not picked or the 16S rRNA gene was not successfully amplified using the PCR primers.

Chapter Six presents whole genome analysis of six novel bacterial species. The isolation of novel species with industrially relevant enzymes, illustrates the potential of the chicken gut as an untapped resource for the discovery of a multitude of enzymes with varied use. The microbiota of the caecum are involved in fermentation of NSPs, therefore are already equipped to digest multiple targets. Setting up a gut model could help isolate more species and test hypotheses of co-culture, competition between species and probiotics in the chicken gut.

The 16S rRNA gene-fragment sequencing in Chapter Four has saturated the discovery of OTUs in the samples. As a large amount of data was produced, the use of a copy number normalisation was utilised without compromising the depth. This is something that has not been applied to chicken 16S rRNA gene-fragment data previously and should provide a more accurate account of the microbial community in the chicken. The development of next-generation long-read technologies such as Oxford Nanopore MinION will benefit future 16S

rRNA gene sequence studies. The capability of obtaining almost full length 16S rRNA gene sequences increases the taxonomic resolution and this has been demonstrated on a small scale [282]. If this can be developed for more complex samples it could be a useful tool in a bacterial census.

This is the deepest 16S rRNA gene-fragment study of the chicken gut to date. Whilst the aim of this study was to identify the microbiota of the chicken gut and discover difference between diets, similar approaches could be used to identify high and low FCR associated OTUs as completed by some previous low-powered studies. This would require a different experimental set-up where the amount of feed available to the chickens would be monitored.

The sequencing results generated in this study largely agree with those found in literature. The chicken gut was dominated by Firmicutes and the lactobacilli were the most abundant in the majority of organs. A difference to the results from other studies was the low abundance of Bacteroidetes, with Proteobacteria the second most abundant phylum instead [24, 119]. The caecum was the most diverse organ with Lachnospiraceae and Ruminococcaceae families the most abundant which is in agreement of other studies [24, 29, 115, 119, 120]. However, from previous studies on the chicken gut microbiota, the results have been varied, especially in the caeca. This could be due to genotypic and geographical differences between flocks of chickens, in addition to feed and antibiotic use. Therefore the results generated from the authors' study may not be universal and may only apply to this specific set of chickens. To test this hypothesis, the caecal samples of multiple chickens, fed different diets and in

different locations could be analysed through high-throughput sequencing and culture-based methods to try and establish a core microbiome of the chicken.

Phytase has been shown to increase the abundance of lactobacilli in the ileum and this was also discovered in this study, however there was not a significant difference in diversity between the diets [112]. The effect of phytase supplements on the microbiota of other organs has not previously been studied, but it was found there was no significant difference in diversity in any organ but the caecum. One hypothesis for this difference could be the lactate produced by the more abundant lactobacillus in the ileum selecting for organisms that can utilise the lactate and produce other volatile fatty acids such as butyrate downstream in the caecum [136].

The study of the gut microbiota is a continuing challenge for microbiologists and has been since the 1800's [36]. Early culturing efforts were limited by specific media and limited anaerobic methods, restricting early pioneers to the discovery of a minute quantity of organisms and failing to capture the true diversity of these complex communities. Whilst more recent culturing approaches to the gut microbiota have led to the discovery of a wide array of new species, it is still a developing technique.

The application of 16S rRNA gene sequencing, initially through clone sequencing and later high-throughput methods, revealed a more complex and diverse environment than previously thought. The advent of high-throughput DNA methods has resulted in the generation of gigabases of data and an almost complete bacterial census of the gut. However, there are still downsides to using high-throughput sequencing. Bias is introduced at many stages of the

sequencing process; DNA extraction, PCR amplification and data analysis. Furthermore, the use of OTUs in 16S rRNA gene-fragment sequencing at ≥97% limits means that some species will be not be discovered through clustering. Different species that have 16S rRNA gene identities above this threshold risk being clustered together; therefore the true diversity of an environment may be higher than estimated.

This study has further shown that a culture-dependent and culture-independent approach to gut microbiota is prudent. The identification of many organisms through culturing helped to support the 16S rRNA gene-fragment sequencing data and their potential roles in the microbiota that would not be possible through 16S gene-fragment sequencing alone. Also the discovery of organisms that were present but not detected through sequencing raises the question of unknown unknowns. Without the culturing data to back up the sequencing data, these organisms would have been missed. The abundance of these organisms in the gut has not been calculated and further analysis would be required to determine if it was the DNA extraction technique, PCR amplification or low abundance behind they were not detected. The cultured isolate sequences that were too short for comparison with the data generated in Chapter Four should have their entire 16S rRNA gene sequenced.

Whilst a metagenomic study of the chicken gut would have reduced PCR bias and could have led to the greater taxonomic identification and perhaps generated whole or partial genome sequences of some of the more abundant organisms, this would have been a more expensive approach. The use of 16S rRNA gene-fragment sequencing allows the multiplexing of multiple samples

whilst still generating enough depth to provide answers. The abundance of chloroplast reads in the majority of chicken gut organs generated in this study shows that non-targeted sequencing will lead to generation of data that might not be of use. This is why microbiome studies have only focussed on the caecum where bacterial density is highest and presence of DNA from the digesta is lowest [2, 29, 68]. Thus, 16S rRNA gene sequencing is still a very useful tool and especially when targeting just the bacteria in biological samples.

A different approach to culturing novel species in the chicken gut could be achieved through the study of metagenomic data. If metagenomic sequencing provided enough data to generate whole or partial genome sequences of novel species, the identification of antibiotic resistances and metabolic pathways could help to design a selective medium to aid the isolation of that organism. Whilst this would apply to only the most abundant species in a sample, this would still be beneficial for samples with a large proportion of novel species such as the chicken caeca.

For a full census of the chicken gut microbiome, a three-pronged approach of 16S rRNA gene sequencing, metagenomic sequencing and intense culturing would need to be completed on multiple chickens. The identification of novel species should lead to full biochemical test and whole-genome sequencing to try and identify their roles within the microbiome.

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